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Field amplified sample injection in high-performance capillary electrophoresis

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ABSTRACT

A simple on-column concentration technique in high-performance capillary electrophoresis (HPCE) is reported. In conventional electro-injection in HPCE, samples are prepared in a buffer solution which has the same concentration as that inside the capillary column. The amount of ions injected into the column under this condition is limited. By preparing samples in a low-conductivity solution, *e.g.*, water, and injecting the sample solution electroosmotically into the column, one can achieve a field enhancement at the injection point. The amount of ions injected will then be proportional to this enhancement factor. However, if one samples by switching the column directly from the high-conductivity buffer reservoir to the low-conductivity sample solution, the buffer boundary at the end of the column is disturbed and the electric field at the injection point might not be amplified properly. By injecting a short plug of water before sample introduction, one can provide a high electric field strength from the beginning of the injection. Several hundred-fold enhancements in the amount of injection were confirmed experimentally.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) has become a major analytical tool for separating charged compounds because of its high resolution capability [1–3]. To preserve the high resolution, samples must be introduced into the capillary column with the minimum volume in a very short time. The small volume of material introduced into the column makes it difficult to detect low concentrations of the material. Therefore, one of the major challenges in HPCE is to improve the injection technique to achieve high sensitivity of detection without sacrificing resolution.

There are a number of methods for injecting a small volume of sample into the column. The two principle techniques are electrokinetic and hydrostatic injection. The electrokinetic injection is performed through pumping activity from the combination of electroosmosis and electrophoresis. A bias towards more positive ions is seen because of the faster ion mobility of the species [4]. The hydrostatic injection is characterized by physically introducing samples into the capillary column and might be referred to as suction, pressure or gravity injection. The hydrostatic injection will increase zone broadening due to laminar flows generated during the injection which can degrade the separation efficiency in HPCE. Comparison of electrokinetic and

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hydrostatic injection shows that each technique has its advantages and disadvantages [5,6]. Nevertheless, the short optical path length demands a high sample concentration in all injection methods.

Several techniques have been reported for performing on-column concentration to enhance the detectability in HPCE. The sample stacking technique, which is well known in electrophoresis [7–9], was first introduced in HPCE by Mikkers *et al.* [2]. In sample stacking, a large plug of sample dissolved in water is introduced hydrostatically into the capillary. The sample ions will form into a narrow band when they migrate into the region with more concentrated background electrolyte. Moring *et al.* [10] reported an increase of a factor of 10 in detectability in HPCE with sample stacking. We have recently studied the optimization of peak variance in sample stacking in an uncoated column. We found that the peak broadening process due to the laminar flow generated by the mismatch of electroosmotic flows between different concentrations is the limiting factor in sample stacking [11].

The second on-column concentration technique is to apply isotachophoresis before HPCE. In an isotachophoretic process, the concentrations of species in their migrating zones will be adjusted to the concentration of the leading electrolyte. Several groups have combined isotachophoresis with zone electrophoresis to achieve an enhancement in signal detectability [12–15]. However, a careful choice of leading, terminating and background electrolytes is required in order to perform this isotachophoretic preconcentration step.

An alternative and simpler method for enhancement of signals is to use electro-injection with samples prepared in a highly diluted buffer or water [4,16–18]. In conventional electro-injection for HPCE, samples are prepared in a buffer solution which has the same concentration as that used in the separation. The amount of ions injected into the column under this condition is limited. However, if the sample is prepared in a diluted buffer, which has the same composition as the background buffer inside the column, an enhanced electric field strength at the injection point will exist as the high voltage is applied. This field amplified sample injection, first used in zone electrophoresis in a glass powder column by Haglund and Tiselius [16], can yield a large enhancement in the amount of ions injected into the column.

However, manipulation of the column during the injection process can produce a physical disturbance at the end of the capillary which causes improper field amplification at the injection point. In this paper, we propose the injection of a short plug of water into the column prior to sample introduction to insure proper field amplification. This field amplified sample injection with water plug can give a 100-fold enhancement in the amount of ions injected without losing the high resolution feature of HPCE. In addition to this enhancement, we also show that the peak narrowing effect due to sample stacking allows one to inject samples using a higher voltage or a longer injection time. Therefore, another order of signal enhancement compared with conventional electro-injection can be obtained.

THEORY

All of the common phenomenon in electrophoresis are based on the Kohlraush equation [19]. A complete theory of the effect of electrophoretic migration of ions on the concentration distributions in free zone electrophoresis has also been developed by

Mikkers *et al.* [20]. In this paper, we present a simplified model for electro-injection of samples prepared in a low-concentration buffer into a column filled with the same buffer of higher concentration. Our model is based on the electric field strength distributions across a pseudo-stationary boundary resulting from different buffer concentrations [21], the contribution from the sample ions being assumed to be very small.

According to Ohm's law, the local field strengths at the injection end $E^{(i)}$ and the remainder of the column $E^{(c)}$ are given by

$$E^{(i,c)} = \frac{\rho^{(i,c)} E_0}{\rho^{(i)} x + \rho^{(c)}(1 - x)} \tag{1}$$

where $E_0 = V/L$ is the field strength of a uniform system with voltage V applied across the column length L, x is the ratio of the length of the low ionic strength region inside the capillary column with respect to L and $\rho^{(i)}$ and $\rho^{(c)}$ are the resistivities in their respective regions.

In general, if different buffers have the same composition and the sample concentration is very low, the resistivity is simply inversely proportional to the buffer concentration as

$$\frac{\rho^{(i)}}{\rho^{(c)}} = \frac{C_b^{(c)}}{C_b^{(i)}} \equiv r \tag{2}$$

where $C_b^{(i)}$ and $C_b^{(c)}$ are the buffer concentrations at the injection point and in the column, respectively. For a sample prepared in water or highly diluted buffer, the effect of impurities and sample ions on the total resistivity also has to be considered.

Substituting eqn. 2 into eqn. 1, we obtain

$$E^{(i)} = \frac{rE_0}{rx + (1 - x)} \tag{3}$$

and

$$E^{(c)} = \frac{E_0}{rx + (1 - x)} \tag{4}$$

For a short injection time such that $x \ll 1$ and $rx \ll 1$, the electric field in the column changes very little from the original uniform field and the electric field at the injection point is enhanced by the factor r, thus, $E^{(c)} = E_0$ and $E^{(i)} = rE_0$. In the case of very large r such that $x \ll 1$ but $rx \gg 1$, eqns. 3 and 4 give $E^{(c)} = 0$ and $E^{(i)} = (1/x)E_0$. The field enhancement at the injection end is inversely proportional to the plug length of the low-concentration buffer.

The total amount of ion species i injected into the column is given by

$$N_i = \int_0^t AC_i^{(i)}[v_{eo}(t) + v_{epi}(t)]\mathrm{d}t \tag{5}$$

where $C_i^{(i)}$ is the concentration of ion species i in the sample reservoir, A is the cross-sectional area of the capillary, t is the length of injection time, v_{epi} is the electrophoretic velocity for ion species i and v_{eo} is the electroosmotic velocity of the bulk solution. The electrophoretic velocity for ion species i at the injection point is the product of its electrophoretic mobility and the local electric field at that point, *i.e.*, $v_{epi} = \mu_{epi}E^{(i)}$. On the other hand, the velocity of the bulk solution during injection changes by only a small amount from the electroosmotic velocity of the pure buffer system, *i.e.*, $v_{eo} = \mu_{eo}E_0$, where μ_{eo} is the electroosmotic mobility of the high-concentration buffer inside the column [21]. Thus, for $E^{(i)} > E_0$, the ions will probably be injected more rapidly into the colunn than the neutral solution.

To calculate the total ions and the plug length injected into the column using eqn. 5, a complete knowledge of the concentration distribution and the dependence of v_{eo}, $E^{(i)}$ and $E^{(c)}$ with respect to the injection time t is required. For a short injection time and very low sample concentration, those parameters could be assumed to be constant, as mentioned earlier. Hence, we can rewrite eqn. 5 as

$$N_i = C_i^{(i)}A(v_{eo} + v_{epi})t$$

$$= C_i^{(i)}A(\mu_{eo} + r\mu_{epi})E_0t \tag{6}$$

where we use the approximation $v_{epi} = r\mu_{epi}E_0$.

If the electrophoretic velocity is much faster than the electroosmotic velocity at the injection point, some of the sample ions will soon pass the buffer concentration boundary and move into the low-field region. Once the sample ions pass the concentration boundary, they will slow down and stack together to a higher concentration. Inside the capillary column, the injected sample ions themselves will now distribute into two different concentrations in the two regions separated by the buffer concentration boundary. In the region limited by the electroosmotic flow, the sample ions have the same concentration as in the original solution $C_i^{(i)}$. In the region past the concentration boundary, the sample ion concentration $C_i^{(c)}$ is enhanced by the same factor r, thus, $C_i^{(c)} = rC_i^{(i)}$ [22]. Eqn. 6 can now be written as

$$N_i = C_i^{(i)}AX_i^{(i)} + C_i^{(c)}AX_i^{(c)} \tag{7}$$

where $X_i^{(i)} = \mu_{eo}E_0t$ and $X_i^{(c)} = \mu_{epi}E_0t$ are the plug lengths of sample ions in low and high buffer concentration regions, respectively.

After sample injection, the end of the column is returned to the buffer reservoir and a high voltage is applied to perform separation. The electric field distribution inside the column follows eqn. 1. The ion species i in the plug of low-concentration region will eventually all migrate into the high-concentration region and stack together into a single zone with concentration $C_i^{(c)}$. Accordingly, eqn. 7 changes to

$$N_i = C_i^{(c)}AX_i \tag{8}$$

Neglecting the diffusion, the effective plug length, or the width of the sample zone during separation, for ion species i can be obtained from eqns. 6 and 8 as

$$X_i = (\mu_{eo}/r + \mu_{epi})E_0t \tag{9}$$

For large r, the contribution from the electroosmotic flow can be neglected and the effective plug length is simply proportional to the electrophoretic mobility.

On the other hand, the plug length using conventional electro-injection is

$$X_i = (\mu_{eo} + \mu_{epi})E_0 t \tag{10}$$

If μ_{eo} is larger than μ_{epi}, the plug length will then be dominated by the electroosmotic flow in conventional HPCE.

Comparing eqns. 9 and 10, we can see that the effective sample plug length using field amplified sample injection is narrower than the plug length using conventional electro-injection because of the stacking effect. Consequently, one can further enhance the sample introduction under the enhanced field by injecting much longer in time or at a higher voltage without much peak broadening from the longer plug length.

We have assumed that the effect of sample ions on the conductivity of the water plug and the column buffer is negligible. This is usually true for sample concentrations less than 10^{-5} M; however, for higher sample concentrations, the field enhancement factor is reduced and the assumption is no longer valid. In addition, we have assumed that the conductivities of the water plug and the sample buffer are constant during sample injection. This is obviously not true as the migration of co-ions and counter ions causes a change in either the conductivity and/or pH, which further complicates our model. A full-scale computer simulation will be necessary to perform an accurate calculation [20,23–25]. However, the simple model allows us to made several predications which are supported by experimental data.

EXPERIMENTAL

Instrumentation

Experiments were performed using an in-house constructed CE system similar to that reported by Jorgenson and Lukacs [3]. Electrophoresis was carried out in a 100 cm × 75 μm I.D. × 365 μm O.D. fused-silica capillary column (Polymicro Technologies, Phoenix, AZ, USA). A high-concentration electrolyte was supplied to the capillary column from a reservoir at the inlet end. A reservoir at the outlet end of the column collects the leaving electrolyte. Two more reservoirs were used for sample introduction, one filled with low-concentration electrolyte or water and the other with the sample solution prepared in the low-concentration electrolyte or water.

A laboratory-made electronic box was connected to the back of a high-voltage power supply (Glassman, Whitehouse Station, NJ, USA) to control the voltage between injection (−5kV) and separation (−30 kV). The high-voltage end of the power supply was connected to a platinum wire dipped into the reservoir filled with a high-conductivity buffer at the outlet end of the column. The ground end of the power supply was connected to a platinum wire at the inlet end of the column and dipped into the reservoir filled with a low-concentration electrolyte or the reservoir filled with the sample solution during injection. After sample introduction, this ground electrode end and the inlet end of the column were both dipped into the reservoir with a high-concentration buffer. The high voltage was then switched to −30 kV and the separation began.

Detection was accomplished by on-column absorption using a high-perfor-

mance liquid chromatographic UV detector (Varian, Palo Alto, CA, USA). The distance from the injection point to the detector was held at 75 cm. In addition to the optical signal, we also monitored the electrophoresis current by measuring the voltage drop across a 10-kΩ resistor in series with the capillary column. Both the optical and electrical signals were then sent to a two-channel analog-to-digital converter board in a Compaq 386 computer. Data were collected and analyzed first by the Varian LC/STAR system and converted later to ASCII files for further processing by computer programs written in-house.

Chemical and electrolytes

To reduce heating effects, a buffer of 2-N-(morpholino)ethanesulfonic acid (MES) and histidine (HIS) at pH 6.2 was chosen for our study. A stock solution of 100 m*M* with respect to both MES and HIS was prepared. A stock solution containing 2.1 mg PTH-arginine and 2.0 mg PTH-histidine in 10 ml of water was prepared. The sample solution was further diluted to about 10^{-4}, 10^{-5} or 10^{-6} *M*, respectively, in both water and MES–HIS buffer. All reagents were purchased from Sigma (St. Louis, MO, USA).

RESULTS AND DISCUSSION

According to eqn. 2, the enhancement factor could easily be several hundred if one injects the sample ions prepared in water, which has very high resistivity, into the column filled with 100 m*M* MES–HIS buffer. However, a much smaller than prediced signal enhancement is usually found when one switches the column directly between the high-conductivity buffer reservoir and the low-conductivity aqueous sample solution. It is possible that during sampling the buffer boundary at the end of the column is disturbed and the electric field at the injection point may not be amplified properly. By injecting a short plug of low-concentration buffer or water before sample introduction, one definitely establishes a high electric field at the injection point from the very beginning of injection.

Fig. 1 compares electropherograms obtained using three different injection methods: (a) conventional electro-injection with $5 \cdot 10^{-5}$ *M* sample dissolved in 100 m*M* MES–HIS buffer; (b) field amplified injection with sample dissolved in water; (c) field amplified injection with a water plug in front of the sample–water. All three injections were done at -5 kV for 10 s. Peaks A and B in Fig. 1 are the two positive ions, PTH-arginine and PTH-histidine, respectively. Peak C is the neutral species and water signal. It is evident from our electropherograms that we achieved a large field enhancement at the injection point. Table I lists the peak heights of these two ions using various injection techniques; the results are normalized to the gravity injection. A two orders of magnitude signal enhancement is obtained for positive ions using field amplified sample injection with a water plug compared with the use of conventional electro-injection.

It is well known that electro-injection will introduce bias in the amount injected. As a reference, Fig. 2 shows the resulting electropherogram using gravity injection. Comparison of Figs. 1 and 2 clearly shows the bias in the amount of PTH-arginine ions relative to PTH-histidine ions. Table II lists the ratio of peak heights normalized to gravity injection of PTH-arginine to PTH-histidine ions using various electro-injections. The expected bias factor is also listed in Table II.

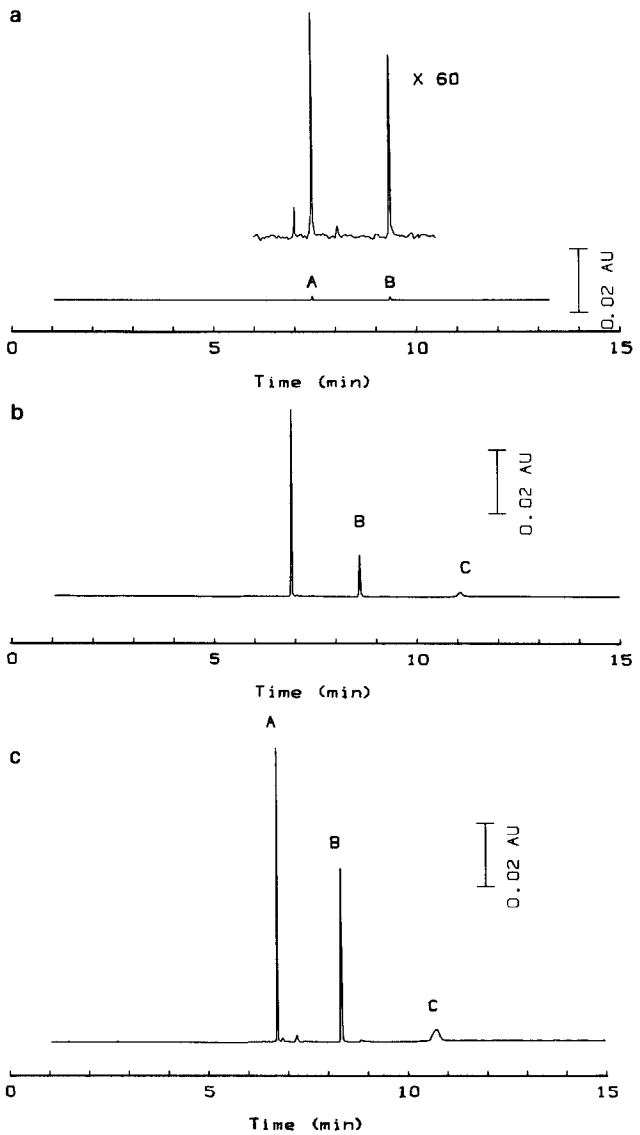

Fig. 1. (a) Electropherogram using conventional electro-injection. The column was filled with 100 m*M* MES–HIS buffer and the sample prepared in 100 m*M* MES–HIS buffer was injected into the column at −5 kV for 10 s. (b) Electropherogram using electro-injection as (a) except the sample was prepared in water. (c) Electropherogram using field amplified sample injection: a short plug of water was injected into the column first by gravity and the sample prepared in water was then injected into the column at −5 kV for 10 s. All experiments were operated with a −30-kV separation voltage. Peaks A, B and C correspond to PTH-arginine, PTH-histidine and neutral marker, respectively.

In conventional electro-injection, this bias factor is proportional to the inverse of the retention time [4]. In field amplified sample injection, as the electroosmotic velocity is much smaller than the electrophoretic velocity during injection, the bias factor is

TABLE I

COMPARISON OF PEAK HEIGHTS FOR PTH-ARGININE AND PTH-HISTIDINE IONS USING VARIOUS INJECTION METHODS

All peaks are normalized with respect to the gravity injection.

Method	PTH-arginine	PTH-histidine
Gravity injection	1	1
Conventional electro-injection sample in 100 m*M* MES–HIS	0.311	0.225
Field amplified sample injection without a water plug in front of the sample	16.96	3.38
Field amplified sample injection with a water plug in front of the sample	28.04	13.44

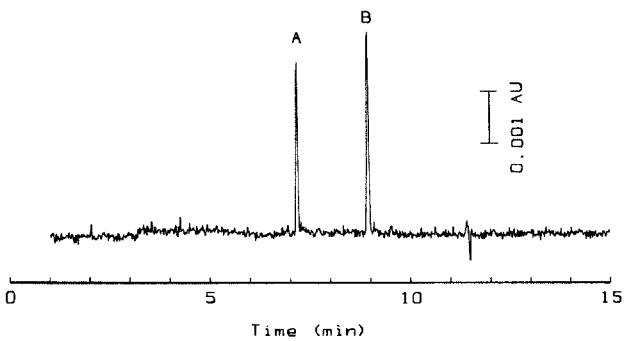

Fig. 2. Electropherogram using gravity injection. The sample prepared in 100 m*M* MES–HIS buffer was injected into the column by raising the sample reservoir to 7.6 cm high for 10 s.

directly proportional to the electrophoretic mobility, which can be calculated from the difference in retention times of sample ions and the neutral marker. Comparison of columns 2 and 3 shows good agreement between theoretical and experimental bias factors for conventional electro-injection and for field amplified sample injection with

TABLE II

COMPARISON OF BIAS FACTORS BETWEEN PTH-ARGININE AND PTH-HISTIDINE IONS USING VARIOUS INJECTION METHODS

Method	Peak A/peak B[a]	Calculated bias factor
Conventional electro-injection sample in 100 m*M* MES–HIS	1.37	1.26[b]
Field amplified sample injection without a water plug in front of the sample	5.02	2.06[c]
Field amplified sample injection with a water plug in front of the sample	2.09	2.06[c]

[a] Ratio of peak heights is normalized with respect to the gravity injection results.
[b] Calculated from the ratio of retention times.
[c] Calculated from the electrophoretic mobilities.

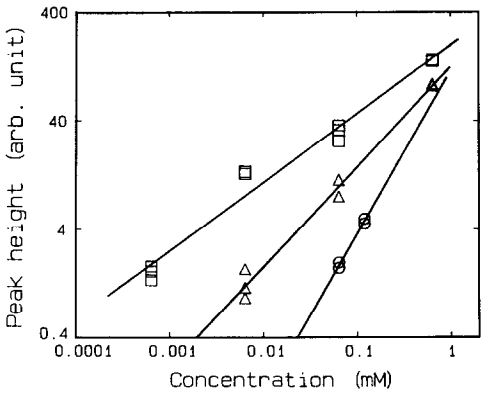

Fig. 3. Peak height of PTH-arginine ions as a function of sample ion concentration. ○ = Results from conventional electro-injection; △ = results from electro-injection with samples prepared in water; □ = results from field amplified sample injection with a short plug of water ahead of the sample.

a water plug. However, a large discrepancy exists in the bias factor for field amplified sample injection without a water plug. An obvious reason is that a large amount of arginine ions, which has an higher mobility, stacks up at the concentration boundary at the inlet of the column when there is no water plug. This high concentration of injected arginine ions will decrease the local electric field at the injection point. Consequently, the amount of injected histidine ions which has a smaller mobility will be reduced. By introducing a water plug before sample injection, we provide not only a high field strength at the injection point, but also a void region for injecting the sample.

Similarly, the sample ion concentration also has an effect on the signal enhancement. Fig. 3 shows the peak height of PTH-arginine ions at various concentrations with three different injection methods. At high concentration, the conductivity of the sample will decrease the effective electric field strength and reduce the signal enhancement. By lowering the sample concentration, an order of magnitude improvement in detection limit is obtained between field amplified sample injection without a water plug and conventional electro-injection. Another order of magnitude improvement in detection limit is obtained between field amplified sample injection with and without a water plug. Instead of $1 \cdot 10^{-5}$ M for conventional electro-injection, the detection limit for PTH-arginine is now less than $1 \cdot 10^{-7}$ M using electro-injection with a water plug.

Field amplified sample injection not only introduces a large amount of ions into the capillary column, it will also perform on-column concentration at the same time. Eqns. 9 and 10 predict that the effective injected plug length of sample ions will be even shorter than using conventional electro-injection. For a rectangular injection profile, the peak variance, σ_i, due to the effective plug length from injection, is equal to $X_i^2/12$. The total variance of a peak is then given by

$$\sigma_t^2 = \sigma_d^2 + \sigma_i^2 \tag{11}$$

where σ_d^2 is the variance due to diffusion, $2Dt$. If the final observed peak shape is

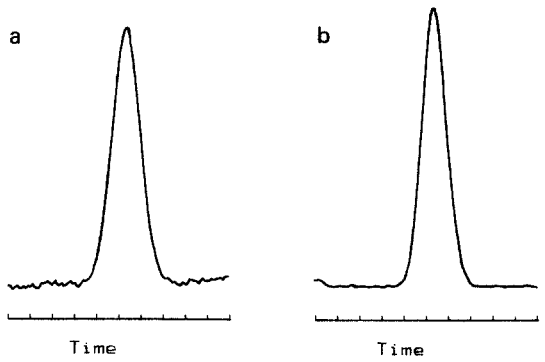

Fig. 4. Comparison of peak shape of PTH-arginine signals between using (a) conventional electro-injection and (b) field amplified sample injection, both at 5 kV for 10 s.

Gaussian, the $X_{1/2}$ (full width at half-maximum) of the peak is equal to $(5.545)^{1/2}\sigma_t$. Substituting $X_{1/2}$ and eqn. 9 or 10 into eqn. 11, we obtain

$$X_{1/2}^2 = 5.545[\sigma_d^2 + (\mu' E_0 t)^2/12] \tag{12}$$

where $\mu' = (\mu_{eo}/r + \mu_{epi})$ in field amplified sample injection and $\mu' = (\mu_{eo} + \mu_{epi})$ in conventional injection. The spatial width of a peak can also be converted to the temporal width by using

$$t_{1/2} = X_{1/2}(t_m/L_d) \tag{13}$$

where t_m is the retention time of the peak and L_d is the column length to the detector.

Eqn. 12 shows that in the case of a short injection time, the peak width in both field amplified sample injection and conventional injection will approach the diffusion limited value. As the injection time increases, the peak width in conventional injection will increase much faster than in field amplified injection. Fig. 4 is a comparison of peak shape of PTH-arginine signals between using conventional electro-injection and field amplified sample injection, both at -5 kV for 10 s. As the ratio of the absolute

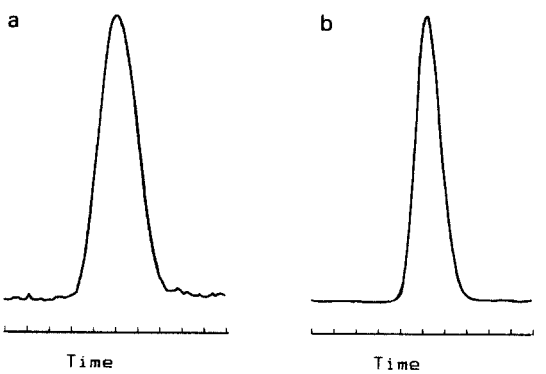

Fig. 5. Comparison of peak shape of PTH-arginine signals between using (a) conventional electro-injection and (b) field amplified sample injection, both at -5 kV for 30 s.

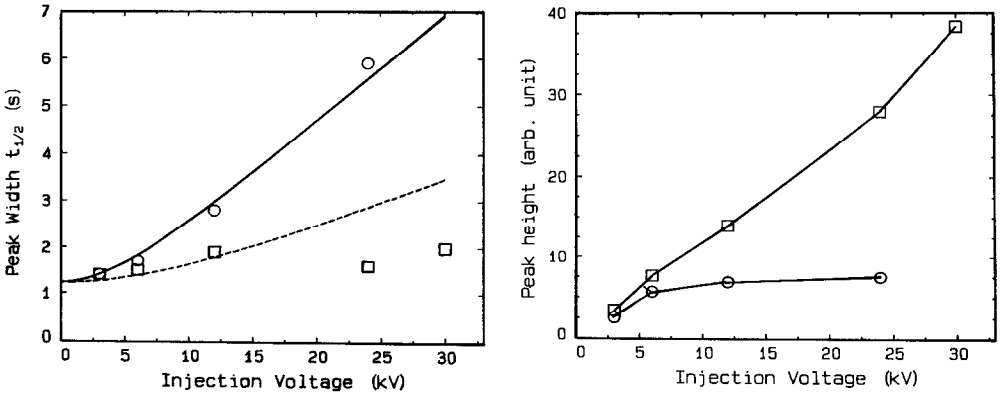

Fig. 6. Full width at half-maximum of PTH-arginine peaks as a function of the injection voltage for (○) conventional electro-injection and (□) field amplified sample injection. The solid and dashed lines were calculated using eqn. 12.

Fig. 7. Peak height of PTH-arginine peaks as a function of the injection voltage for (○) conventional electro-injection and (□) field amplified sample injection. The sample concentration in field amplified sample injection experiments is an order of magnitude lower than that in conventional electro-injection so that the peak heights are of the same order of magnitude at low voltage.

signal differs by two orders of magnitude, both peaks are normalized to their respective peak maxima. The result shows that the $t_{1/2}$ of the peak drops from 1.5 s in conventional injection to the diffusion limited peak width of 1.3 s in field amplified sample injection. As the injection time is increased to 30 s, the $t_{1/2}$ in conventional injection increases rapidly to 2.0 s as expected, while the $t_{1/2}$ in field amplified sample injection remains at 1.3 s as shown in Fig. 5. Figs. 4 and 5 clearly show that one can achieve an even larger enhancement in signal detectability while preserving high resolution just by using a longer injection time.

In addition to a longer injection time, we can also use a higher injection voltage. Fig. 6 shows the plot of $t_{1/2}$ of PTH-arginine signal peaks *vs.* the injection voltage for both conventional injection and field amplified sample injection. The solid and dashed lines are the results for conventional injection and field amplified sample injection, respectively, obtained from eqn. 12 with the mobilities calculated from the retention times. Once again, the $t_{1/2}$ in conventional injection increases rapidly from 1.4 s at 3 kV to 5.9 s at 24 kV. The $t_{1/2}$ in field amplified sample injection, on the other hand, increases very slowly at first from the diffusion-limited value of 1.3 s at 3 kV to 1.8 s at 12 kV. Then, surprisingly, the peak width decreases to 1.5 s as injection voltage increases to 24 kV. We believe that this extra narrowing effect, which is similar to isotachophoresis, is probably due to changes in buffer conductivity at high sample concentration. In field amplified sample injection at high voltage, a tremendous amount of sample ions moves into the running buffer and tries to stack up in front of the water boundary. As the sample concentration becomes higher, the local conductivity of the stacking region will increase, which causes a further decrease in the electric field strength. Consequently, the leading edge of the sample region will slow down more and further enhance the stacking effect.

As the peak width remains very narrow, the peak height will increase accordingly

as one injects more samples into the column. Fig. 7 shows the peak height *vs.* the injection voltage for PTH-arginine ions using both conventional injection and field amplified sample injection. The sample concentration in field amplified experiments is an order of magnitude lower than that in conventional electro-injection so the peak heights are of the same order of magnitude at low voltage. While the conventional injection shows a saturation in peak height beginning around 5 kV, field amplified sample injection shows a continuous increase in the peak height. Another order of magnitude increase in signal enhancement is obtained by using an injection voltage of 30 kV compared with the normal 3-kV injection.

In conclusion, simple on-column concentration in HPCE is achieved using field amplified sample injection. Large amounts of sample ions can be injected into the capillary column while the high resolution feature of HPCE is retained. In addition, only a small amount of neutral plug is introduced into the column, which means that the peak broadening caused by the laminar flow originated from the mismatch of electroosmotic velocities is minimized. By injecting a short plug of water before sample introduction, the minimum detectable concentration of sample injected is of the order of 10^{-8} *M*. This sensitivity is now comparable to the best results obtained from high-performance liquid chromatography.

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