Journal of Chromatography, 589 (1992) 315–319
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 677

Radial pH distribution during capillary electrophoresis with electroosmotic flow

Analysis with high ionic strength buffers

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(First received June 18th, 1991; revised manuscript received August 8th, 1991)

ABSTRACT

The surface of a fused-silica capillary contains ionized silanol groups when in contact with a liquid solution at pH >2. Positive counter ions obeying the Boltzmann distribution law, including H^+, are responsible for the electroosmotic flow and a radial pH gradient. The pH value at the capillary wall can be more than 2 pH units lower than the bulk pH at the tube axis. If analysis is performed at a bulk pH slightly above the isoelectric point of analyte, the pH gradient can result in a net positively charged analyte near the wall. Severe analyte peak tailing or even total adsorption on the wall can occur. The pH gradient can be reduced by increasing the ionic strength of the buffer. Results at various pH values and buffer concentrations are presented.

INTRODUCTION

The characterization of polypeptides and proteins is a very promising area of capillary electrophoresis (CE) [1–6]. These substances, however, have a tendency for adsorption on the capillary wall owing to coulombic interactions between positive moieties of the polypeptide/protein and the negatively charged fused-silica capillary surface. Severe peak tailing or even total adsorption can result. There are several ways of minimizing these "chromatographic effects" [1,4,7–10], one of which is to perform the CE analysis at pH values above the isoelectric point (p*I*) of the polypeptide/protein [4].

Above the p*I* the analyte is net negatively charged, thus being repelled from the likewise negatively charged capillary wall. However, at pH values slightly above the analyte p*I* there are two factors that can cause severe peak tailing. First, even though the analyte is net negatively charged, eventual positive moieties of the molecule are still electrostatically attracted by the fused-silica surface. Second, in all capillaries with an electroosmotic flow, a pH gradient is established between the tube axis and the wall. Hence, when analysis is performed only slightly above the polypeptide/protein p*I*, the radial pH gradient can lead to a net positively charged analyte close to the wall and thereby cause analyte adsorption.

Here we present calculations of the pH gradient as a function of electroosmotic flow and temperature. Electropherograms illustrating the use of high ionic strength buffers to suppress the radial pH gradient are also shown.

0021-9673/92/$05.00

THEORY

One of the characteristics of CE in uncoated capillaries is a strong electroosmotic flow under typical experimental conditions. Electroosmosis is usually explained by the Gouy–Chapman model as revised by Stern [11]. At pH values above *ca.* 2 the silanol groups of the fused-silica capillary wall are ionized, thus making the inner surface negatively charged. Hydrated positive counter ions in the bulk solution are responsible for the electroosmotic flow when a voltage is applied. Hence analytes are transported through the capillary tube by the combined action of electroosmosis and electrophoresis.

The positive counter ions, including H^+, are distributed as a function of radial position in accordance with the Boltzmann equation. The Boltzmann distribution of H^+ ions results in a radial pH gradient in the double layer region, hence of the order of a few nanometres from the capillary surface. A maximum concentration of H^+ ions is obtained close to the wall, whereas the maximum pH is at the capillary tube axis.

The concentration ratio of H^+ ions at the tube wall, $[H^+]_0$, to that at the tube axis (bulk pH), $[H^+]$, is calculated as

$$[H^+]_0/[H^+] = \exp(ze\zeta/kT) \quad (1)$$

where z is the number of charges (= 1), e is the elementary charge (= $1.602 \cdot 10^{-19}$ C), k is the Boltzmann constant (= $1.38 \cdot 10^{-23}$ J K^{-1}) and T is the absolute temperature. The zeta potential, ζ, is calculated by using the equation for electroosmotic mobility, μ_{EO}:

$$\mu_{EO} = v_{EO}/E = \zeta\varepsilon_0\varepsilon_r/\eta \quad (2)$$

where v_{EO} is the electroosmotic velocity (cm s^{-1}), E is the electric field strength (V cm^{-1}) and ε_0 is the permittivity of vacuum (= $8.854 \cdot 10^{-12}$ C^2 J^{-1} m^{-1}). The relative permittivity, ε_r, is calculated as [12,13]

$$\varepsilon_r = 309.78\exp(-0.004605T) \quad (3)$$

while the viscosity, η, is approximated by [12]

$$\eta = (1/800)\exp(1958/T) \quad (4)$$

Combining eqns. 1–4 yields the pH gradient from the capillary tube axis to the wall:

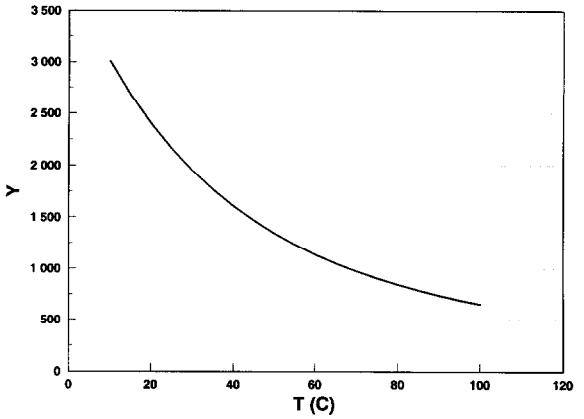

Fig. 1. $Y = \Delta pH/\mu_{EO}$ *vs.* temperature (T, °C). Y is calculated using eqn. 5b. See the text for details.

$$\Delta pH = \log([H^+]_0/[H^+]) = \frac{528.67\exp(1958/T + 0.004605T)}{\ln(10) \cdot T}\mu_{EO} \quad (5a)$$

$$\Longleftrightarrow$$

$$\Delta pH = Y(T)\mu_{EO} \quad (5b)$$

Hence ΔpH is proportional to μ_{EO}, the proportionality constant Y being a function of the temperature, T.

$Y(T)$ is depicted in Fig. 1, and Fig. 2 shows ΔpH *vs.* μ_{EO} at four different temperatures. The pH gradient decreases at decreasing μ_{EO} values (a low bulk pH and/or a high buffer ionic strength suppresses the electroosmotic flow) and increasing

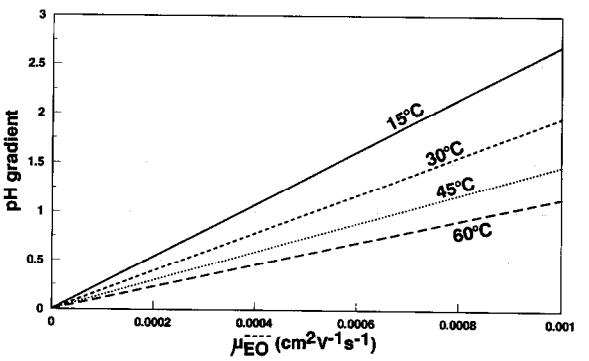

Fig. 2. Radial pH gradient (ΔpH) *vs.* the electroosmotic mobility (μ_{EO}) at four selected temperatures. ΔpH was calculated using eqn. 5a. Under typical experimental conditions such as μ_{EO} = 0.0006 cm^2 V^{-1} s^{-1} at 30°C, ΔpH exceeds unity. See the text for details.

temperatures. It is interesting that pH gradients exceeding unity are often met under typical experimental conditions such as $\mu_{EO} = 0.0006$ cm^2 V^{-1} s^{-1} at 30°C.

EXPERIMENTAL

Biosynthetic human growth hormone (B-hGH) was obtained from Novo Nordisk (Gentofte, Denmark), 2-(N-morpholino)ethanesulphonic acid (MES) from Sigma (St. Louis, MO, USA) and N-[tris(hydroxymethyl)methyl]glycine(tricine) from Fluka (Buchs, Switzerland). A 50 μm I.D. × 192 μm O.D. capillary from Polymicro Technologies (Phoenix, AZ, USA) was cut to a 50-cm total length; the effective length from the introduction end to the detector was 25 cm.

All experiments were performed on an Applied Biosystems Model 270A CE instrument. Except for the electropherogram in Fig. 8, the experimental conditions were as follows. A 0.5 mg/ml B-hGH sample was introduced in 2.0s by means of a 16.8-kPa vacuum; 8 kV was applied during analysis (160 V/cm). Detection was accomplished at 200 nm with a 0.5-s detector rise time. The capillary tube was thermostated at 30°C.

Three experimental series were performed, with different pH values of the MES running buffer. In series 1 the buffer solution had a pH of 6.5 and the MES concentration was varied in the range 12.5–625 m*M*, in series 2 the buffer pH was 6.0 and the MES concentration was varied in the range 25–625 m*M* and in series 3 a 625 m*M* MES buffer at pH 5.5 was used. A 625 m*M* MES stock solution was adjusted to the desired pH by addition of NaOH. The MES concentration was varied by diluting the 625 m*M* MES stock solution with distilled water.

The specific conductivity of the buffer solutions was measured with a CDM 83 conductivity meter (Radiometer, Copenhagen, Denmark) at 21°C. The results are shown in Fig. 3.

RESULTS AND DISCUSSION

Human growth hormone (hGH) is a protein consisting of 191 amino acids [14]. The molecular weight is 22 125 dalton and the isoelectric point (p*I*) is close to pH 5.0.

CE analysis was carried out at three pH values (5.5, 6.0 and 6.5) above the hGH p*I* and at various MES concentrations (12.5–625 m*M*). Fig. 4 shows the measured μ_{EO} values (eqn. 2) *vs.* the measured specific conductivity (mS/cm) of the buffer solutions. μ_{EO} decreases exponentially with increasing specific conductivity. Further, μ_{EO} increases with increasing pH as a larger proportion of the silanol groups are ionized.

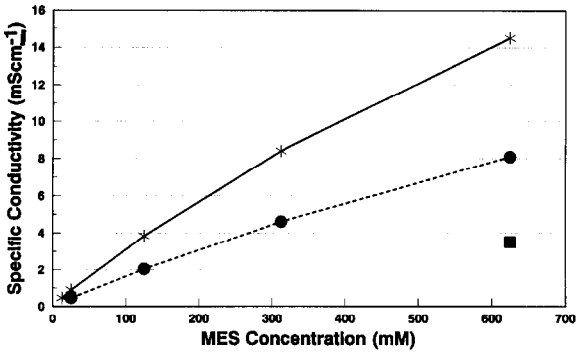

Fig. 3. Measured specific conductivity *vs.* MES concentration in the three experimental series. The pH was adjusted with NaOH, while a 625 m*M* MES stock solution was diluted to the desired final concentration with distilled water. pH: ✳ = 6.5; ● = 6.0; ■ = 5.5.

Converting the measured μ_{EO} values to radial pH gradients in accordance with eqn. 5a (T = 303.15 K) yielded Fig. 5. At low MES concentrations the pH gradient exceeds unity. When the MES concentration is increased the electroosmotic mobility (and the double layer thickness) decreases, thus decreasing the radial pH gradient.

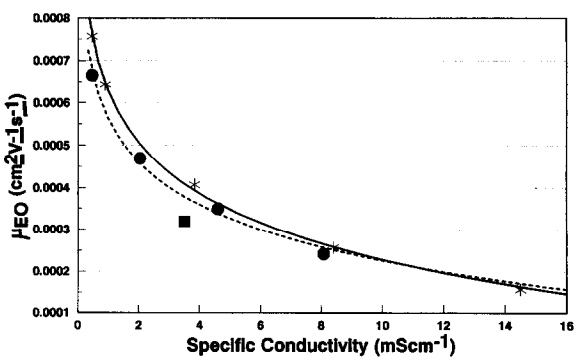

Fig. 4. Measured electroosmotic mobilities (μ_{EO}) *vs.* the measured specific conductivity for each of the buffers used in the experiments. The lines are exponentially fitted to the experimentally obtained values. pH: ✳ = 6.5; ● = 6.0; ■ = 5.5.

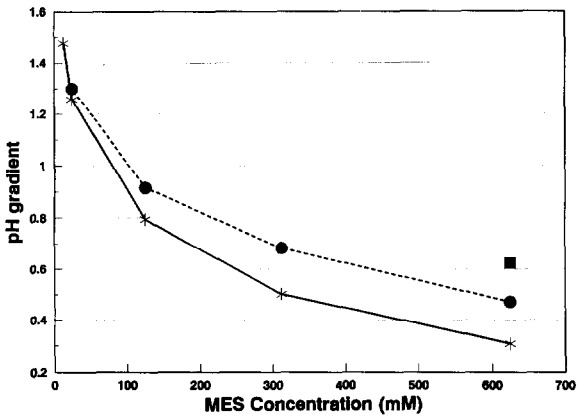

Fig. 5. Calculated radial pH gradient (ΔpH) *vs.* MES concentration for each experiment. ΔpH was calculated by inserting the measured μ_{EO} values in eqn. 5a (T = 303.15 K). pH: ✳ = 6.5; ● = 6.0; ■ = 5.5.

On replacing the pH gradients by the pH values at the capillary wall, Fig. 6 results. In two of the experiments the pH close to the silica surface was calculated to be below the B-hGH p*I* value of *ca.* 5. In those two experiments B-hGH did not elute at all, thus indicating total adsorption on the capillary wall.

All the electropherograms are shown in Fig. 7. With decreasing buffer concentrations the radial pH gradient increases and peak tailing becomes more pronounced. Only at the highest experimental buffer

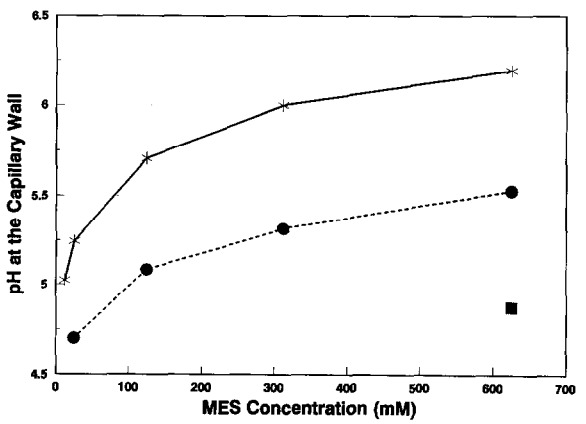

Fig. 6. Calculated pH value at the capillary wall (bulk pH − ΔpH) *vs.* MES concentration for each experiment. In two of the experiments the pH close to the capillary surface was below the hGH p*I* ≈ 5. pH: ✳ = 6.5; ● = 6.0; ■ = 5.5.

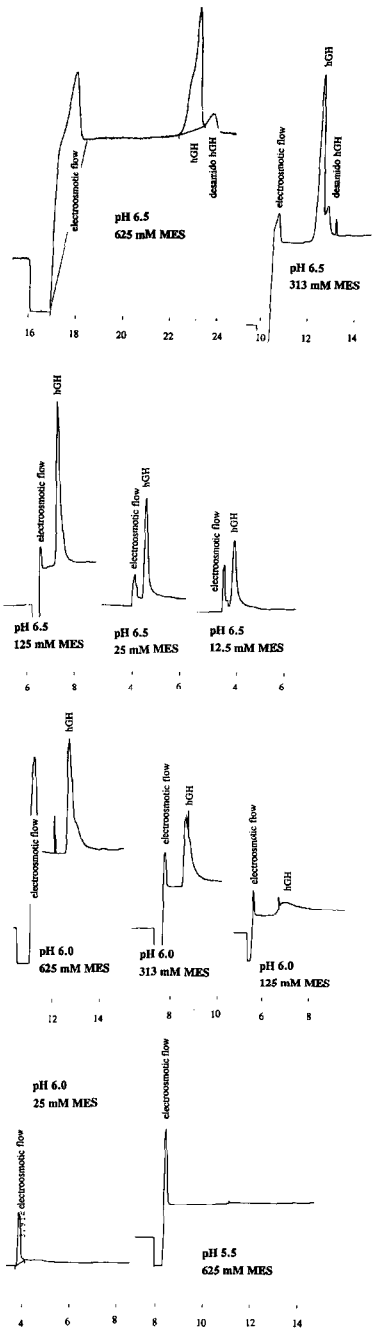

Fig. 7. All the electropherograms obtained during the three experimental series (bulk pH 5.5, 6.0 or 6.5). All retention times are in minutes. Increasing the MES concentration reduces peak tailing. For experimental conditions, see the text.

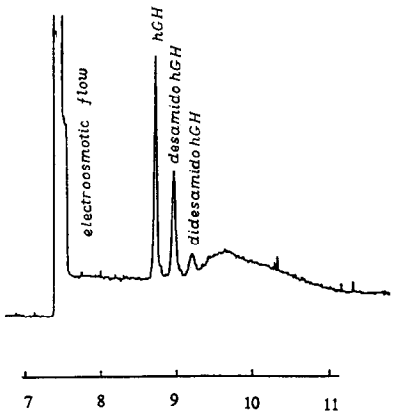

Fig. 8. Electropherogram showing baseline separation of hGH, desamido-hGH and didesamido-hGH [15]. The pH of the running buffer was 8.0. Retention times are in minutes. The experimental conditions were as follows: buffer, pH 8.0, 10 m*M* tricine; sample, 0.1 mg/ml B-hGH (which was allowed to degrade for several weeks at room temperature), introduced in 1.0 s by a 16.8-kPa vacuum; detection at 200 nm, 0.5-s detector rise time; capillary length, 100 cm (total), 75 cm (effective); I.D., 50 μm; 20 kV applied; temperature, thermostated at 27°C.

concentrations and pH values was a separation of hGH and its deamidated form observed, although it is poor. Baseline separation of hGH, desamido-hGH and didesamido-hGH is easily accomplished however, by increasing the buffer pH even further above the analyte p*I*, as shown in Fig. 8. The buffer pH was 8.0.

In addition to suppressing the pH gradient, at increasing buffer concentrations positive moieties in the protein molecule are more effectively shielded, thus decreasing coulombic wall–protein attractions. It is trivial to note that at pH values above the analyte pH increasing the bulk pH increases the capillary wall pH (and the net negative charge on the analyte), which also decreases the wall–protein attraction. Both phenomena contribute to less peak tailing.

CONCLUSIONS

If CE analysis of a polypeptide/protein is performed at a pH value slightly above its isoelectric point, it shows a tendency for adsorption on the fused-silica capillary wall owing to, among other factors, a radial pH gradient. The pH gradient can easily exceed unity under typical experimental conditions. Using high ionic strength buffers suppresses the pH gradient, thus decreasing excessive peak tailing. As an example, in order to keep ΔpH below 0.5, μ_{EO} should not exceed $2.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at 30°C according to Fig. 2.

ACKNOWLEDGEMENT

The Danish Academy of Technical Sciences is acknowledged for financial support.

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