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Capillary zone electrophoresis of two synthetic neuropeptides: examination of detectability and resolution as a function of peptide concentration and buffer concentration

Huey G. Lee[a], Dominic M. Desiderio[a,b,c,*]

[a]The Charles B. Stout Neuroscience Mass Spectrometry Laboratory, The University of Tennessee, Memphis, Memphis, TN 38163, USA

[b]Department of Neurology, The University of Tennessee, Memphis, Memphis, TN 38163, USA

[c]Department of Biochemistry, The University of Tennessee, Memphis, Memphis, TN 38163, USA

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Abstract

The capillary zone electrophoretic behavior of substance P and methionine enkephalin, when the amount of peptide was increased at a constant injection volume, was examined using a 50-μm I.D. capillary. Peak-shape distortion (asymmetry) increased when the concentration of these two peptides exceeded 1% of the buffer concentration. Increasing the buffer concentration increased the peak height and decreased the peak width, leading to higher detectability and resolution, respectively. Peak area *versus* analyte concentration remained linear, even under the overloading conditions that distorted the peak shape.

1. Introduction

Since the publications of Jorgenson and Lukacs [1,2] in the early 1980s, capillary zone electrophoresis (CZE) has become a popular technique for the separation and analysis of peptides. The technique is based on each peptide's charge and mass parameters [3–14]. High electrophoretic resolution can be obtained using high voltage (10–30 kV). A high voltage may be applied because efficient heat dissipation can be achieved by using very narrow, thin-walled fused-silica capillaries (<100 μm I.D.). Other advantages of CZE include rapid analysis, high selectivity and high detection sensitivity, on-line detection, interfacing to a mass spectrometer (MS), long column life, low sample/reagent consumption, and automation.

The applications of CZE in biomedical research have been reviewed recently [15–17]. For many years, this laboratory has been involved in the analysis of neuropeptides extracted from biological sources, including human tissues and fluids, using multi-dimensional reversed-phase high-performance liquid chromatography (RP-HPLC) for sample preparation [18], and using radioimmunoassay (RIA) [19], MS [20], and tandem MS (MS–MS) [21] for qualitative and quantitative analyses [22]. CZE is an electrophoretic technique that may be used to substitute

* Corresponding author.

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for, or to complement, gel-permeation (GP) and RP-HPLC for the purification of biological samples prior to RIA, MS, and MS–MS detection.

In order to fully utilize CZE in biomedical research, not only its above-mentioned advantages, but also its limits must be explored. For example, in an early study of CZE, Mikkers *et al.* [23] have theorized that the ionic strength of the analyte must be $< 1\%$ that of the buffer in order to avoid distortion of the band shape. This requirement is particularly stringent, because at high analyte concentration, migrational dispersion predominates over diffusional dispersion due to boundary differences in the conductivity and in the electric field between the analyte and the buffer, leading to a distortion of the electric field and thus a distortion of shape of the analyte peak. More recently, this electrokinetic dispersion effect has been described in great detail [24]. Other factors such as injection volume, longitudinal diffusion, and analyte–wall interactions may also contribute to peak broadening in CZE [25]. In our opinion, these other factors do not present a major problem because samples can be easily concentrated and desalted (allowing the use of a low injection volume), analyte–wall interactions can be minimized using an acidic (*e.g.* pH 2.5) buffer, and longitudinal diffusion is already minimized by the use of high voltage.

Accordingly, in the present study, the analyte concentration and the buffer concentration were varied in a rational way, and the loadability (a term defined here as the loading limit that does not produce apparent peak-shape distortion) of two synthetic neuropeptides, substance P (SP) and methionine enkephalin (ME), was investigated in CZE using a 50-μm I.D. capillary. This loadability factor should be determined when analyzing samples extracted from biological tissues and fluids, because one aims to achieve maximal loading without significant loss in resolution and to maximize sample collection for further identification with methods such as MS. Thus, the effect of the amount of SP and ME loaded onto the capillary at various concentrations of each peptide was examined with respect to electropherographic peak height, peak width, and peak area, using three different buffer concentrations.

2. Experimental

2.1. Reagents and materials

SP, ME, and bovine hemoglobin (Hb) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and used without further purification. The borate buffer (pH 10) was prepared from sodium borate (Fisher Scientific Co., Fair Lawn, NJ, USA), and titrated with sodium hydroxide (Mallinckrodt, Paris, KY, USA). CZE buffers were prepared from ammonium formate (J.T. Baker, Phillipsburg, NJ, USA), and titrated to pH 2.5 with trifluoroacetic acid (TFA; Pierce Chemical Co., Rockford, IL, USA). Fused-silica capillary with 50 μm I.D. and 360 μm O.D. (155 μm wall thickness) was purchased from Polymicro Technologies, Phoenix, AZ, USA. Distilled water was used.

2.2. Instrumentation

An ISCO Model 3140 Electropherograph (ISCO, Lincoln, NE, USA) outfitted with an IBM Personal System/2 Model 30 286 computer (IBM, Armonk, NY, USA) was used. The electropherograph has four vacuum levels: two for sample injections and two for column washing. Vacuum is applied from the outlet beaker through the column to the inlet beaker and is measured as a pressure difference [(Δp (Pa)] between the column inlet (ambient atmosphere pressure) and the column outlet (vacuum). According to the manufacturer, Δp is 3.44, 6.89, 13.77, and 27.55 kPa for vacuum levels 1–4, respectively. The operator, *via* the computer, controls the length of time that the vacuum is applied. Thus, ISCO measures injection volume in units of vacuum time, or kPa s units. Vacuum level 1 is recommended for injections ≤ 50 kPa s, and for injections > 50 kPa s vacuum level 2 is used. Settings 3 and 4 are the two higher vacuum levels that are used for column washing. These instrumental vacuum settings must be calibrated (see below) in terms of the corresponding volume that is injected.

Peptides were detected by UV absorption at 200 nm using the built-in variable wavelength UV detector. Operation of the electrophero-

graph and data collection/analysis were controlled by the ISCO ICE 3.1.0. software. A Varian DMS 200 UV-Vis Spectrophotometer (Varian Associates, Sunnyvale, CA, USA) was used for absorbance measurement of the Hb solutions.

2.3. Methods

Injection volume calibration

To calibrate the injection volume, a new spectrophotometric method was developed using bovine Hb solutions and measuring absorbance at 410 nm. A standard curve was used to interpolate and extrapolate the total weight of the Hb collected in the outlet beaker from multiple vacuum injections. This total weight of Hb was converted to the corresponding volume of the Hb solution used for injections.

A Hb solution of 90 mg/ml was made by dissolving 10.6 mg Hb in 0.107 ml (calculated from its mass = 107 mg) of the buffer (100 m*M* sodium borate, pH 10). For the standard curve, dilutions of this 90 mg/ml solution were made to obtain a Hb concentration of 0.225 mg/ml, which was further diluted to prepare a series of Hb solutions of 0.112, 0.057, 0.028, and 0.014 mg/ml. (These concentrations were all determined from weighing the transferred and diluted volumes for greater accuracy.) Duplicate samples of each concentration were prepared and absorbance (A) was measured. A standard curve of A (in the range of 0–0.6 absorbance units) *versus* mg/ml of Hb was plotted, and a best-fit straight line was obtained.

For CZE vacuum injection, ten consecutively-programmed injections (repeated 5 ×; total of 50 injections) using the concentrated 90 mg/ml Hb solution at a vacuum level corresponding to 20 kPa s (as read on the computer) were performed at 26°C; this temperature, below the 30°C limit, is used to minimize sample evaporation. After each set of ten consecutive injections, the injected Hb solution was transferred to the outlet beaker, which contained 1.00 ± 0.03 ml of borate buffer, by applying high vacuum (level 4) for 10 min. For every set of ten injections, a new sample vial containing the Hb solution was used to minimize any evaporation that might have concentrated the sample. The resulting Hb concentration in the beaker after the five sets (50 injections) had been collected was determined by using the spectrophotometer and standard curve. The outlet beaker [weighed with no contents (8.388 ± 0.013 g; $n = 4$)] plus borate buffer (1 ml added) plus the solution used to transport Hb through the capillary (*ca.* 4 μl) was weighed (9.412 ± 0.020 g; $n = 4$). The weight (and thus the corresponding volume) of the buffer plus the Hb solution collected (1.024 ± 0.014 g; $n = 4$) was calculated from the difference. Knowing that volume, one readily obtains the total mass of Hb collected. That mass, divided by 50 (the total number of injections) gives the average mass of Hb in each injection. The same procedure for calibration of the injection volume was followed for the vacuum injections at 40, 80, and 160 kPa s.

Capillary zone electrophoresis

The fused-silica capillary used was 98 ± 0.2 ($n = 3$) cm long, with a 68 ± 0.2 ($n = 3$) cm distance from the injection point to the middle of the detector window. The internal volume of the capillary is *ca.* 2 μl. Visual observation of absorbance shows that, at vacuum level 4 (Δp = 27.55 kPa), the injected solvent reaches the end of the column in *ca.* 300 s. Prior to daily use, the column was preconditioned with the following sequence of solvents by applying 10 min of high vacuum (setting 4) for each solvent: water, 1 *M* NaOH, water, 0.1 *M* HCl, water, and finally the appropriate buffer for the experiment. The volume calculated for that 10 min (600 s) corresponds to *ca.* two column washes. A volatile buffer system similar to that described by Johansson *et al.* [26] was chosen so that samples could be processed further for MS detection. The buffer was 20, 50, or 100 m*M* ammonium formate, titrated to pH 2.5 with TFA. Different concentrations of the synthetic peptides SP and ME were prepared by five-fold serial dilution of the original peptide solution with 5 m*M* ammonium formate. Thus, the buffer capacity maintained the peptides positively-charged even for the most concentrated samples of SP (1.78 m*M*) and ME (4.18 m*M*). [Only the SP (1.78

m*M*) sample was incompletely (but still 80%) buffered.]

The applied voltage was 27 (±1%) kV; the current was *ca.* 20, 40, and 80 μA for the 20, 50, and 100 m*M* buffer, respectively. The temperature was regulated to 30 ± 0.5°C by the electropherograph's built-in air-circulating system.

Data analysis

Electropherograms were obtained at the appropriate UV sensitivity so that peaks were recorded within the appropriate absorbance unit full scale (AUFS). Peak height, peak width, and peak area (peak height × peak width, divided by 2) were calculated manually from the computer printouts. Peak height was correlated to absorbance units. (The absolute measurement of peak height is not important in this research, because relative values are compared). The molecular mass of SP and of ME was based on their chemical weights.

3. Results and discussion

Two general modes of sample injection are used in capillary electrophoresis (CE): hydrodynamic and electrokinetic. The former mode was chosen for this study because the injected amount can be more easily and directly quantified, whereas the latter mode gives an injection volume that is dependent on the electrophoretic mobilities of the sample components and on the ionic strength of both the sample and the sample buffer. Even though the injection volume is more easily and directly quantified *via* the hydrodynamic method, experimental quantification of the injection volume in CE is particularly difficult to achieve because the injection volumes normally used are in the low-nanoliter range. Therefore, the Poiseuille equation has often been used to estimate the injection volume hydrodynamically [9,14,27]:

$$V_c = (\Delta p \pi r^4 t)/(8\eta L)$$

where V_c is the calculated injection volume, Δp is the pressure (vacuum) gradient across the capillary length, r is the capillary radius, t is the time that the vacuum is applied, η is the viscosity of the electrolyte, and L is the capillary length [27]. All units are in the MKS system. The viscosity is temperature dependent, and at 26°C equals 0.871×10^{-3} N s m^{-2} for water [28].

It was necessary to independently and experimentally validate this approach for the calculation of the volumes for our CE instrument; thus, a new spectrophotometric method was designed to measure the amount of Hb that is injected and transported through the capillary in the 0.1 *M* sodium borate (pH 10) buffer, in which the Hb molecules should be negatively charged (in accordance with several published p*I* values of other Hbs; *e.g.* human Hb, p*I* = *ca.* 7) [29] and thus should experience charge repulsion against the negatively-charged fused-silica inside wall at this basic pH. All of the Hb injected into the capillary should thus be collected.

The plot of the standard curve (not shown) obtained from this new method produced a best-fit straight line [corresponding equation is $A = 5.14 \cdot [\text{Hb}] - 0.021$ ($r^2 = 1.000$; $n = 2$)], which was used to determine the total amount of Hb collected in the outlet beaker. Knowing the total amount of Hb collected and the initial concentration of the stock solution that was used for the injection, the injection volume was determined. The measured volume (average; $n = 2$) and the volume calculated from the Poiseuille equation [27] are plotted together in Fig. 1 for comparison. Within the range of injection vacuum levels tested (20–160 kPa s), the calculated volumes and the measured volumes both give straight-lines with similar slope and intercept values, suggesting that, for most practical purposes, the calculated volumes are good estimates of the experimentally measured volumes under these experimental conditions. Therefore, the calculated volumes at the appropriate temperature were used for the following experiments. The calculated injection volumes were 15.7 nl for SP and 3.9 nl for ME. The starting concentration of ME was higher, and thus a lower injection volume was used. In either case, the injection volume was < 1% of the capillary volume. As a rule of thumb, the injection volume should not

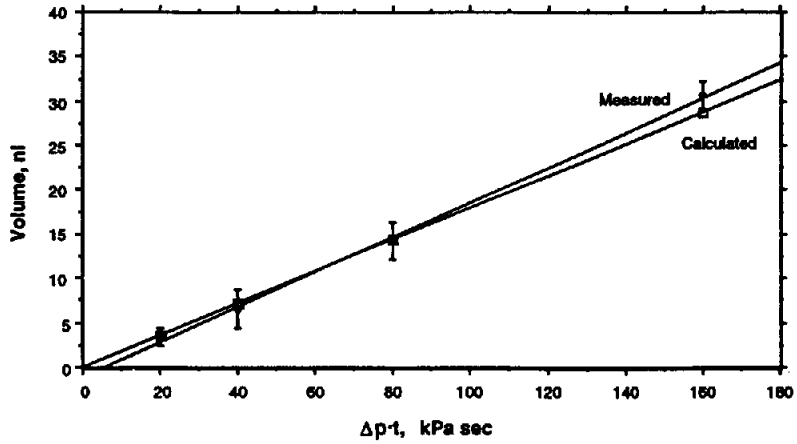

Fig. 1. The plot of injection volume *versus* ISCO kPa s units, and a comparison of the injection volumes obtained from the experimental measurements and the calculated values. The experimental measurements ($r^2 = 0.998$) represent the averages determined on two different days. The vertical bar gives the range of upper and lower values determined. The calculated volumes derive from the Poiseuille equation [27].

exceed 2% of the capillary volume [30]. Typical reproducibility of the volume obtained by vacuum injection, based on peptide peak height, was $< 8\%$ (R.S.D.; $n = 5$). This variation does not significantly affect the objectives of our study.

Fig. 2 shows the electropherograms of four different amounts of SP injected when 20 m*M* buffer was used for electrophoresis. In general, peak height decreases and peak width increases as the amount of SP increases, but not until sample No. 3 (1.12 pmol) did the peak begin to visibly deteriorate (non-symmetrical peak). These observations agree well with the theoretical consideration that the ionic strength of the analyte must be less than 1% that of the buffer to avoid any distortion of the peak shape due to migrational dispersion effects [23]. The initial SP concentration in the sample plug of the capillary, after injection Nos. 3 and 4, was $7.13 \cdot 10^{-5}$ *M* and $3.57 \cdot 10^{-4}$ *M*, respectively. One percent of the buffer concentration within the entire capillary column was $2 \cdot 10^{-4}$ *M*. According to these calculations, apparent band-broadening should occur by No. 4, as observed experimentally in Fig. 2. In Fig. 3 the electrophoretic behavior of ME was also tested using 20 m*M* CZE buffer with increasing amount of loading. Apparent peak-shape distortion is visible in electropherogram No. 4, which represents an initial ME concentration of $8.36 \cdot 10^{-4}$ *M* in the sample plug of the capillary. Again, this band-broadening

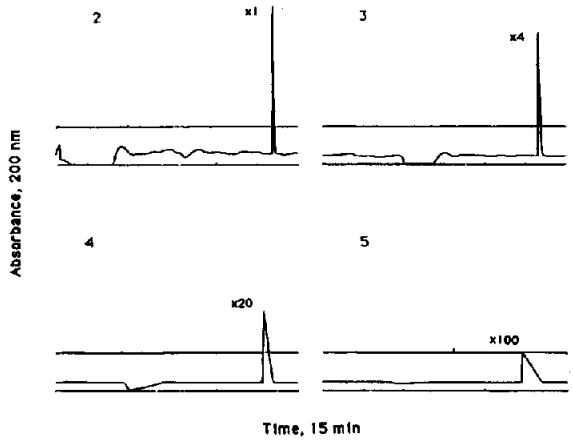

Fig. 2. The electropherograms of four different amounts of SP injected at a fixed injection volume. The calculated injection volume was 15.7 nl. The electrophoretic buffer concentration was 20 m*M*. Only the electropherograms (Nos. 2–5), representing the four highest concentrations of the five SP samples, are shown. AUFS for electropherograms Nos. 2–5 were 0.005, 0.02, 0.1, and 0.5 (see corresponding multiplication factors of ×1, ×4, ×20, and ×100), respectively. The amounts of SP injected were 0.22 pmol (No. 2), 1.12 pmol (No. 3), 5.59 pmol (No. 4), and 27.95 pmol (No. 5). The total electrophoretic time was 15 min. The horizontal line in each electropherogram represents the monitored current (*ca.* 20 μA).

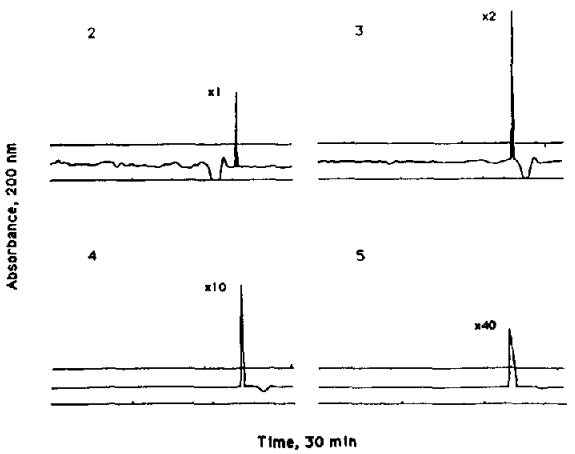

Fig. 3. The electropherograms of four different amounts of ME injected at a fixed injection volume. The calculated injection volume was 3.9 nl. The electrophoretic buffer concentration was 20 m*M*. Only the electropherograms (Nos. 2–5), representing the four highest concentrations of the five ME samples, are shown. AUFS for electropherograms Nos. 2–5 were 0.005, 0.01, 0.05, and 0.2 (see corresponding multiplication factors of ×1, ×2, ×10, and ×40), respectively. The amounts of ME injected were 0.13 pmol (No. 2), 0.65 pmol (No. 3), 3.26 pmol (No. 4), and 16.31 pmol (No. 5). The total electrophoretic time was 30 min. The horizontal line in each electropherogram represents the monitored current (*ca.* 20 μA).

occurred when ME exceeded 1% of the buffer ionic strength.

Together, Figs. 2 and 3 suggest that 1–2 pmol of a synthetic neuropeptide such as SP and ME is the practical upper loading limit before any significant peak-shape distortion and loss of resolution are observed under these experimental conditions. In contrast, the detection limit for these two peptides in our instrument using the 50-μm I.D. capillary was <40 fmol (data not shown).

Figs. 4A and B illustrate the effect of increasing CZE buffer concentration (20, 50, and 100 m*M*) on the peak height of SP and ME, respectively. As predicted, the results demonstrate the increasing detection sensitivity with higher buffer concentration [23,31] because peak heights increase, especially at the high analyte concentrations shown by Nos. 4 and 5 in Figs. 2 and 3. Higher buffer concentration should improve the stacking phenomenon (an effect that occurs when the electrophoretic velocity of an analyte is higher in the sample plug than in the electrophoretic buffer), leading to peak-height improvement in CZE [32]. Furthermore, and more importantly, increasing buffer concentration commensurately raises the 1% limit for the analyte concentration [23], and thus the loadability. The combination of these factors improves the peak height and peak shape. For SP (Fig. 4A), the use of 50 m*M* buffer increases peak heights at high analyte concentrations, compared to those obtained using 20 m*M* buffer (Nos. 4 and 5, Fig. 2). In 100 m*M* buffer, the peak heights increase further. This improvement in peak height using the 100 m*M* buffer represents a *ca.* five-fold increase in loadability, in good agreement with the theoretical consideration of Mikkers *et al.* [23] that predicts a loadability enhancement of five-fold because of the five-fold increase in the ionic strength of the buffer.

For ME (Fig. 4B), the effect of increasing the buffer concentration from 20 m*M* to 50 m*M* is less significant, and not until the highest sample concentration is a significant peak-height increase observed for the ME peak using the 100 m*M* buffer. Overall, the increased detection enhancement with higher buffer concentration was less for ME than for SP. The low injection volume (<10 nl) used for ME samples might have made the stacking effect less important [30]. The increases in the Joule heating and in the current when using higher buffer concentrations, and the relatively long migration time (*ca.* 28 min in the 50 m*M* buffer; *ca.* 32 min in the 100 m*M* buffer) of ME, might combine to offset the beneficial effect of using higher buffer concentration. Furthermore, because ME is a more neutrally-charged peptide than SP [14], the migrational dispersion due to the electric field distortion in the zone boundary might not be as pronounced.

The effect of increasing buffer concentration on the peak width of SP and ME was also examined. For SP (Fig. 5A), at low (<1 pmol) analyte concentration ($<1\%$ of the CZE buffer concentration), the peak widths cluster near each other at *ca.* 0.2 min. However, at the highest (28 pmol) analyte concentrations ($>1\%$ of the CZE

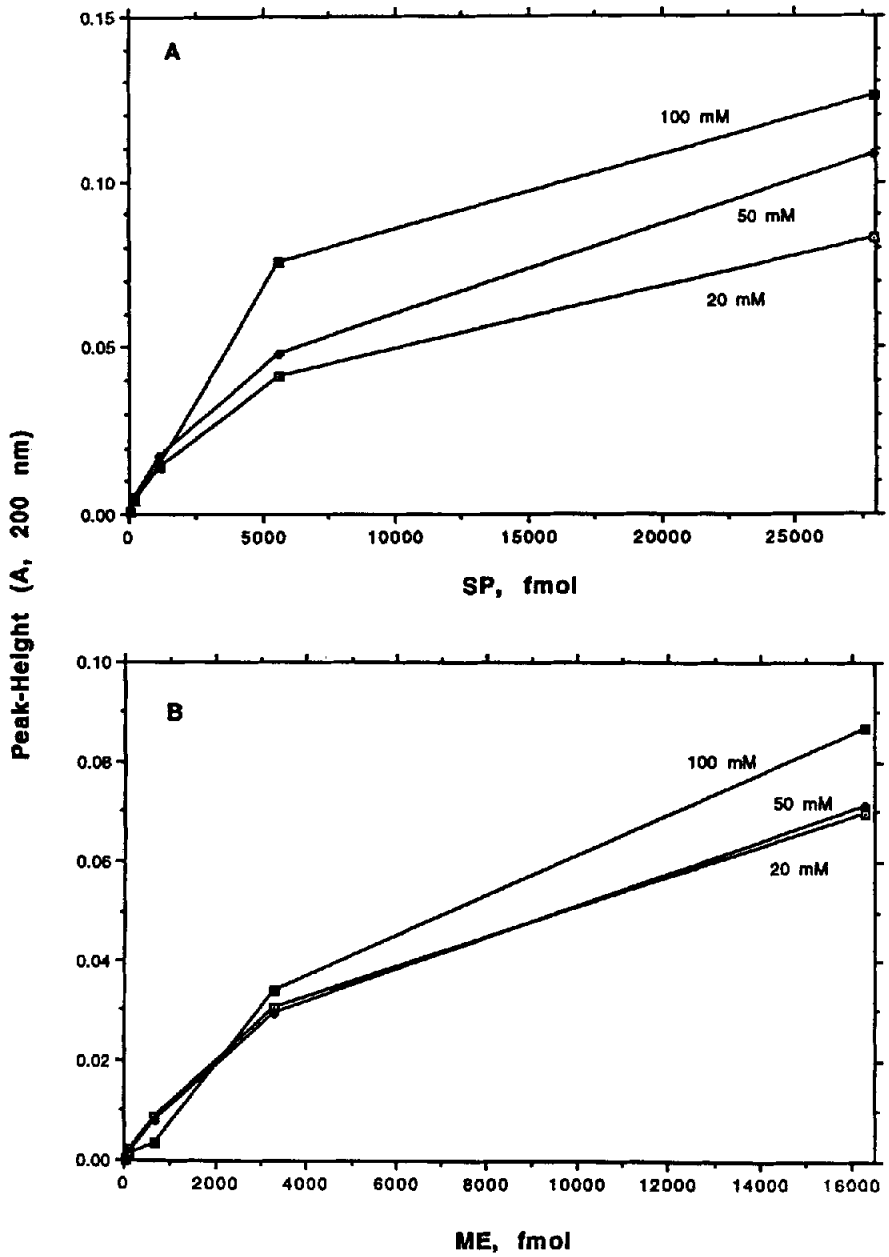

Fig. 4. Plot of peak height (as absorbance units) *versus* amount (fmol) of SP (A) and ME (B). Three buffer concentrations (20, 50, and 100 m*M*) are plotted.

buffer concentration), peak width (0.9–1.3 min) increases significantly, leading to a significant loss of resolution. The ME data (Fig. 5B) cluster at *ca.* 0.3 min peak width for a sample amount of <1 pmol. On the other hand, resolution also significantly decreases as peak-width increased to 0.8–1.05 min for the sample at 16.3 pmol. However, increasing the buffer concentration suppresses these peak-width increases, especially in the region of the highest analyte concen-

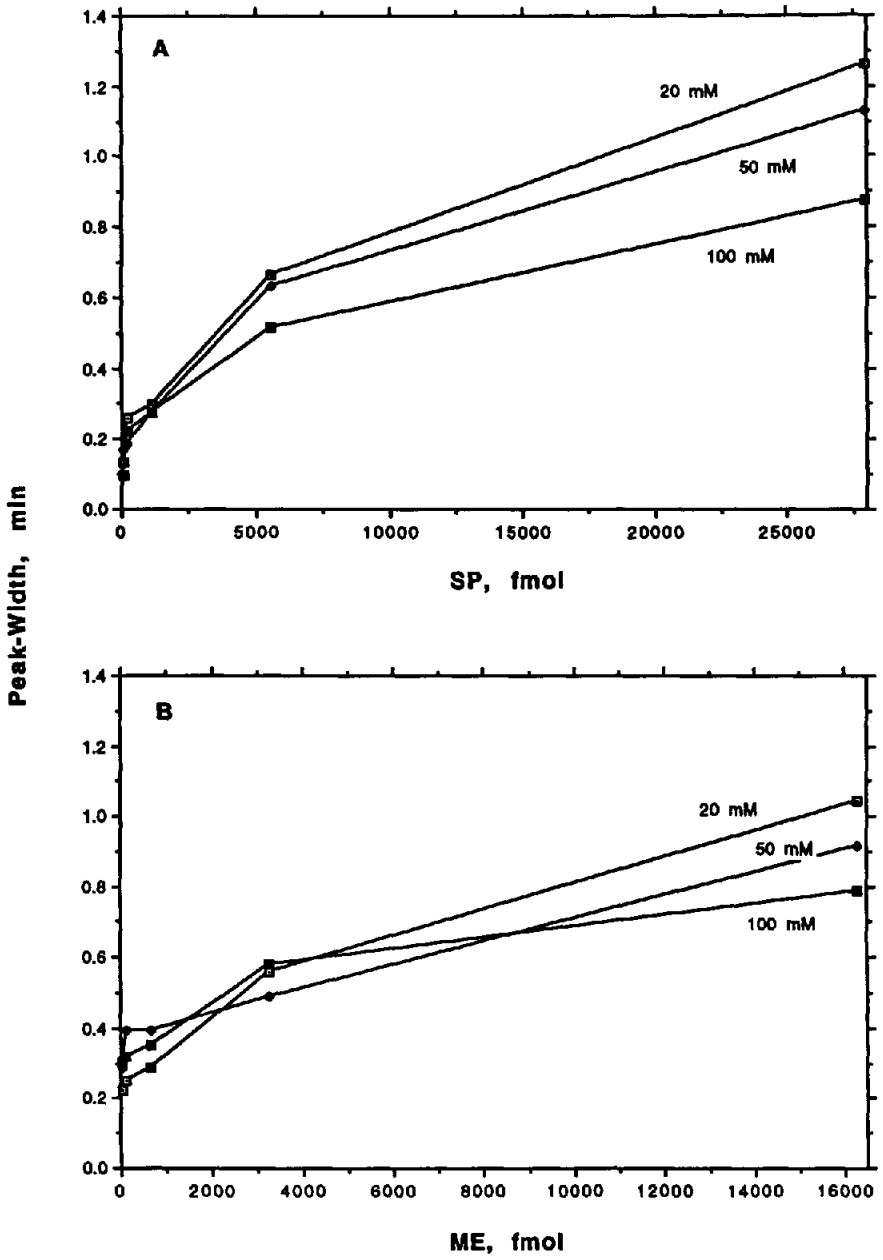

Fig. 5. Plot of peak width (min) *versus* amount (fmol) of SP (A) and ME (B). Three buffer concentrations (20, 50, and 100 m*M*) are plotted.

tration. Thus, whereas the data in Fig. 4 (peak height) demonstrate that increases in the buffer concentration improve the detection sensitivity, the data in Fig. 5 (peak width) demonstrate an improved resolution when the buffer concentration is increased.

Finally, Figs. 6A and B show that, although the buffer and the analyte concentrations have

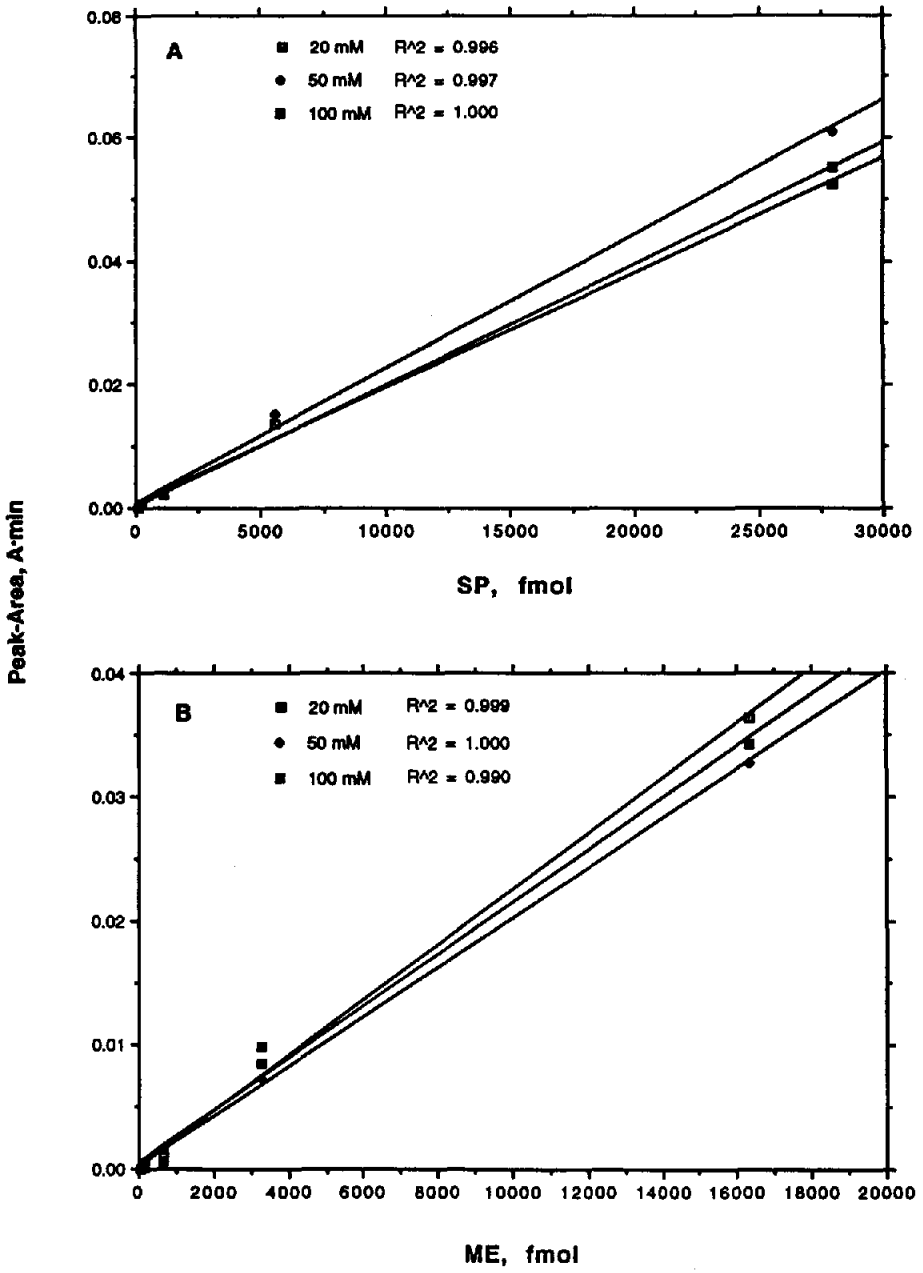

Fig. 6. Plot of peak area (absorbance × min) *versus* amount (fmol) of SP (A) and ME (B). Three buffer concentrations (20, 50, and 100 m*M*) are plotted.

significant effects on peak height and peak width, the peak area is unaffected for SP and for ME at all buffer concentrations and at all analyte concentrations. This behavior is illustrated by the linear relationship (all r^2 values ≥ 0.99) obtained between peak area and the wide range of the amount of SP or ME injected at the three buffer concentrations tested. Thus, the data in

Fig. 6 suggest that recovery appears to be high, even if the capillary column is overloaded, within the range of this study. The high recovery observed here may be attributed to the fact that the analyte–wall interaction was indeed minimal with the use of the acidic (pH 2.5) buffer. The upper loading limit when the peak area becomes non-linear was not examined, because when one aims to achieve maximal loading with a minimal loss of resolution, resolution, as represented by peak width, is the major concern.

4. Conclusions

A new method of calibrating the injection volume for CE hydrodynamic injection is described. Hb is proven spectrophotometrically to provide sufficient sensitivity for the measurement of injection volumes in the nanoliter range. The use of the Poiseuille equation for calculating the injection volume is validated empirically for our instrument and experimental protocol, thus enabling us to investigate sample loading more accurately and confidently.

In this study, the electrokinetic dispersion effect of the two selected peptides is demonstrated to be a major limiting factor in loading concentrated samples into the capillary. Our experimental observations agree well with the theoretical consideration that peak-shape symmetry degrades when the loaded analyte concentration approaches 1% of the buffer concentration. Increasing the buffer concentration should enhance directly the loadability, because peak height increases and peak width decreases, leading to higher detectability and resolution, respectively, and thus less peak-shape distortion. For example, in the ideal case, increasing the buffer concentration five-fold would correlate to a five-fold loadability while maintaining the detectability, resolution, and peak-shape symmetry. This ideal relationship was observed for the highly-charged SP peptide and to a lesser extent for the nearly neutrally-charged ME. The limit of this approach is the eventual detrimental increase in current using the high buffer concentration which results in excess Joule heating. One could also use a capillary with a larger cross-sectional area (*e.g.* 75–200 μm I.D. capillary) to enhance the loading capability, although the resolution may be less and the current will also increase. Therefore, the question of how much resolution is needed for further sample analysis, *e.g.* with MS, will influence how one approaches the loading problem. Successful preparative applications of CZE will rely heavily on achieving the maximal loading level possible.

5. Acknowledgement

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6. References

[1] J.W. Jorgenson and K.D. Lukacs, *Anal. Chem.*, 53 (1981) 1298–1302.
[2] J.W. Jorgenson and K.D. Lukacs, *Science*, 222 (1983) 266–272.
[3] F. Nyberg, M.D. Zhu, J.L. Liao and S. Hjerten, in C. Shaefer-Nielsen (Editor), *Electrophoresis '88*, VCH Publishers, New York, 1988, pp. 141–150.
[4] Z. Deyl, V. Rohlicek and R. Struzinsky, *J. Liq. Chromatogr.*, 12 (1989) 2515–2526.
[5] Z. Deyl, V. Rohlicek and M. Adam, *J. Chromatogr.*, 480 (1989) 371–378.
[6] J. Frenz, S.-L. Wu and W.S. Hancock, *J. Chromatogr.*, 480 (1989) 379–391.
[7] P.D. Grossman, J.C. Colburn and H.H. Lauer, *Anal. Biochem.*, 179 (1989) 28–33.
[8] T.A.A.M.V.D. Goor, P.S.L. Janssen, J.W.V. Nispen, M.J.M.V. Zeeland and F.M. Everaerts, *J. Chromatogr.*, 545 (1991) 379–389.
[9] E.C. Rickard, M.M. Strohl and R.G. Nielsen, *Anal. Biochem.*, 197 (1991) 197–207.
[10] J.R. Florance, Z.D. Konteatis, M.J. Macielag, R.A. Lessor and A. Galdes, *J. Chromatogr.*, 559 (1991) 391–399.
[11] H.-J. Gaus, A.G. Beck-Sickinger and E. Bayer, *Anal. Chem.*, 65 (1993) 1399–1405.
[12] V.J. Hilser, Jr., F.D. Worosila and S.E. Rudnick, *J. Chromatogr.*, 630 (1993) 329–336.
[13] H.J. Issaq, G.M. Janini, I.Z. Atamna, G.M. Muschik and J. Lukszo, *J. Liq. Chromatogr.*, 15 (1992) 1129–1142.
[14] H.G. Lee and D.M. Desiderio, *J. Chromatogr.*, 667 (1994) in press.

[15] B.L. Karger, A.S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585–614.
[16] Z. Deyl and R. Struzinsky, *J. Chromatogr.*, 569 (1991) 63–122.
[17] J.P. Landers, R.P. Oda, T.C. Spelsberg, J.A. Nolan and K.J. Ulfelder, *BioTech.*, 14 (1993) 98–111.
[18] J.L. Lovelace, J.J. Kusmierz and D.M. Desiderio, *J. Chromatogr.*, 562 (1991) 573–584.
[19] X. Zhu and D.M. Desiderio, *J. Chromatogr.*, 616 (1993) 175–187.
[20] J.J. Kusmierz, C. Dass, J.T. Robertson and D.M. Desiderio, *Int. J. Mass Spectrom. Ion Proc.*, 111 (1991) 247–262.
[21] D.M. Desiderio, J.J. Kusmierz, X. Zhu, C. Dass, D. Hilton, J.T. Robertson and H.S. Sacks, *Biol. Mass Spectrom.*, 22 (1993) 89–97.
[22] D.M. Desiderio, in D.M. Desiderio (Editor), *Mass Spectrometry of Peptides*, CRC Press, Boca Raton, FL, 1990.
[23] F.E.P. Mikkers, F.M. Everaerts and Th.P.E.M. Verheggen, *J. Chromatogr.*, 169 (1979) 1–10.
[24] G.O. Roberts, P.H. Rhodes and R.S. Snyder, *J. Chromatogr.*, 480 (1989) 35–67.
[25] X. Huang, W.F. Coleman and R.N. Zare, *J. Chromatogr.*, 480 (1989) 95–110.
[26] I.M. Johansson, E.C. Huang, J.D. Henion and J. Zweigenbaum, *J. Chromatogr.*, 554 (1991) 311–327.
[27] J. Harbaugh, M. Collette and H.E. Schwartz, Technical Bulletin TIBC-103, Palo Alto, CA, Beckman Instruments, Spinco Division, 1990.
[28] R.C. Weast (Editor), *CRC Handbook of Chemistry and Physics*, 56th ed., CRC Press, Cleveland, OH, p. F-49, 1975.
[29] D. Malamud and J.W. Drysdale, *Anal. Biochem.*, 86 (1978) 620–647.
[30] S.E., Moring, J.C. Colburn, P.D. Grossman and H.H. Lauer, *LC GC*, 8 (1990) 34.
[31] G. McLaughlin, R. Biehler, K. Anderson and H.E. Schwartz, Technical Bulletin TIBC-106, Palo Alto, CA, Beckman Instruments, Spinco Division, 1991.
[32] D.S. Burgi and R.-L. Chien, *Anal. Chem.*, 63 (1991) 2042–2041.