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Cetyltrimethylammonium chloride as a surfactant buffer additive for reversed-polarity capillary electrophoresis–electrospray mass spectrometry

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ABSTRACT

The rapid analysis of picomole quantities of various cationic molecules (laser dyes, tripeptides and larger bioactive peptides) has been achieved by on-line capillary electrophoresis–electrospray mass spectrometry (CE–ES-MS). Use of the cationic surfactant, cetyltrimethylammonium chloride (CTAC) in the CE buffer greatly facilitated the analyses. Under reversed-polarity conditions (negative potential at the source vial), CTAC induces electroosmotic flow towards the mass spectrometer, presumably due to the creation of a cationic layer on the inner surface of the fused-silica capillaries. CTAC diminishes analyte–capillary wall interactions, allowing efficient separations and symmetrical peak shapes. It may be used over a wide range of pH values without loss of electroosmotic flow. Added selectivity, provided by the surfactant properties of CTAC, played a critical role in resolving closely related tripeptides as well as larger (five to thirteen amino acid units) peptides. Above the critical micelle concentration of CTAC, interactions with the pseudostationary micellar phase increased selectivity even for ionic analytes.

INTRODUCTION

When dealing with cationic species, either singly or multiply charged, the diminution of analyte–silica wall interactions is imperative to achieving high-efficiency separations in capillary electrophoresis (CE). An effective approach in this regard is to reverse the charge on the silica surface from negative to positive by chemical derivatization procedures, or by modification of the CE buffer system. These approaches require reversed-polarity operation in order to direct electroosmotic flow towards the detector.

Recently, aminopropyl-silylated (APS) fused-silica capillaries have been used by Moseley *et al.* [1] for on-line capillary zone electrophoresis–electrospray mass spectrometry (CZE–ES-MS) analysis of peptides. The capillary modification process involves the chemical bonding of (3-aminopropyl)trimethoxysilane to surface silanol sites. This afforded a positively charged column at pH 3.4 and below, corresponding to the pK_a value for APS. The number of derivatized sites was increased by pretreating the fused-silica with strong acid [2]. The use of APS columns not only reversed the direction of electroosmotic flow relative to standard fused-silica columns (corrected by reversing the polarity), but also increased the flow-rate relative to the latter.

The rate of electroosmotic flow in standard fused-silica capillaries can also be altered by varying the composition of employed buffer systems. Moreover, this represents an alternative approach to reversing the direction of electroosmotic flow. Cationic surfactants belonging to the alkyltrimethylammonium class of compounds have proven to be particularly effective in this regard. Terabe *et al.* [3] first reported the use of cetyltrimethylammonium bromide (CTAB) which formed a positively charged layer on the inner wall of the capillary and induced the

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reversal of flow. In addition, under otherwise equivalent conditions, Altria and Simpson [4] showed that the rate of this reversed flow was one order of magnitude greater for capillaries filled with CTAB (2 m*M*), as compared to bare silica capillaries containing phosphate buffers (2 m*M*). Interestingly, a ten-fold dilution of the CTAB concentration only diminished the flow-rate by 36%. Addition of acetonitrile (1:1, v/v) to a solution of the surfactant changed the direction of flow once again, allowing normal-polarity operations at low flow-rates.

When employing surfactants in CE systems, the mechanism of separation may involve contributions from several components. Above the critical micelle concentration (CMC) of the surfactant, partitioning into the micellar pseudophase contributes significantly to overall selectivity, as practiced in micellar electrokinetic capillary chromatography (MECC) [5]. Below the CMC, analyte–surfactant interactions are more poorly defined.

The analytical utility of performing CE separations utilizing cationic surfactants has been well documented. Separation of anionic polystyrene "nanospheres" has been demonstrated utilizing the CTAB surfactant [6]. In that study, particle–capillary wall interactions were postulated to retain larger particles to a higher degree. Kasper *et al.* [7] also exploited properties afforded by CTAB (20 m*M*), including ion-pairing interactions, to efficiently separate linear DNA fragments. Selectivity was found to improve in the presence of urea (4 *M*). Liu *et al.* [8] demonstrated the separation of a series of angiotensin peptides consisting of seven to ten amino acid units in a buffer system containing dodecyltrimethylammonium bromide (DTAB). Selectivity was clearly shown to be superior when the concentration of DTAB was above its CMC value (14 m*M*), indicating that micellar interactions can play an important role in separations of charged components. Tetradecyltrimethylammonium bromide (TTAB) was used by Huang *et al.* [9] for the rapid separation of a series of low-molecular-mass carboxylic acids. Since electrophoresis and electroosmotic flow occurred in the same direction, analysis times were very short (<3.5 min).

We report the utilization of cetyltrimethylammonium chloride (CTAC) as a primary buffer system for on-line CE–ES-MS. The surfactant additive has been used to aid in the analysis of a laser dye (Rhodamine 6G), tripeptides, and larger bioactive peptides (five to thirteen amino acid units). The CMC value for CTAC has been reported to be 1.3 m*M* using the equivalent conductance method [10].

EXPERIMENTAL

Chemicals

All peptides and proteins were purchased from Sigma (St. Louis, MO, USA). Rhodamine 6G (99% purity) was purchased from Eastman Kodak (Rochester, NY, USA). Cetyltrimethylammonium chloride (CTAC) was purchased from Aldrich (St. Louis, MO, USA).

Capillary electrophoresis

Both CE–UV and on-line CE–ES-MS were performed using the Dionex CE System I (Dionex, Sunnyvale, CA, USA). For all applications reported, a constant negative voltage (reversed-polarity) was employed. Vitreous silica capillaries were used in all analyses; 100 μm I.D., 235 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Electrospray mass spectrometry

A Vestec 201 electrospray mass spectrometer (Houston, TX, USA) was employed for all mass spectral analyses. Collisionally induced dissociations were minimized in all experiments by maintaining a low skimmer-collimator voltage difference.

CE–ES-MS interface

The in-house construction and optimization of the interface has been detailed elsewhere [11]. The interface allows for the delivery of a "make-up" fluid and an electrical contact at the capillary exit, in an analogous fashion to the sheath flow set-up described by Smith *et al.* [12]. For the analysis of the tripeptides and the larger peptides, the silica tip was maintained 0.1 to 0.2 mm outside the stainless-steel needle in order to

achieve maximum sensitivity [11]. However, in the case of the laser dye (strong cation) ES-MS sensitivity was not an issue, hence, the silica capillary was maintained within the stainless-steel needle to afford greater stability [11] and to improve mixing of the CE eluent and sheath flow liquid.

Prior to each CE–ES-MS analysis, the interface was optimized by electroosmotically infusing the analyte mixture and tuning the mass spectrometer on a particular analyte ion. This exercise not only maximized the signal intensity, but also allowed operating parameters (chamber temperatures, liquid flow-rates, and applied potentials) to equilibrate. In order to maintain the optimum probe position and operating conditions, the pressure inject facility on the CE instrument was utilized to clean and fill the capillary with fresh buffer while maintaining the probe inside the mass spectrometer. All reconstructed ion electropherograms contained in this report display raw data with no post-acquisition processing.

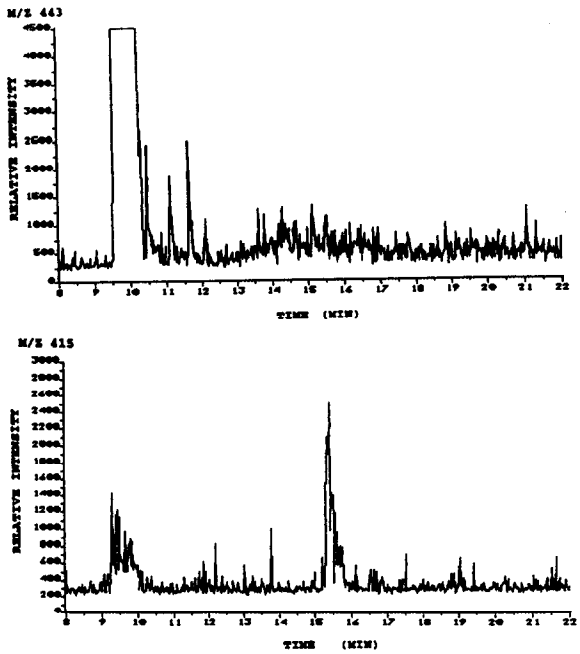

Fig. 1. Selected-ion electropherograms of m/z 443 (top) and m/z 415 (bottom) from the CE–ES-MS analysis of Rhodamine 6G (1000 ng/μl solution/64 pmol on column). Buffer: CTAC (0.5 mM) and NaCl (5 mM), pH 6.2; capillary: 100 μm I.D., 235 μm O.D., 100 cm in length; sample injection: 100 mm/10 s; CE voltage: 20 kV; ES voltage: 2.8 kV; MS scan rate: 1.21 s/scan (m/z 300 to 600).

RESULTS AND DISCUSSION

Analysis of Rhodamine 6G

Initial normal-polarity CE–ES-MS attempts to evaluate the purity of Rhodamine 6G, a widely used laser dye, were thwarted by high silica wall–analyte interactions which proved detrimental to achieving high-efficiency separations [11]. The incorporation of CTAC (0.5 mM)–NaCl (5 mM) into the buffer system was effective in generating greater efficiencies, and excellent ES-MS signals were observed for the parent ion (m/z 443) at picomole levels (6.4 pmol). The presence of isomeric compounds was also evident, although signal-to-noise ratios were poor for these low level impurities. When the same analysis was performed with greater loading (64 pmol on column) the data displayed in Fig. 1 were obtained. Even though the selected ion electropherogram of m/z 443 indicates overloading of the main component, the desired effect of increasing the ES-MS response for isomeric components as well as for analogues (m/z 415) was achieved. Clearly, three isomeric m/z 443 peaks (Fig. 1, top) eluting between 10.5 and 12.5 minutes are present in the mixture.

The appearance of two m/z 415 components (Fig. 1, bottom) having vastly different retention times was also observed. The early eluting compound is likely to correspond to a hydrolyzed form of Rhodamine 6G where the ethyl ester has been converted to the carboxylic acid form, thus reducing its positive character at the operating pH of 6.2. The zwitterionic nature of this species could be responsible for early elution. The later eluting peak (Fig. 1, bottom) may correspond to the replacement of an ethyl group with a hydrogen atom at either nitrogen.

The identity of the early eluting m/z 415 peak was further clarified by performing mild alkaline hydrolysis (NaOH, pH 8.5) of Rhodamine 6G (100 ng/μl, structure shown in Fig. 2). At given time intervals, aliquots were removed for analysis and diluted ten-fold with the operating buffer (CTAC 0.5 mM, NaCl 5 mM, pH 6.2). The

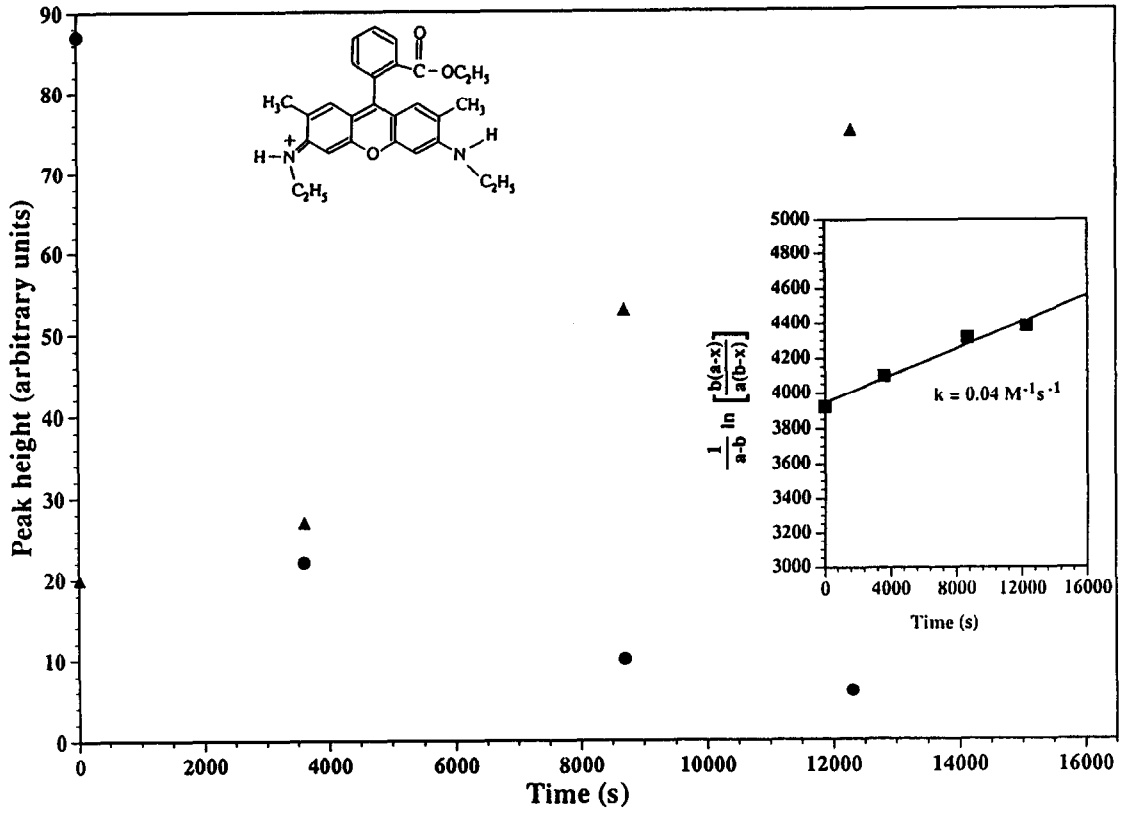

Fig. 2. Plot indicating the change in concentration of (●) the ethyl ester (structure at top) and (▲) the carboxylic acid forms of Rhodamine 6G (100 ng/μl) as functions of time under mild alkaline hydrolysis conditions (NaOH, pH 8.5). At given time intervals, aliquots were removed for analysis and diluted ten-fold with the operating buffer (CTAC 0.5 mM, NaCl 5 mM, pH 6.2). The reaction was monitored by CE–fluorescence detection (λ_{Ex}: 515 nm, λ_{Em}: 550 nm). Inset: second-order kinetics plot to determine 'k', the rate constant for alkaline hydrolysis of Rhodamine 6G: a = initial [Rhodamine 6G]; b = initial [OH^-]; x = [product].

reaction was monitored by CE–fluorescence detection (λ_{Ex}: 515 nm, λ_{Em}: 550 nm). The hydrolysis product was observed to elute just before Rhodamine 6G. Depletion of the main component (ethyl ester form) with rapid growth of the hydrolysis product (carboxylic acid form) was evident, as shown in Fig. 2. The plot of second-order kinetics expected for alkaline hydrolysis (inset, Fig. 2) yields the rate constant, $k = 0.04\ M^{-1}s^{-1}$ for the reaction. This experiment adds proof that the early eluting m/z 415 peak in Fig. 1 does represent the carboxylic acid (hydrolyzed) form of Rhodamine 6G.

Analysis of tripeptides

The ability to separate component peptides in a mixture using capillary electrophoresis with CTAC-based buffers is aided significantly by analyte interactions with the cationic surfactant. Overall selectivity can be augmented by varying the operating pH quite independently from the CTAC concentration, in order to invoke net

TABLE I

TRIPEPTIDES SUBJECTED TO CE–ES-MS USING THE CTAC BUFFER ADDITIVE

Peptide	M_r	Quantity injected (pmol)
Gly-DL-Leu-DL-Ala	259.3	64
Glu-L-Cys-L-Gly (Glutathione)	307.3	54
Gly-L-Phe-L-Leu	335.4	50
Gly-L-Phe-L-Phe	369.4	45

charge differences between peptides. Two tripeptides (listed third and fourth in Table I) were separated utilizing the CTAC (1 m*M*) buffer at pH 2.9. As the buffer concentration was below the CMC, no micelles were expected to be present. The peptides (both at 250 ng/μl) were dissolved in the same CTAC buffer (pH 2.9), ensuring both protonation of these molecules and cationic coating of the capillary wall. Single-ion monitoring electropherograms (Fig. 3) display the signals for the protonated species.

The larger tripeptide (M_r 369) was found to elute approximately 1 min before the smaller one (M_r 335). Under the employed operating conditions, both tripeptides appear to exhibit similar behavior toward acquiring charge (protonation). The smaller peptide is retained longer, indicating a higher electrophoretic mobility against the strong electroosmotic flow.

Within these CTAC systems, pH may be varied readily without losing electroosmotic flow. A CTAC buffer system (2.5 m*M*, above the CMC) was utilized at pH 4.3 to separate the four tripeptides listed in Table I, including the two referred to earlier. Displayed in Fig. 4 are the selected-ion electropherograms, representing protonated forms of peptides having molecular masses ranging from 259 to 369. Baseline resolution of all components was achieved within 8 min. Glutathione (M_r 307) was found to elute first, while the order of elution for the three remaining tripeptides was according to increasing molecular mass. The elution order for the two larger peptides is opposite to the situation illustrated in Fig. 3. Moreover, retention times are lower by approximately a factor of two, indicating changes in their net charge.

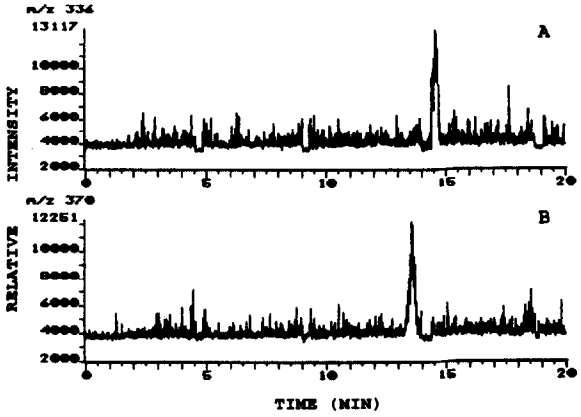

Fig. 3. Single-ion monitoring electropherograms from the CE–ES-MS of (A) Gly-L-Phe-L-Leu (MH^+, *m/z* 336) and (B) Gly-L-Phe-L-Phe (MH^+, *m/z* 370). Sample concentration: 250 ng/μl each/25 pmol and 23 pmol injected on column, respectively. Buffer: CTAC (1 m*M*) and glacial acetic acid (1%, v/v), pH 2.9; capillary: 100 μm I.D., 235 μm O.D., 85 cm in length; sample injection: 100 mm/10 s, CE voltage: 18 kV; ES voltage: 2.84 kV; MS scan rate: 0.63 s/scan.

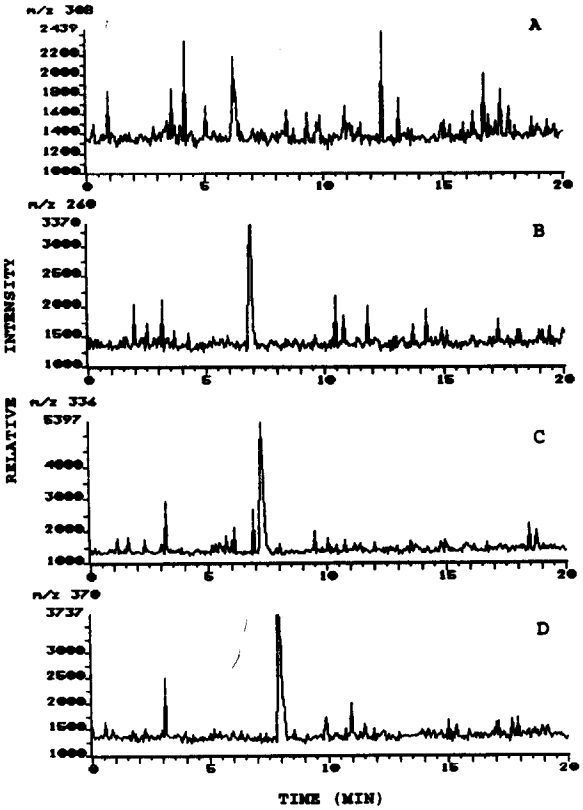

Fig. 4. Selected-ion electropherograms from the CE–ES-MS analysis of tripeptides: (A) Glu-L-Cys-L-Gly (MH^+, *m/z* 308), (B) Gly-DL-Leu-DL-Ala (MH^+, *m/z* 260), (C) Gly-L-Phe-L-Leu (MH^+, *m/z* 336), (D) Gly-L-Phe-L-Phe (MH^+, *m/z* 370). Buffer: CTAC (2.5 m*M*), pH 4.3; capillary: 100 μm I.D., 235 μm O.D., 85 cm in length; sample injection: 100 mm/10 s; CE voltage: 18 kV; ES voltage: 2.71 kV; MS scan rate: 3.1 s/scan.

The elution of glutathione prior to the other components suggests that the molecule possesses more negative character than the others, most likely originating from dissociation of its glutamic acid residue. As the concentration of CTAC was maintained above the CMC, interaction with micelles now becomes a factor in the

overall separation scheme. If the remaining three peptides exhibit overall negative character (highly unlikely at pH 4.3), then elution with increasing molecular mass could be rationalized on the basis of electrophoretic mobility. It is more plausible that the three remaining tripeptides have positive character, and that micellar interactions play the dominant role in affording selectivity. For this series, the degree of hydrophobicity of the amino acid side chains increases with increasing molecular mass, hence, interaction with the pseudostationary micellar phase is expected to increase, causing the larger molecules to be retained longer.

Off-line ES of certain peptides indicated that in the presence of CTAC, sensitivity fell by a factor of three to four relative to optimium conditions in the absence of CTAC. The CTAC buffer is being continuously infused into the ES ion source during CE–ES-MS operation, hence, the absolute sensitivity for the separated peptide zones is lower than it would have been, if the separation had been conducted in volatile buffers. A major portion of the total electrospray ion current is carried by the cationic surfactant, thus suppressing the protonated peptide signal. As depicted in Fig. 4, the signal-to-noise ratios for all peaks were satisfactory despite the presence of CTAC, which allowed the generation of mass spectrometer scan data (m/z 250 to 280 and m/z 290 to 400). The quadrupole was not scanned over the CTAC cation (m/z 284) in order to avoid "saturation" of the electron multiplier detector.

Analysis of larger peptides

A mixture of larger peptides (five to thirteen amino acid units, see Table II) were prepared in CTAC (2.5 mM, pH 4.3) and subjected to analytical conditions similar to those employed for the tripeptides. The utilization of an acidic sheath flow liquid (methanol-water-glacial acetic acid 80:10:10, pH 2.8) proved particularly favorable in increasing the analyte ion currents in comparison to the use of pure methanol. Nevertheless, it became apparent that ES-MS sensitivities for these larger peptides were not as high as those observed for the tripeptides. For this reason, the mass spectrometer was operated

TABLE II

LARGER PEPTIDES ANALYZED BY CE–ES-MS USING THE CTAC BUFFER ADDITIVE

Peptide	M_r	Quantity injected (pmol)
Leucine enkephalin	555.6	10
β-casomorphin	789.9	7
Ile-Ser-bradykinin	1260.5	5
Angiotensinogen	1644.9	4

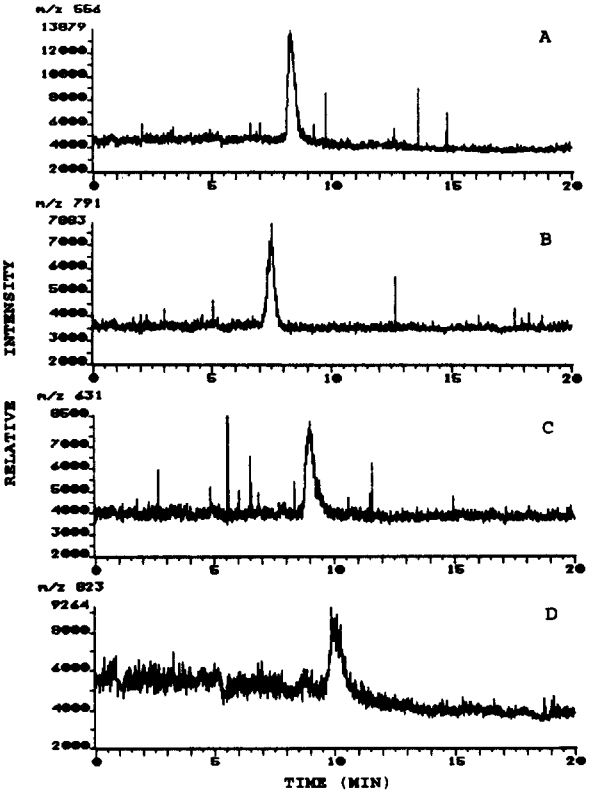

Fig. 5. Single-ion monitoring electropherograms from the CE–ES-MS analysis of larger peptides (five to thirteen amino acid units): (A) leucine enkephalin (MH^+, 556), (B) β-casomorphin (MH^+, m/z 791), (C) Ile-Ser-bradykinin [$(M+2H)^{2+}$, m/z 631] and (D) angiotensinogen [$(M+2H)^{2+}$, m/z 823]. Buffer: CTAC (2.5 mM), pH 4.3; capillary: 100 μm I.D., 235 μm O.D., 85 cm in length; sample injection: 50 mm/4 s, CE voltage: 18 kV; ES voltage: 2.84 kV; MS scan rate: 0.66 s/scan.

in the single ion monitoring (SIM) mode during on-line CE–ES-MS. Off-line ES data for the largest peptides in the series: Ile-Ser-bradykinin and angiotensinogen in the presence of CTAC (pH 4.3), afforded primarily the doubly-charged species $(M+2H)^{2+}$ at m/z 631 and m/z 823, respectively; hence, these ions were chosen to be monitored. The ion currents for leucine enkephalin and β-casomorphin were mainly in the form of the singly charged (protonated) species (m/z 556 and m/z 791, respectively).

The SIM electropherograms are displayed in Fig. 5. From a theoretical standpoint, the CE separation efficiencies are non-ideal; nevertheless, the buffer system has afforded sufficient selectivity to allow baseline resolution of all components. As with the tripeptides, the buffer pH (4.3) and CTAC concentration (2.5 m*M*) play a critical role in achieving this selectivity. The elution profile is influenced by the basicity of the component amino acids which dictate the charge state at the operating pH. The hydrophobicity of the peptide side chains also plays a role in determining selectivity via micellar interactions [8].

Since leucine enkephalin (M_r 555) elutes after β-casomorphin (M_r 790), interaction with the pseudostationary phase does not appear to be the dominant mechanism for determining selectivity (β-casomorphin has more hydrophobic substituents). The elution order is more consistent with charge-to-size considerations governing electrophoretic mobilities of similarly charged (positive) species. Ile-Ser-bradykinin and angiotensinogen (M_r 1261 and 1645, respectively) were found to elute after the smaller peptides. At the buffer pH of 4.3, these larger peptides carry more protons than the smaller species, which can result in longer retention in the column. The contribution of diffusion processes to band broadening was amplified by the long (100 cm) column length. Reducing the sample loading to improve the efficiency was not feasible due to sensitivity limitations. Increasing the ionic strength of the buffer would also have improved efficiencies, but instability caused by increased conductance down the capillary precluded this possibility.

CONCLUSIONS

The presence of the quarternary ammonium surfactant CTAC in the CE buffer effectively reduces adsorption of cationic species onto the interior of the fused-silica wall while also providing a strong electroosmotic flow. The latter is particularly important for on-line CE–MS where the use of unusually long capillaries may be required, thus increasing analysis times. The non-bonded cationic surfactant layer is constantly regenerated by the buffer system, thus diminishing the problem of fouling of the interior of the capillary, and enabling excellent reproducibility of electropherograms. The strong cationic nature of the surfactant enables operation over a wide pH range without losing electroosmotic flow, and without degradation of any phase. This feature enables rapid sequential analyses which can be exploited for real-time monitoring of fast reactions. No time was spent preparing bonded-phase columns which may have limited operational pH ranges (*e.g.*, pH 3.4 and below for APS columns). Slow degradation of these types of bonded-phase columns limit column lifetime and analytical reproducibility. The main drawback of the surfactant additive approach for on-line CE–MS is the sensitivity limitation caused by the bulk flow of charged reagent into the mass spectrometer ion source. Adsorption of surfactant onto the counterelectrode (nozzle) diminishes sensitivity over time, necessitating cleaning after each full day of use.

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