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Capillary electrophoresis–atmospheric pressure ionization mass spectrometry for the characterization of peptides

Instrumental considerations for mass spectrometric detection

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ABSTRACT

On-line capillary electrophoresis (CE) separations are shown for a synthetic peptide mixture and a tryptic digest of human hemoglobin in an uncoated fused-silica capillary with detection using atmospheric pressure ionization mass spectrometry (API-MS). The CE system utilized a 1-m capillary column of either 75- or 100-μm I.D. These somewhat larger inside diameters allow higher sample capacities for MS detection, and the 1-m length facilitates connecting the CE column to the liquid junction–ion spray interface and MS system. Low volatile buffer concentrations (15–20 m*M*) of ammonium acetate or ammonium formate, and high organic modifier content (5–50%) of methanol or acetonitrile facilitates ionization under electrospray conditions.

This study shows that peptides separated by CE may be transferred to the API-MS system through a liquid junction coupling to the pneumatically assisted electrospray (ion spray) interface at low buffer pH when the electroosmotic flow is low (0–0.04 μl/min). CE–MS as described herein is facilitated by features in modern CE instrumentation including robotic cleaning and pressurization of the capillary inlet. The latter is particularly useful for repetitive rinsing and conditioning of the capillary column between analyses in addition to continuous 'infusion' of sample to the mass spectrometer for tuning purposes.

In addition to facile molecular weight determination, amino acid sequence information for peptides may be obtained by utilizing on-line tandem MS. After the tryptic digest sample components enter the API-MS system, the molecular ion species of individual peptides may be focussed and transmitted into the collision cell of the tandem triple quadrupole mass spectrometer. Collision-induced dissociation of protonated peptide molecules yielded structural information for their characterization following injection of 10 pmol of a tryptic digest from human hemoglobin.

INTRODUCTION

The interest and use of capillary electrophoresis (CE) as a separation technique has expanded considerably in recent years. Separations are based on the principles of

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the electrically driven flow of ions in free solution. High separation efficiency, short analysis times and a low amount of sample consumed makes CE a very attractive separation technique. On-column detection with UV absorbance or fluorescence is commonly used. The technique has recently been reviewed [1].

Peptides belong to a group of compounds where CE may be very useful both to separate bioactive peptides when the sequences differ by only one amino acid and for peptide mapping from proteolytic digests of proteins [2]. At low pH the uncoated fused-silica capillary wall carries few negative charges, so the electroosmotic flow is low. Positively charged peptides migrate towards the negatively charged cathode at the capillary exit. A decrease in electroosmotic flow observed under low pH conditions provides a wider separation window for the positively charged peptides. Good separations have been obtained for dipeptides in low-pH phosphate buffer [3] and for the protease V8 digest of β-lactoglobulin in citric acid pH 2.5 [4] in uncoated fused-silica capillaries with UV detection.

It is desirable to obtain high-quality electropherograms with mass spectrometry (MS) as the detection system. An advantage for MS detection is that molecular weight information may be obtained in addition to the migration time for each component peak in a mixture. It is also possible to obtain structural information of the mixture components by tandem mass spectrometry (MS–MS). In fact, the mixture analysis capability of the latter may even provide structural information from co-eluting components (see below). Sodium citrate, phosphate and borate buffers that are commonly used in CE separations are, however, not generally suitable for CE in combination with MS. The production of gas-phase ions during the electrospray process is facilitated by using volatile buffers at low concentration to obtain a high analyte ion current response. In addition, organic solvents such as methanol and acetonitrile, which reduce the surface tension of electrospray droplets, are desirable and facilitate the formation of gas-phase ions in the ion spray process [5]. In this work we discuss factors that pertain to the CE system when a mass spectrometer equipped with an atmospheric-pressure ionization source is used as the detector (API-MS). A review of the API-MS instrumentation used in this work has been reported by Huang *et al.* [6].

CE–MS with ionization at atmospheric pressure has previously been demonstrated for a variety of compounds [7–15]. Developments in CE–MS interfacing are based either on the electrospray–ion spray process where gas-phase ions are formed at atmospheric pressure (API) or continuous-flow fast atom bombardment (CF-FAB) [16–18]. For the latter the CE–MS interface must accommodate very low flow-rates and differing buffers used in CE in addition to the introduction of 10–25% glycerol for the CF-FAB process. The make-up liquid is introduced either by a liquid junction [8,12–14,17,18] or as a sheath-flow arrangement [9–11,15,16]. The use of a liquid junction or a sheath flow has provided preliminary results for CE–MS characterization of peptides. Here we have used a pneumatically assisted electrospray (ion spray) interface with the liquid junction coupling described previously by Lee *et al.* [14]. The buffer in the liquid junction is typically but not necessarily the same as the separation buffer for CE, and provides suitable make-up buffer flow to sustain a stable spray to the API mass spectrometer.

Earlier investigations with the ion spray interface have shown that CE–MS performs well with buffers of pH 4.8–6.9 and 50–90% acetonitrile in the running

buffer which produces a bulk electroosmotic flow between 0.4–1.8 μl/min [8,12–14]. In this work we demonstrate that analyte ions are transferred from the CE capillary via the liquid junction to the ion spray interface and the API-MS system under conditions where the electroosmotic flow is very low.

Increased interest in CE is also reflected by the rapid development of commercial CE instrumentation. We present the advantages of interfacing a commercially available CE instrument to API-MS and show the utility of CE–MS and CE–MS–MS techniques for the determination of peptides for characterizing protein tryptic digests. Several CE–MS experimental parameters are assessed as they relate to MS detection using the described system.

EXPERIMENTAL

Chemicals

Fused-silica capillary tubes with dimensions of 50, 75 and 100 μm I.D. and 365 μm O.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). HPE calibrators were obtained from Bio-Rad Labs. (Richmond, CA, USA). The HPE calibrator consists of the following nine peptides: (1) bradykinin; (2) angiotensin II; (3) α-melanocyte stimulating hormone (α-MSH); (4) thyrotropin releasing hormone (TRH); (5) luteinizing hormone-releasing hormone (LHRH); (6) oxytocin; (7) leucine enkephalin; (8) methionine enkephalin and (9) bombesin. The individual proteins, human hemoglobin and trypsin, were obtained from Sigma (St. Louis, MO, USA). The enzyme that was used to digest hemoglobin was trypsin treated with L-tosylamide-2-phenylethyl chloromethyl ketone (TPCK). The electroosmotic flow marker used in the study with UV detection was a heptapeptide (Arg–Lys–Arg–Ser–Arg–Lys–Glu) obtained from Applied Biosystems (Santa Clara, CA, USA). Sequencing-grade trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Optima-grade acetonitrile and methanol were obtained from Fisher Scientific (Flair Lawn, NJ, USA). Water was purified in-house with a Barnstead Nanopure system. All other chemicals were of analytical or reagent grade and used without further purification.

Capillary electrophoresis instrumentation

Two commercial CE systems were used in this work. System A was an Applied Biosystems (Foster City, CA, USA) Model 270A while system B was a Beckman (Palo Alto, CA, USA) Model P/ACE 2000. Most of the off-line work with UV detection at 210 nm was performed with system A. In system A the total length of the capillary was 72 cm with a length of 50 cm between the inlet end and the UV detector. All CE–MS experiments in this report were performed with system B. For CE–MS work the total length of the capillary was 100–104 cm with the UV detector located 20 cm from the inlet end. Two modifications were made on the commercial Beckman instrumentation (System B) to facilitate CE–MS operation. The first was installation of a switch to bypass the safety lock between the capillary ends. Measurement of the current across the capillary by system B could not be made during CE–MS operation with this modification. The current through the system was measured by an external meter. The second modification involved bypassing the capillary cartridge temperature control system during CE–MS operation. Since most of the CE capillary ex-

tended through ambient air between the CE system and the mass spectrometer, the normal temperature control system would have been ineffective.

In system B, the first 20 cm of the capillary were housed in a cartridge holder. This device was also used in the CE–MS work, but the capillary extended beyond the UV detector to reach the mass spectrometer. A slit was made in the upper portion of the cartridge holder to allow extension and connection of the capillary end to the liquid junction coupling and the ion spray interface. Of the 100 cm total length, 36 cm of the capillary were inside the cartridge holder while 64 cm were held in air outside this device. The temperature in system B is usually regulated by a circulating liquid inside the cartridge holder during CE operation. This liquid, a mixture of C_{5-18} perfluorocompounds containing sodium, had a very high response in the API-MS such that a small leakage of the liquid from the cartridge holder into the running buffer vial was sufficient to interfere with the analyte signal. Considerable difficulty was experienced rebuilding the cartridge holder, so it was more convenient to bypass the circulating liquid and thereby the temperature control of the capillary. Part of the capillary (64 cm) was positioned in air so there was no temperature control for that part of the capillary. Therefore, removal of the temperature control fluid from the first portion of the capillary had little effect on the separation. Both CE systems A and B employ a high-voltage power supply with a maximum of 30 kV.

Mass spectrometry

A Sciex TAGA 6000E triple quadrupole mass spectrometer equipped with an API source was used in this work (Sciex, Thornhill, Canada). The capillary exit end from the CE system was coupled to the API-MS via a liquid junction and a pneumatically assisted electrospray (ion spray) interface [5]. The same liquid junction and ion spray interface have been described by Henion and co-workers [8,12–14]. The only modification in this work was that the inner capillary in the ion spray interface consisted of uncoated fused silica instead of stainless steel. The I.D. of the fused-silica capillary in the ion spray interface was either 75 or 100 μm while the corresponding I.D. was used in the separation capillary. In the present study the liquid junction along with the ion spray interface were floated at +3.5–4.2 kV while the mass spectrometer was operated in the positive-ion mode of detection. When the high voltage required for the CE separation was maintained at 30 kV, the overall potential difference across the separation capillary was actually 26 kV.

Gas-phase ions generated from the ion spray interface via the ion evaporation mechanism [19] were sampled into the mass spectrometer by a potential difference of about 3 kV set between the ion spray interface and the ion sampling orifice. The sampling orifice was a 100-μm-diameter hole in the end of a conical skimmer extended towards atmosphere. To minimize solvent cluster formation a curtain of ultrapure nitrogen was applied at the atmospheric side of the skimmer. For full-scan CE–MS work the first quadrupole (Q1) was scanned from either m/z 250 or m/z 350 to m/z 850 in about 5 s with a mass scan step of 1 dalton. For CE–MS–MS work, ultrapure argon (AIRCO, The BOC Group, Murray Hill, NJ, USA) was used as the collision gas and was introduced into the collision cell (Q2) at a target gas thickness of $200 \cdot 10^{12}$ atoms/cm^2. The collision energy used for all MS–MS experiments described was 70 V.

Methods

Rinsing or conditioning of the capillary was performed by pressurizing the inlet vial to 20 p.s.i. in system B or by the built-in vacuum system at 10 in.Hg in system A. The latter was not deemed practical for connection to the ion spray interface due to the need to disconnect the capillary end from the ion spray interface during the capillary rinsing and sample injection process. New capillaries were flushed with 15 column volumes of 1 *M* sodium hydroxide, 5 column volumes of distilled water, 5 column volumes of 0.1 *M* hydrochloric acid, 5 column volumes of distilled water and finally with 10 column volumes of electrophoretic buffer. Between analyses the capillaries were usually either rinsed with 2–5 column volumes of 0.1 *M* sodium hydroxide followed by 8–10 column volumes of buffer or only with buffer. In some experiments the sodium hydroxide solution was replaced by 1 *M* NH_4OH.

The buffers were prepared from an aqueous solution of ammonium acetate. The pH of these solutions was adjusted to pH 5.0 with acetic acid or to pH 2.5 with trifluoroacetic acid (TFA). The final concentrations in this work were related to the total concentration of ammonium acetate. The electrophoretic running solutions were filtered through a 0.45-μm Miller-HV filter unit prior to use (Millipore, Bedford, MA, USA).

For CE–MS work one bottle of the synthetic peptide test mixture was dissolved in 50 μl of 1 m*M* ammonium acetate buffer to give a concentration of 500 μg/ml of each peptide. For CE–UV work one bottle of the synthetic peptide mixture was dissolved in 500 μl of 1 m*M* ammonium acetate buffer. Sample volumes ranging from 6 to 32 nl were introduced into the column using the automatic injection system. In system B the injection was made by pressurizing the inlet side of the capillary to 0.5 p.s.i. while a vacuum of 5.0 in.Hg applied at the capillary exit end was used to make the injection in system A.

Human hemoglobin was digested with TPCK-treated trypsin for 18 h at 37°C. A substrate-to-enzyme ratio of 50:1 (w/w) was used in 50 m*M* ammonium bicarbonate buffer solution where the pH had been adjusted to pH 8.5 with 1 *M* ammonium hydroxide. The digestion was stopped by adding acetic acid to the solution and the sample solution was kept at −20°C until CE–MS analysis was performed. The concentration of the hemoglobin tryptic digest was 2.5 mg/ml (154 pmol/μl). The injection was made by means of electromigration with a voltage of 10 kV and an injection time of 20–25 s.

The electroosmotic flow, v_{eo}, was calculated by measuring the migration time, t_0, from an electropherogram containing a neutral marker which was either benzyl alcohol or mesityloxide where $t_0 = V/v_{eo}$ and V is the volume of the capillary [20]. V was calculated where the capillary length (l) was the distance between the inlet end of the capillary and the UV detector; $l = 20$ cm in the CE–MS mode and $l = 50$ cm for the off-line work with the UV detector only. The calculation for column efficiency was based on $N = 5.54\ (t/w_{0.5})^2$, where N is the number of theoretical plates, t is the migration time for the compound and $w_{0.5}$ is the peak width at half peak height.

The extent of bulk flow generated by a deliberate "siphon" effect was investigated by elevating the capillary inlet (anode) relative to the buffer surface level in the liquid junction reservoir. The bulk flow was measured by moving the capillary inlet to an aqueous solution of benzyl alcohol while monotoring the "breakthrough" curve with UV detection at 210 nm and with the ion spray interface in operation. When the

surface of the electrode solution at the anode was at the same level as the surface of the makeup liquid in the liquid junction at the cathode, no "breakthrough" curve was observed over a period of 15 h. These conditions were normally used during CE–MS work reported herein.

The ion spray interface was positioned within 1 cm of the conical ion sampling orifice of the atmospheric pressure ionization mass spectrometer. The position of the spray from the ion spray interface relative to the ion sampling orifice of the API-MS system in addition to mass spectrometric tuning parameters were optimized by pressurized delivery ("infusion") of a bradykinin solution ($2 \cdot 10^{-4}$ *M* dissolved in the electrophoretic buffer) through the capillary using the 0.5 p.s.i. pressurized injection feature of system B. The doubly charged ion at m/z 531 for bradykinin was monitored while these parameters were adjusted to give optimal ion current signal from the API-MS system. The mass resolution for both Q1 and Q3 was maintained such that the ion peak width at half height was 1 dalton throughout this work.

RESULTS AND DISCUSSION

CE with UV detection

A synthetic mixture of nine model peptides was selected for the investigation of changes in electrophoresis conditions needed to connect a commercial CE instrument originally designed for UV detection to our liquid junction coupling ion spray API-MS system. Buffer compositions were studied which contained additives known to be favorable for good ion spray API-MS performance. The peptide mixture could be separated using 20 m*M* ammonium formate buffer adjusted to pH 2.5 with TFA. The UV electropherogram is shown in Fig. 1A. The first peak in the electropherogram in Fig. 1A–D is the positively charged heptapeptide marker used in system A to relate migration times to the electroosmotic flow at low pH. This was done by determining the electrophoretic mobility of the peptide standard in the buffer system used compared against a neutral marker [21]. The separation was made in an uncoated 50-μm I.D. fused-silica capillary with UV detection at 200 nm using system A. Although the peptide solution contained 50 μg/ml, only 6 nl were injected into the CE column corresponding to a sample loading of 0.3 ng or 0.18–0.97 pmol of each peptide.

The electropherogram in Fig. 1A is comparable to that obtained for the same peptide mixture using sodium citrate buffer at pH 2.5 [22]. We have used ammonium formate because this buffer is volatile and more compatible with ionization under electrospray conditions. The separation may be maintained with an increased percentage of methanol while holding the buffer concentration constant. The UV electropherograms resulting from using 5, 15 and 25% methanol in the electrophoresis buffer are shown in Fig. 1B–D. The ammonium formate concentration in the buffer was maintained at 20 m*M* in this experiment. The migration times increased for the peptides with increased methanol composition in the buffer. The applied voltage was 0.375 kV/cm capillary. The current dropped from 36 μA without methanol (Fig. 1A) to 21 μA with 25% methanol (Fig. 1D) in the buffer. No change in migration order for the peptides was observed. The additional methanol favors an increase in the ion current response in the API-MS system (*vide infra*). An electroosmotic flow of 0.02 μl/min was measured under conditions shown in Fig. 1A. When the content of methanol in the buffer increased, the electroosmotic flow decreased. The same observation

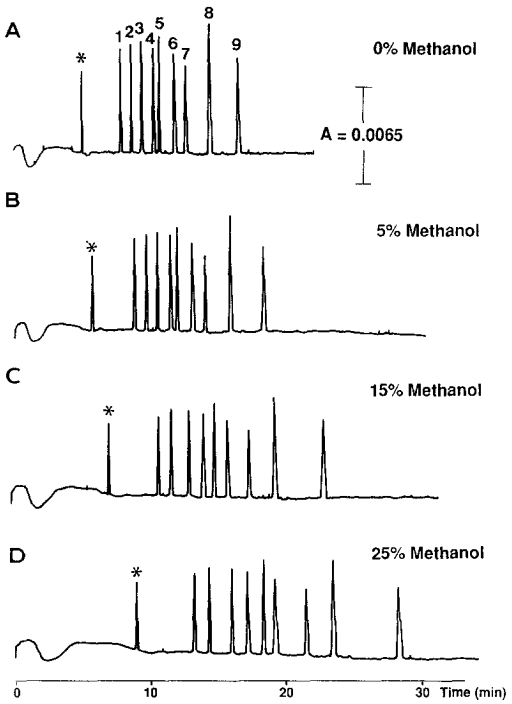

Fig. 1. CE–UV electropherogram of a synthetic peptide mixture. Capillary: uncoated fused-silica 72 cm × 50 μm I.D., 50 cm to the UV detector; detection: 200 nm; temperature: 30°C; voltage: 27 kV; buffer: 20 m*M* ammonium formate with TFA to pH 2.5 with 0–25% methanol; injection: 6 nl of a 50-μg/ml solution (0.3 ng or 0.18–0.97 pmol) of each peptide. Peaks: * = peptide standard; 1 = bradykinin; 2 = angiotensin II; 3 = α-MSH; 4 = TRH; 5 = LHRH; 6 = oxytocin; 7 = leucine enkephalin; 8 = methionine enkephalin and 9 = bombesin.

has been described by Altria and Simpson [23]. They also found that a methanol content higher than 25% reduced the buffer heat capacity enough to cause the methanol to boil within the CE capillary whereupon an electrical current breakdown in the system occurred. We observed the same phenomenon.

Changes for API-MS detection

There were some other changes required in the CE system which must be considered to integrate the CE system successfully with the present API-MS instrumentation. The overall capillary length must extend beyond the UV detector to facilitate connection to the API-MS system. The capillaries used for API-MS in this work were 100–104 cm long. Mass spectrometric detection of the substances was made at the exit end of the capillary via the liquid junction–ion spray interface so the migration distance for the substances was at least twice that with UV detection (50 cm in Fig. 1A–D). Another change implemented for CE–MS was the use of a wider-bore capillary. Thus, a 75-μm I.D. CE capillary was used in contrast to earlier reports using UV or fluorescent detection [1,21]. A wider-bore capillary increases the sample capacity of

the separation column which facilitates API-MS detection of mixture components. The increased capillary column diameter also reduced the separation efficiency as would be expected.

CE–MS analysis of the standard nine-component peptide mixture using 15% methanol in 15 m*M* ammonium acetate buffer, pH 2.5, with 15% methanol is shown in Fig. 2A with the API-MS system operated under selected-ion monitoring (SIM) conditions. The amounts injected were 3–16 pmol for each compound while the most abundant protonated molecule ion for each of the nine peptides was monitored. The applied voltage was 0.26 kV/cm when the maximum applied voltage was 30 kV and the interface was maintained at a potential of +4 kV. The potential drop across the length of the CE capillary was thus 26 kV. There are, however, only seven peaks in the electropherogram. Oxytocin, *m/z* 504 (5-pmol sample) and leucin enkephalin, *m/z* 556 (8 pmol injected) are below the detection limits for this experiment. For reasons not yet entirely understood, ammonium acetate used in this experiment provided greater sensitivity than ammonium formate under our ion spray conditions, and was used in place of the latter. A decrease in the ionic strength of the buffer from 20 m*M* to 15 m*M* gave a two-fold increase in the response from the API-MS system. The separation deteriorated to an unacceptable condition with a further decrease to 10 m*M* buffer ionic strength.

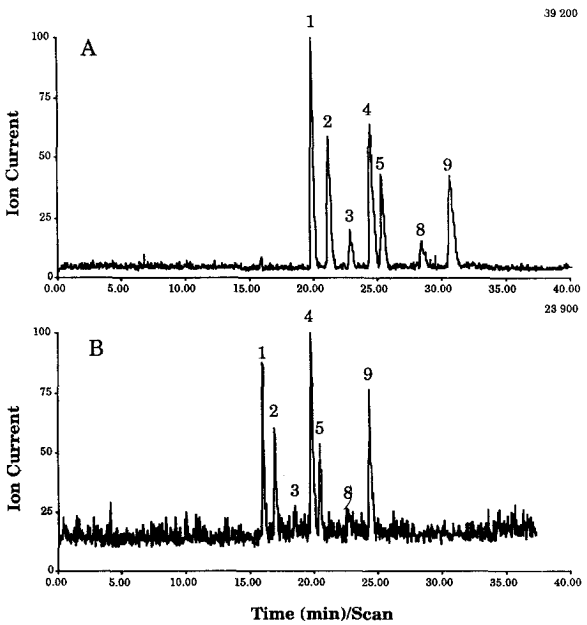

Fig. 2. SIM CE–MS total selected ion electropherogram of a synthetic peptide mixture. Capillary: uncoated fused-silica 100 cm × 75 μm I.D.; buffer: 15 m*M* ammonium acetate and TFA to pH 2.5 with (A) 15% methanol and (B) 15% acetonitrile; voltage: 26 kV; injection 10 nl of a 500-μg/ml solution of each peptide (3–16 pmol). Peaks: 1 = bradykinin, *m/z* 531 $[M + 2H]^{2+}$; 2 = angiotensin II, *m/z* 524 $[M + 2H]^{2+}$; 3 = α-MSH, *m/z* 556 $[M + 3H]^{3+}$; 4 = TRH, *m/z* 363 $[M + H]^{+}$; 5 = LHRH, *m/z* 592 $[M + 2H]^{2+}$; 8 = methionine enkephalin, *m/z* 574 $[M + H]^{+}$; and 9 = bombesin, *m/z* 811 $[M + 2H]^{2+}$.

Organic modifier

Acetonitrile as organic modifier in the separation buffer described for Fig. 2A resulted in a 25% reduction in migration times for the peptides compared to methanol-containing buffers. The comparison is shown in Fig. 2A and B with 15% methanol and acetonitrile, respectively. Peak identification is facilitated by CE–MS due to the ease of distinguishing the individual peptide components by, for example, their molecular weights. No change in selectivity was observed by varying the organic solvent in the 15 m*M* ammonium acetate buffer in Fig. 2A en B. Slightly higher electroosmotic flow was obtained with acetonitrile in the system; v_{eo} = 0.042 μl/min for acetonitrile compared to v_{eo} = 0.036 μl/min for methanol. The shorter analysis time using acetonitrile could be an advantage in certain circumstances where sample throughput is important. Acetonitrile gave sharper component peaks; *e.g.*, theoretical plates, N = 46 000–83 000, compared to N = 27 700–43 600 for methanol in the 100-cm capillary. The response, however, was about 20% lower for acetonitrile compared to methanol, and gave a poorer signal-to-noise ratio as observed in Fig. 2B. This change in response was confirmed using the pressurized injection of system B to "infuse" the sample through the capillary using bradykinin as the test analyte. The shorter analysis time for acetonitrile compared to methanol was also observed by Fujiwara and Honda [24] when they studied addition of organic solvent for the separation of positional isomers of substituted benzoic acids. They preferred acetonitrile because of the shorter analysis time and sharper peaks leading to higher sensitivity in the UV detector. The shorter analysis time is likewise a benefit for CE–MS. VanOrman *et al.* [25] studied the effect of methanol and acetonitrile on the electroosmotic flow and found that approximately 25% acetonitrile could be used in the buffer without significant decrease in electroosmotic flow. An additional advantage for using acetonitrile for CE–API-MS is that higher concentrations of acetonitrile may be used while still maintaining a stable electrophoresis system; 90% acetonitrile has been reported for the separation of acidic pesticides [14], for example.

Elevation of the capillary inlet

Migration times for the peptides could also be shortened by elevating the capillary inlet to induce a "siphon" effect for those instances of low or zero v_{eo}. This is illustrated in Fig. 3 where the same sample and buffer conditions as described in Fig. 2B were employed but the capillary inlet was elevated 8 cm. Thus the surface of the electrode solution at the anode was 8 cm higher than the surface of the makeup liquid in the liquid junction reservoir at the cathode. This created a bulk flow of 0.05 μl/min by siphoning through the capillary. The increased bulk flow "compresses" the electropherogram resulting in migration times for the peptides which are about 15% faster. Under these conditions the total-ion electropherogram in Fig. 3 was obtained for 4.5–25 pmol per component with a mass spectrometer scan range from m/z 250 to m/z 850. The buffer composition in Fig. 3 was the same as in Fig. 2B; *i.e.*, 15 m*M* ammonium acetate adjusted with TFA to pH 2.5 and 15% acetonitrile. The first peak in the electropherogram, bradykinin, was 22 s wide, so there were only 3–4 scans over the peak which makes calculation of N impractical. In this instance the "siphon" effect may be used to some degree to decrease analyses times under low electroosmotic flow conditions by increasing the bulk flow of buffer. The latter is practical provided there is adequate separation of the individual components, and the mass spectrom-

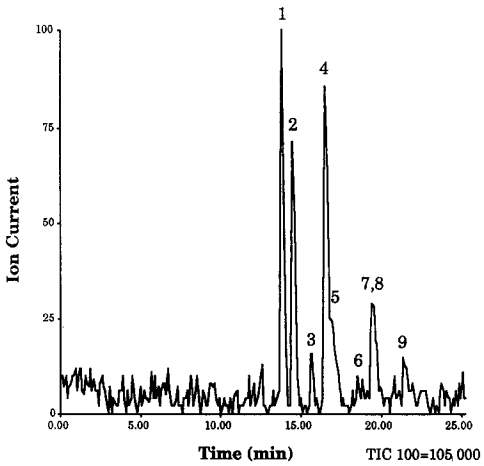

Fig. 3. TIC CE–MS reconstructed full-scan electropherogram of a synthetic peptide mixture. Capillary: uncoated fused-silica 100 cm × 75 μm I.D.; buffer: 15 m*M* ammonium acetate and TFA to pH 2.5 with 15% acetonitrile; the capillary inlet was elevated 8 cm; full-scan acquisition from *m/z* 250 to *m/z* 850, 6 s per scan; voltage: 26 kV; injection: 15 nl of a 500-μg/ml solution of each peptide (5–24 pmol). Peaks same as in Fig. 2, and 6 = oxytocin; 7 = leucine enkephalin.

eter scan rate is sufficient to provide several (8–10) scans across the CE component peaks. The data acquisition rate should be increased to accommodate the faster-eluting CE peaks, but there may be some compromise in sensitivity resulting from the increased mass spectrometric scan rate.

Separation of peptides at pH 5.0

An increase in CE sample loading may be achieved by using larger-I.D. capillaries. An experiment using a wider-bore capillary was performed at pH 5.0 because the pH 2.5 buffer gave excessively high current at the same applied voltage resulting in a breakdown in the electrophoretic process due to the heat generated inside the capillary. Fig. 4A–E shows the CE–MS ion current profile obtained from the analysis of the nine-component synthetic peptide mixture using a 100-μm I.D. capillary containing a buffer of 15 m*M* ammonium acetate–acetic acid, pH 5.0 with 50% acetonitrile. The injected amount was 9.6–52 pmol of each peptide.

The peptides possess a net positive charge at pH 5.0, but the net number of charges are different than at pH 2.5. For example, α-MSH has +3.3 charges at pH 2.5 and +2.1 charges at pH 5.0, resulting in a different migration order. The last two peptides, leucin enkephalin and methionine enkephalin, carry only +0.002 net charges at pH 5.0 as calculated with a computer program based on the aqueous acid dissociation constants for the individual amino acids [26]. Therefore, these two compounds have migration times comparable to a neutral compound. The mass spectrometric scan was from *m/z* 350 to *m/z* 850 because prior infusion experiments with these peptides have shown that their most abundant ions occur within this mass region. Fig. 4A–D shows the extracted ion current profile for the doubly charged ions at *m/z* 592, 811, 833 and 504 for LHRH, bombesin, α-MSH and oxytocin. The extracted ion electropherograms in Fig. 4A–D show that the peptides elute in bands

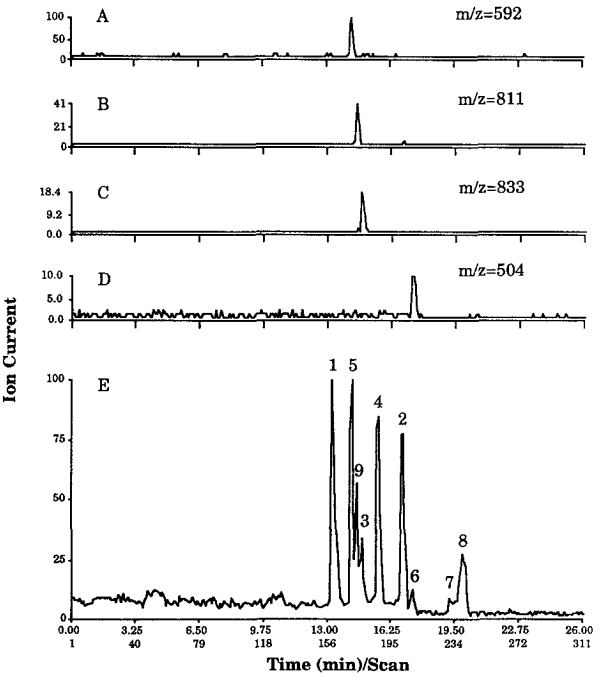

Fig. 4. Reconstructed full-scan CE–MS TIC profile (E) and extracted ion current profiles (A–D) from the analysis of a synthetic peptide mixture. Capillary: uncoated fused-silica 100 cm × 100 μm I.D.; buffer: 15 m*M* ammonium acetate and acetic acid to pH 5.0 with 50% acetonitrile; voltage: 26 kV; injection: 32 nl of a 500-μg/ml solution of each peptide (9.6–52 pmol). (A) LHRH, *m/z* 592 $[M + 2H]^{2+}$; (B) bombesin, *m/z* 811 $[M + 2H]^{2+}$; (C) α-MSH, *m/z* 833 $[M + 2H]^{2+}$ and (D) oxytocin, *m/z* 504 $[+ 2H]^{2+}$; (E) full-scan acquisition from *m/z* 350 to *m/z* 850, 5s per scan. Peaks same as in Fig. 1.

which are 17–22 s wide. The electroosmotic flow in this system was 0.4 μl/min. The smooth, stable total ion current (TIC) profile shown in Fig. 4D demonstrated the performance characteristics which may be expected from this system.

Mass spectra for the peptides at pH 5.0 and pH 2.5

The mass spectrum obtained at pH 2.5 for α-MSH shows the triply charged ion at *m/z* 556 to be the most abundant (Fig. 5A). However, at pH 5.0 the doubly charged ion at *m/z* 833 is the most abundant ion in the mass spectrum (Fig. 5B). A similar observation was made for bradykinin and angiotensin II which suggests that ions with a higher charge state may be preferred in this system under lower pH conditions. The doubly charged ions for bradykinin were the most abundant at both pH 5.0 and 2.5, but a triply charged ion with a relative abundance of 50% was also observed at pH 2.5. This is illustrated in Fig. 6A and B for bradykinin. This change in charge state could not be correlated with any particular structural feature for this peptide.

The peptides form adducts with sodium ions (Na^+) if the latter are present in the system as is illustrated in Fig. 6C for bradykinin. Since sodium hydroxide was used in the capillary rinsing procedure, it was important to change the buffer in the liquid junction before each injection. Fig. 6C shows that some residual sodium ions remained in the system after one rinsing procedure. Some attempts were made to

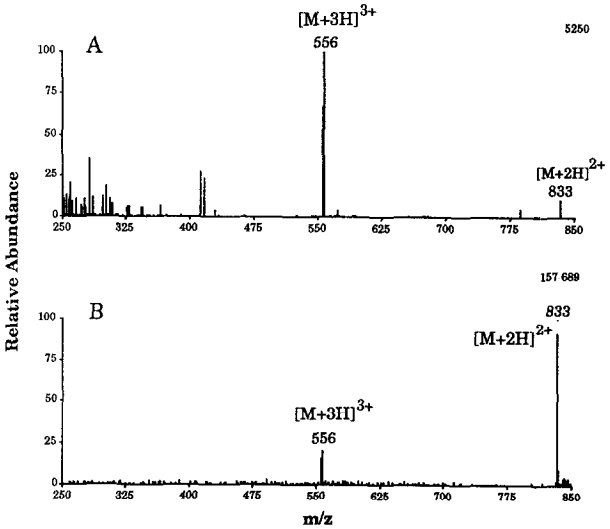

Fig. 5. Mass spectra of α-MSH. (A) pH 2.5 (conditions as in Fig. 3); (B) pH 5.0 (conditions as in Fig. 4).

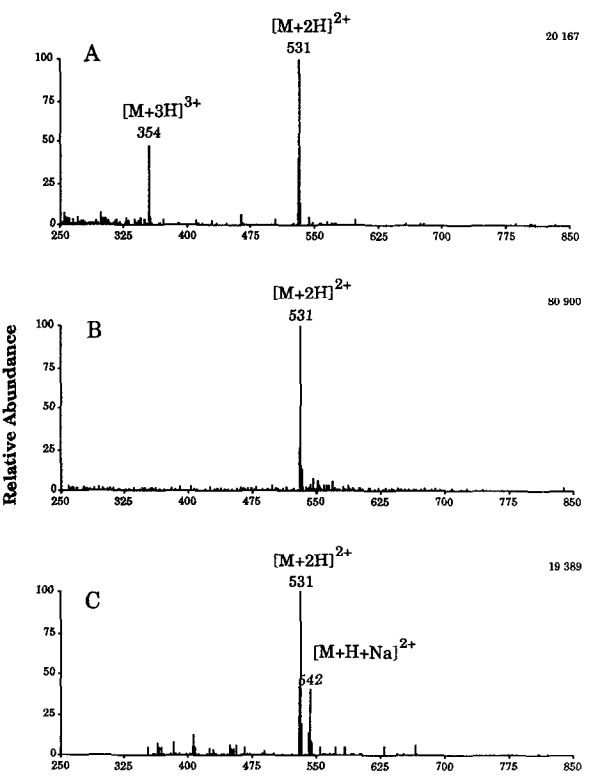

Fig. 6. Mass spectra of bradykinin. (A) pH 2.5 (conditions as in Fig. 4); (B) and (C) pH 5.0 (conditions as in Fig. 5). In (C) the buffer in the interface was not properly changed and bradykinin formed adducts with residual sodium ions remaining from the capillary rinsing solution.

rinse the capillary with 1 *M* ammonium hydroxide instead of sodium hydroxide, but ammonium hydroxide appeared to change the capillary wall to give an increase in v_{eo} while the separation was lost after repeated rinsing with ammonium hydroxide.

Tandem mass spectrometry

Samples ranging from 10–52 pmol loaded onto the capillary at pH 5.0 contained sufficient material to obtain on-line MS–MS spectra to provide structural information for the peptides studied in this work. This is illustrated in Fig. 7 for TRH. By mass selecting the $(M+H)^+$ ion at *m/z* 363 for TRH with the first quadrupole (Q_1), performing collision-induced dissociation (CID) in the second quadrupole (Q_2), and scanning the third quadrupole (Q_3) from *m/z* 10 to 400 in 5 s, one obtains the full-scan total ion electropherogram (Fig. 7A) and its corresponding daughter-ion mass spectrum (Fig. 7B) obtained from 52 pmol of TRH. Daughter ions at *m/z* 84 and 221 are consistent with the sequence of this peptide. The immonium ions at *m/z* 70 for proline and *m/z* 110 for histidine are also observed in Fig. 7B [27]. These results demonstrate that structurally informative peptide mass spectra may be obtained by on-line CE–MS–MS using the described system. Sample loading for the capillary was 52 pmol in this experiment which is half the concentration used in earlier investiga-

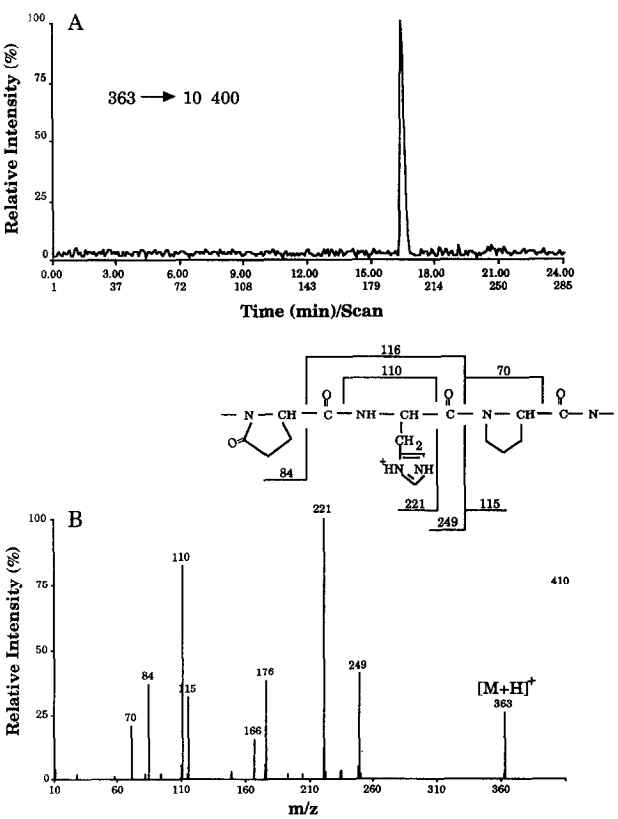

Fig. 7. CE–MS–MS total ion electropherogram (A) and daughter-ion mass spectrum (B) for 52 pmol TRH. Conditions as in Fig. 4.

tions to obtain daughter-ion mass spectra for peptides, *e.g.*, dynorphin 1–9, using microbore liquid chromatography (LC)–MS [28].

Hemoglobin tryptic digest

A total ion current electropherogram obtained from the injection of 10 pmol human hemoglobin tryptic digest is shown in Fig. 8A using a buffer consisting of acetonitrile–15 m*M* ammonium acetate 50:50 (v/v) adjusted to a pH of 5.0 with acetic acid. The sample contained both the α and β chain of hemoglobin which yields a total of 28 different peptides in the digest sample [29]. The injection was made by electromigration, 25 s at 10 kV, from a solution containing 154 pmol/μl hemoglobin tryptic digest. The mass of two tryptic fragments was too small to be detected in the scan range (m/z 250–850 u) used. The CE–MS total ion current electropherogram shown in Fig. 8A results from a buffer compromise imposed by the mass spectrometer. The electrospray conditions used in this work were optimized by using relatively low concentrations (less than 20 m*M*) volatile buffers and acids in addition to higher percentages of organic solvents such as methanol or acetonitrile than is customary for capillary electrophoresis conditions with UV or other detection. Several regions of the electropherogram show evidence for coelution of different peptides. It is, however, possible to obtain mass spectra of the different peaks. Since tryptic peptides typically yield abundant multiply protonated molecules in the scan range measured [30], we can determine their molecular weights. Extracted ion electropherograms may be obtained for individual peptides as is illustrated in Fig. 8B for tryptic fragment T-3

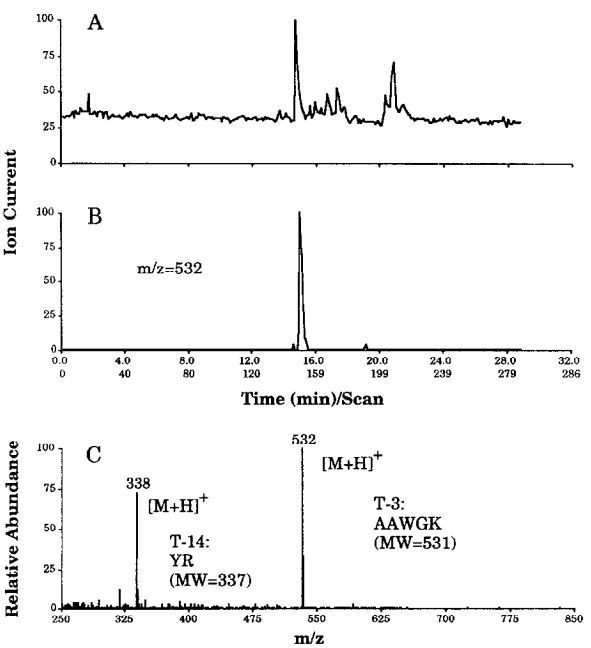

Fig. 8. Full-scan CE–MS TIC profile (A), extracted ion profile for m/z 532 (B) and mixed mass spectrum of T-3 and T-14 (C) from the analysis of 10 pmol human hemoglobin tryptic digest. Injection: 25 s at 10 kV of a 154 pmol/μl solution. Conditions as in Fig. 4. One-letter amino acid codes; MW = molecular weight.

at m/z 532. The appearance of an additional protonated molecule observed in Fig. 8C suggests the T-3 fragment migrates together with another fragment which was later determined to be T-14 by its molecular weight and CID mass spectrum (not shown). The mass spectrum shown in Fig. 8C reveals both peptides which appear as a shoulder in the 15-min peak observed in Fig. 8A. This ability to detect and identify coeluting components by CE–MS demonstrates the considerable analytical advantage of this technique. Comparison of the corresponding mass spectra and extracted ion profiles for particular peaks in the TIC electropherogram allows the detection and molecular-weight determination for all expected tryptic fragments in the hemoglobin tryptic digest. The two largest fragments of the α chain, T-12 with a mass of 2965 dalton and T-9 with a mass of 2996 dalton, appeared as $(M + 5H)^{5+}$ at m/z 594 and 600, respectively.

By monitoring the $(M + H)^+$ ion at m/z 532 for T-3 with the first quadrupole, performing CID in the second quadrupole, and scanning the third quadrupole from m/z 10 to 560 in 5 s, the full-scan total ion electropherogram (Fig. 9A) and the daughter-ion mass spectrum (Fig. 9B) for the T-3 fragment in 10 pmol of injected human hemoglobin tryptic digest were acquired. The daughter-ion mass spectrum shown in Fig. 9B reveals fragments corresponding to A, B and Y″ series ions [31]. In this case, less sample (10 pmol compared to 50 pmol) was used to obtain sequence information for tryptic fragments than was reported previously by microbore LC–MS [31]. However, LC–MS will typically allow injection of larger sample volumes and quantities of sample than is possible with CE–MS.

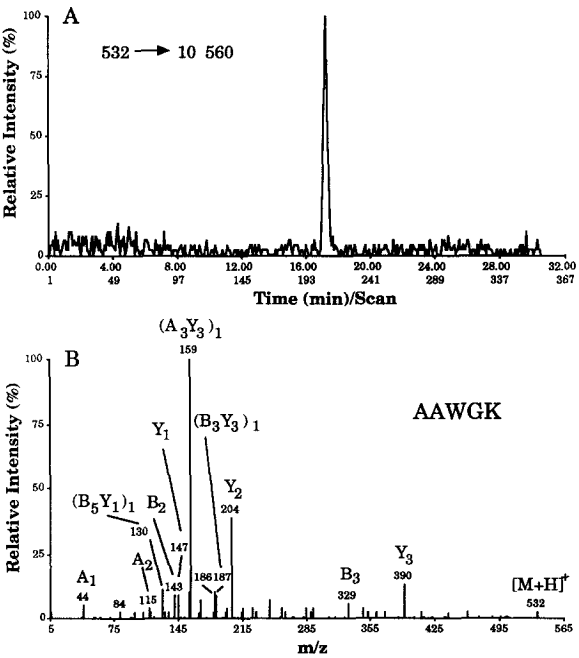

Fig. 9. Full-scan CE–MS–MS (m/z 10–560) product ion TIC electropherogram from the analysis of a human hemoglobin tryptic digest. (A) CE–MS–MS total ion electropherogram and (B) daughter-ion mass spectrum from m/z 532, T-3. Conditions as in Fig. 8.

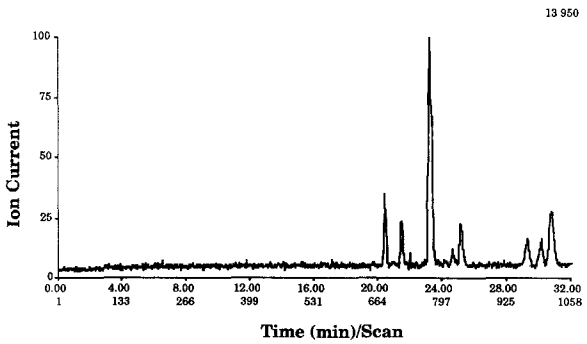

Fig. 10. SIM CE–MS total selected ion electropherogram for eight components in a human hemoglobin tryptic digest. Injection: 20 s at 10 kV of a 154 pmol/μl solution (8 pmol). Conditions as in Fig. 2A.

A SIM CE–MS analysis of the tryptic digest of normal β-chain hemoglobin was made at low pH with a 75-μm I.D. capillary and a buffer of 15 m*M* ammonium acetate adjusted with TFA to pH 2.5 and 15% methanol (Fig. 10). Eight different ions were monitored following an injection of about 8 pmol of sample. The peptides whose characteristic ions were not monitored in this SIM CE–MS experiment are of course not observed in Fig. 10. These data show acceptable separation efficiency, N = 85 000–135 000, from the on-line CE–MS analysis of a relatively low level of sample. The signal-to-noise level is good with excellent ion current stability and a minimum of peak broadening.

SUMMARY AND CONCLUSIONS

The changes which benefit MS detection with on-line CE include using a larger-I.D. capillary to increase the capillary column sample capacity, increased capillary length to connect the CE and MS instrumentation, volatile buffers with lower buffer salt concentration (less than 20 m*M*), and higher content of organic solvent than normally used for CE–UV. This study shows that it is possible to obtain useful CE–MS data for low pmol levels of peptides and a protein tryptic digest including the determination of their molecular weight and characteristic amino acid sequence information. These results also show the convenience of interfacing a commercial CE system equipped with a pressurized injection feature inlet to MS. The latter feature is particularly helpful for rinsing the capillary between analyses and for tuning the mass spectrometer using the entire CE–MS hardware.

The ion spray interface and its associated liquid junction decouples the CE capillary exit from the ion spray interface described in this report. Because the API-MS system does not require introduction of the CE capillary exit into the mass spectrometer vacuum system, both the inlet and exit of the capillary are maintained at atmospheric pressure. This arrangement avoids the extra-column band broadening and suction on the capillary exit which may be associated with the CF-FAB approach [16–18]. However, the ion evaporation ionization which takes place in the approach reported herein imposes some restrictions on the CE buffer which can compromise optimum CE separations. Future developments are needed which provide more flex-

ibility for CE buffers and more sensitive mass spectrometric detection before we may enjoy the full potential of CE–MS.

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