Journal of Chromatography, 541 (1991) 489–495
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 061

Short Communication

Capillary electrophoresis in [^{2}H]water solution

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(First received September 18th, 1990; revised manuscript received November 14th, 1990)

ABSTRACT

Capillary electrophoresis in [^{2}H]water-based buffer solution has been shown to give enhanced resolution of a number of nucleosides and dansyl amino acids, compared to electrophoresis carried out in water solution of the same acidity. These effects are thought to result from a lowering of electroosmotic flow due to the higher viscosity of [^{2}H]water and to a reduction of the zeta potential.

INTRODUCTION

Capillary electrophoresis (CE) has become a popular separation technique as it is complementary to high-performance liquid chromatography (HPLC). The mechanism of separation by these two techniques depends on different physico-chemical properties of solutes. Unlike HPLC, where separation of molecules occurs due to differences in partitioning between the mobile and stationary phases, in CE resolution occurs due to differences in the mobilities of ions in an electric field [1]. CE can be applied for the resolution of a variety of molecules of different sizes: organic [2] and inorganic [3] species, peptides [4], DNA fragments [5] and proteins [6].

A number of experimental parameters can influence retention and resolution in CE. These include applied voltage, length of capillary, pH of buffer and the use of organic modifiers. CE studies reported to date have been largely carried out in water-based buffer solution. In this paper we have explored the effect on some separations by CE after replacement of water (H_2O) by deuterium oxide (^2H_2O). The latter solvent has properties such as viscosity and ionisation which are significantly different to those of water and which can play an important role in CZE separations. Viscosity of ^2H_2O at 25°C is 1.23 times greater than that of H_2O at the same temperature whereas the ionisation constants of H_2O and ^2H_2O are $1.00 \cdot 10^{-14}$ and $1.95 \cdot 10^{-15}$, respectively.

Our preliminary studies have revealed that the analytical quality of CE separations can be improved considerably when carried out in ^2H_2O rather than in water, although the time of analysis is always longer in the former solvent. We describe

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two examples of CE separations where the use of ^2H_2O in place of water has been shown to give a better resolution (R_s) of components in a mixture, due primarily to lowering of electroosmotic flow in agreement with the relationship [7]:

$$R_s = \frac{1}{4}\left(\frac{V}{2D}\right)^{\frac{1}{2}}\left(\frac{l}{L}\right)^{\frac{1}{2}}\frac{\Delta\mu_{ep}}{(\bar{\mu}_{ep} + \mu_{eo})^{\frac{1}{2}}} \quad (1)$$

where V is the applied voltage, D is the diffusion coefficient of the solute, l is the effective length of the capillary from the injection end to the detection portion, L is the total length of the capillary, $\Delta\mu_{ep}$ is the difference in electrophosphoretic mobility of two solutes, $\bar{\mu}_{ep}$ is the average electrophoretic mobility and μ_{eo} is the electroosmotic mobility.

EXPERIMENTAL

Reagents and chemicals

Adenosine-5′-O-thiomonophosphate (AMP-S) and adenosine-5′-O-(2-thiophosphate) (ADP-β-S) were obtained from Calbiochem, adenosine-5′-monophosphate (AMP), adenosine-5′-diphosphate (ADP), adenosine-3′,5′-cyclic monophosphate (cAMP) and cytidine-5′-monophosphate (CMP) were purchased from Sigma, and adenosine-3′,5′-cyclic-O-thiomonophosphate (Rp-cAMP-S) was synthesized at SmithKline Beecham Pharmaceuticals. The dansyl amino acid derivatives of ε-amino-*n*-caproic acid, leucine, glycine, γ-aminobutyric acid (GABA), β-alanine, valine and phenylalanine and reagent-grade tricine were purchased from Sigma. ^2H_2O and 40% (w/w) solution of NaO^2H in ^2H_2O were from Aldrich. AnalaR-grade sodium dihydrogenphosphate and sodium monohydrogenphosphate were supplied by BDH. Water was of distilled-deionised quality (Milli-Q) and all solutions were filtered through 0.2-μm Millipore filters before use for CE.

Apparatus and procedure

The apparatus for CE consisted of a Glassman high-voltage d.c. supply (Whitehouse Station, U.S.A.) and a CE absorbance detector (CV^4, ISCO). CE measurements were carried out within a protective Perspex enclosure with interlock switches. Electrophorerograms were recorded on a Kipp and Zonen chart recorder and data were also acquired on a Perkin-Elmer LIMS/CLAS system. Fused-silica capillaries (Applied Biosystems) of 50 μm internal diameter and of 122 cm total length (L) were used. The separation distance (l) from the anode to the detector was 95 cm. Capillaries were cleaned and filled with solution using a high-pressure syringe pump (Harvard Model 440). Platinum electrodes were used for the connection of the voltage supply to the buffer reservoirs at each end of the capillary. pH and p^2H measurements were recorded on a Radiometer PHM82 pH meter calibrated with standard pH buffers. p^2H was measured by adding 0.4 units to the reading of the meter [8].

Before use for CE, capillaries were cleaned with 0.5 M NaOH or 0.5 M NaO^2H for 10 to 20 min and then flushed with the appropriate buffer for about 15 min. All samples analysed were dissolved in the separation buffer containing a trace of methanol and were loaded electrokinetically by applying 5 kV for 1–5 s.

For the CE separation of nucleosides in H_2O or ^2H_2O, tricine (40 m*M*) buffer was used as the electrolyte and the pH or p^2H was adjusted in the range 8.60 to 9.45 with 0.1 *M* NaOH or 0.1 *M* NaO^2H, as apropriate. The voltage across the ends of the capillary was set at 40 kV and detection of nucleosides was by UV absorption at 254 nm.

The separation of dansyl amino acids was carried out in phosphate buffer made up to 10 m*M* NaH_2PO_4 with 10 m*M* Na_2HPO_4, adjusted to a pH of 7.83 with 0.1 *M* NaOH. In ^2H_2O-based solutions the buffer constituents were as in water and the p^2H was adjusted to 7.85 with 0.1 *M* NaO^2H. The separation voltage and the wavelength of detection were the same as for the CE of nucleosides.

RESULTS AND DISCUSSION

The first set of closely related compounds studied were six nucleosides namely cAMP, Rp-cAMP-S, AMP, AMP-S, ADP, ADP-*β*-S. The CE separation of these molecules together with that of cytindine-5′-monophosphate (CMP) is shown in Fig. 1 where experiments A and B refer to separations carried out in H_2O (pH 9.02) and ^2H_2O (p^2H 9.03) solutions. Under both conditions the adenosine-thiophosphate analogue has been found to be less mobile than the corresponding phosphate derivative. Although the migration time in water is shorter than in ^2H_2O at pH $=$ p^2H $= 9.0$, the resolution of the seven nucleosides is markedly better in the latter solvent. Fig. 2 shows the variation of migration time of the adenosine nucleosides with pH or p^2H. At pH or p^2H values close to 8.5 migration times in H_2O and ^2H_2O are very close. At values above 9 migration times are much lower in water than in ^2H_2O. As the pK_a values of the nucleosides under study is between 5.0 and 6.5 (the thiophosphates have the lower pK_a values) these molecules are expected to be fully ionised at pH values above 8.5 used in this study. For ionised species migration times result from the influence of both electroosmotic flow and electrophoretic flow. In general, higher pH

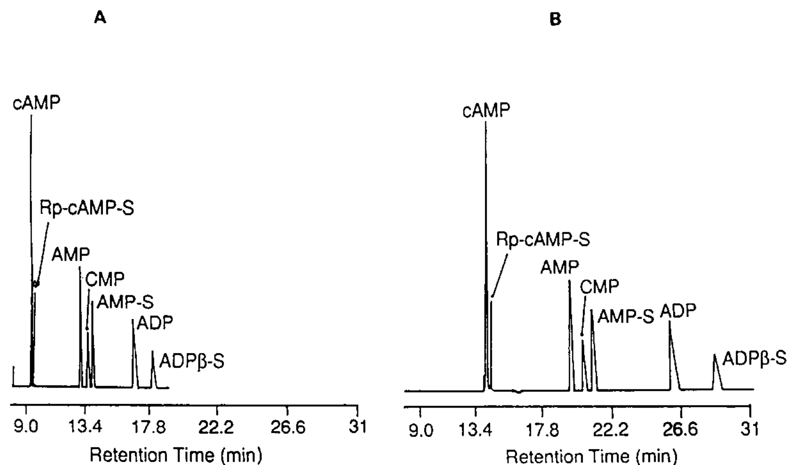

Fig. 1. CE separaton of nucleosides in (A) H_2O- and (B) ^2H_2O-based buffer solutions. Buffer, 40 m*M* tricine in H_2O (pH 9.02) or ^2H_2O (p^2H 9.03); L = 122 cm; l = 95 cm; separation voltage, 40 kV; current, 50 μA; injection voltage, 5 kV for 2 s; detection, UV at 254 nm; temperature, ambient.

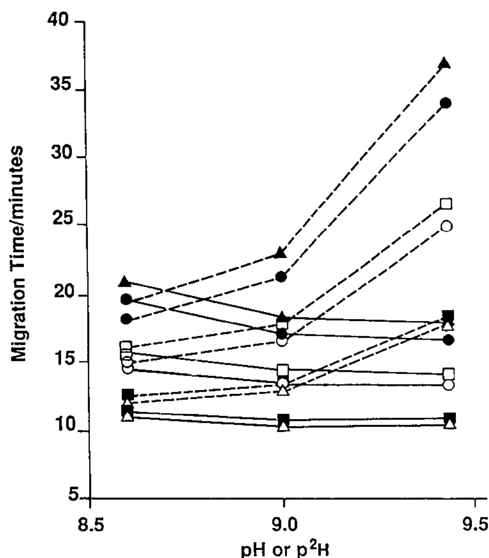

Fig. 2. Variation of the migration time of nucleosides with pH or p^{2}H in H_2O- and ^2H_2O-based buffer solution. ▲ = ADP-β-s; ● = ADP; □ = AMP-s; ○ = AMP; ■ = Rp-cAMP-s; △ = cAMP; solid lines: H_2O; dashed lines: ^2H_2O.

or p^{2}H values give higher electroosmotic flow due to the higher concentration of dissociated Si–OH groups on the inner surface of the capillary increasing the amount of negative surface charge.

The electroosmotic velocity (v_{osm}) at a pH of 9.4 is found to be about 45% higher than that at a p^{2}H of the same value using tricine as a buffer, v_{osm} is usually expressed by the relationship [9]:

$$v_{osm} = \frac{V}{L}\frac{\varepsilon\zeta}{4\pi\eta} \tag{2}$$

where V is the applied voltage across the ends of a capillary tube of length L, D is the dielectric constant, ζ is the zeta potential and η is the viscosity coefficient.

All experiments in this study have been carried out at the same applied voltage (V) using the same length of capillary (L). Moreover, the dielectric constant (D) of water (78.30) at 25°C is almost equal to that of ^2H_2O (78.25) at the same temperature. Thus any differences in electroosmotic flow in water and ^2H_2O can only be accounted for in terms of viscosity and zeta potential. In fact, the viscosity of water (η = 0.890 Nsm^{-2}) is markedly lower (about 23%) than that of ^2H_2O (η = 1.098 Nsm^{-2}) at 25°C and must therefore be a major contributory factor in slowing down the migration of the nucleosides in ^2H_2O compared to water (Figs. 1 and 2).

The zeta potential (ζ) is directly related to the amount of charge per unit surface area on the inner wall of the capillary. The magnitude of ζ will depend both on the concentration of dissociated ions in the buffer solution and the dissociation of the Si–OH groups on the inner surface of the capillary. The pK_a values of both the tricine

buffer and that of the Si–OH groups are expected [10] to be about 0.6 units higher in ^2H_2O than in water. Thus the pK_a of tricine will increase from 8.2 in water to about 8.8 in ^2H_2O, and that of the silyl groups will increase from 9.8 (based on the pK_a of silicic acid) [10] to about 10.4. At a pH or p^{2}H of 9.4 a higher degree of ionisation of the Si–OH groups is expected to occur in H_2O compared to ^2H_2O leading to a correspondingly higher ζ potential. Moreover, a lower amount of negatively charged free base of the tricine buffer will exist in ^2H_2O compared to H_2O when the p^{2}H or pH is set at 9.4, again contributing to a decrease in the ζ potential and hence a decrease in the mobility of the nucleosides in ^2H_2O. The concentration of tricine free base is calculated to be 94 and 80% in water and ^2H_2O, respectively. Finally, the magnitude of the zeta potential may also be affected by the degree of negative charge on the substrates. In fact, slow down in mobility is greatest for the multi charged nucleosides at the pH or p^{2}H under study.

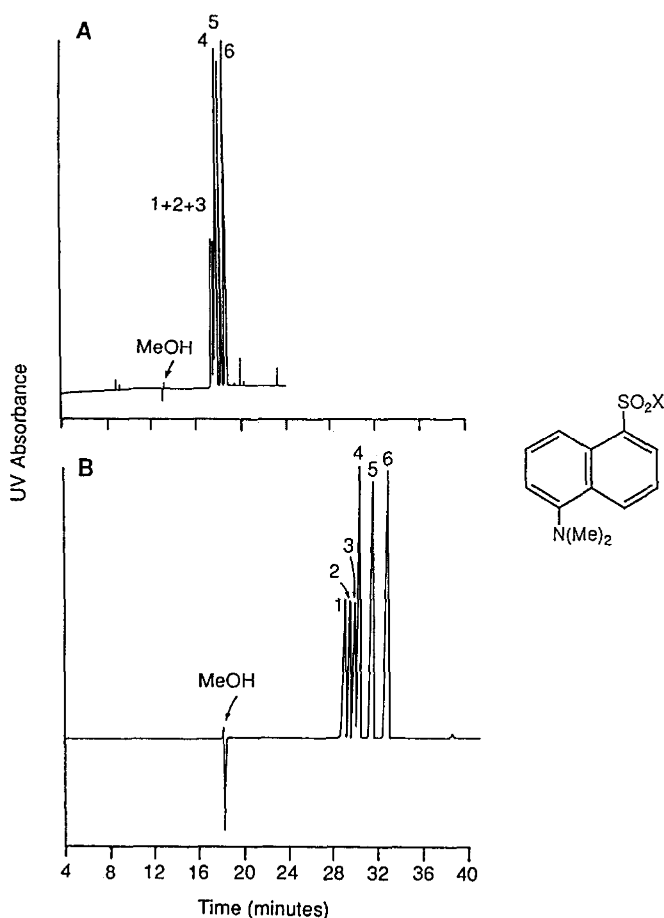

Fig. 3. CE separation of dansyl amino acids in (A) H_2O (pH 7.83) and (B) ^2H_2O (p^{2}H 7.85). L = 122 cm; l = 95 cm; buffer, 20 mM NaH_2PO_4–Na_2HPO_4, p^{2}H or pH $\approx$ 7.8; separation voltage, 40 kV; current, 50 μA; injection voltage, 5 kV for 1 s; detection, UV at 254 nm; temperature, ambient. Peaks (see structure): X = (1) ε-amino-*n*-caproic acid; (2) Leu; (3) Gly; (4) GABA; (5) β-Ala; (6) Val. Me = Methyl.

The second set of model solutes that were initially investigated for separation in H_2O and ^2H_2O were the dansyl derivatives of the following six amino acids: ε-aminocaproic acid, glycine, valine, leucine, β-alanine and GABA. The separation of these solutes in phosphate buffer at pH 7.83 is poor as shown in Fig. 3a. In comparison, almost baseline resolution is obtained for all the six compounds at a p^2H of 7.85 using the same concentration of phosphate buffer in ^2H_2O. Having obtained such excellent analytical improvement dansyl phenylalanine was added to the original solute mixture. Partial separation of this dansyl derivative from the remaining compounds was only obtained in ^2H_2O (Fig. 4). No attempt was made to improve this separation further.

The electroosmotic velocity in phosphate buffer was found to be about 35% lower at p^2H 7.85 compared to pH 7.83. As in the case of the CE separation of the nucleosides in these two solvent media, the increase in viscosity in ^2H_2O has to be a major cause for the longer migration time. The pK_a of phosphoric acid and deutrophosphoric acid have been reported [11] as 7.2 and 7.8 respectively. Thus, at a pH or p^2H around 7.8 the percentage of phosphate free base is 80% in H_2O compared to 50% in ^2H_2O, again indicating that differences in the concentration of the ratio of free acid to free base plays some part in affecting the mobility of the dansyl substrates in H_2O and ^2H_2O solution.

In both the case of the CE separation of the nucleosides and dansyl amino acids the enhanced resolution observed can be ascribed to a reduction in electrophoretic mobilities according to eqn. 1. However, an increase in viscosity would also mean

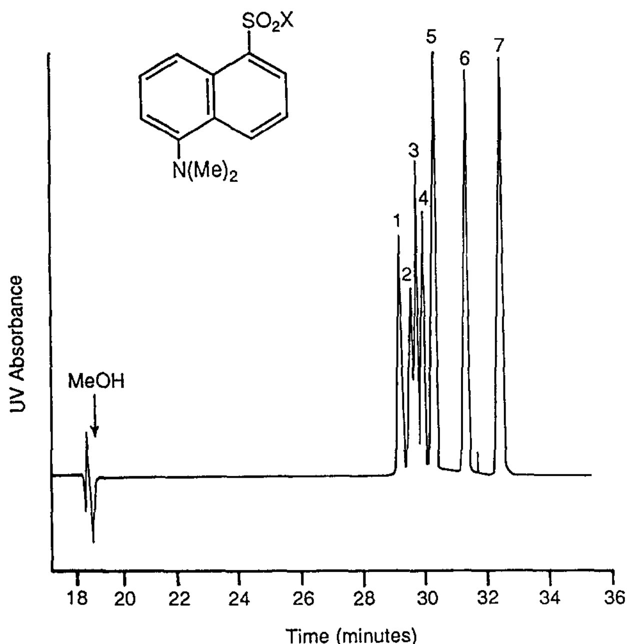

Fig. 4. CE separation of dansyl phenylalanine from the other dansyl derivatives shown in Fig. 3. Peaks (see structure): X = (1) ε-amino-*n*-caproic acid; (2) Leu; (3) Phe; (4) Gly; (5) GABA; (6) β-Ala; (7) Val.

a reduction in electrophoretic mobility because the average electrophoretic velocity υ is related [12] to μ_{ep} and μ_{eo} by eqn. 3:

$$\upsilon = (\mu_{ep} + \mu_{eo})/(V/L) \quad (3)$$

Thus, the enhanced resolution in ^2H_2O rather than H_2O-based buffer solutions must be certainly ascribed to a reduction in electroosmotic flow primarily due to a reduction in zeta potential.

In conclusion, we have shown that CE separation in ^2H_2O-based buffer solutions can considerably improve electrophoretic resolution compared to H_2O solution. Carrying out CE separations both in H_2O and ^2H_2O can also provide insight on the influence of electroosmotic flow on the electrophoretic mobility of the compounds under study. Moreover, the use of ^2H_2O may have the advantage in that it can be used to control electroosmotic flow without the aid of the addition of other components to the buffer system, in particular ionic surfactants [13] or organic modifiers [14].

REFERENCES

1 D. J. Rose and J. W. Jorganson, *Anal. Chem.*, 60 (1988) 642.
2 T. Tsuda, K. Nomura and G. K. Nakagawa, *J. Chromatogr.*, 248 (1982) 241.
3 T. Tsuda, K. Nomura and G. K. Nakagawa, *J. Chromatogr.*, 264 (1983) 385.
4 J. W. Jorgenson and K. D. Lukas, *J. Chromatogr.*, 218 (1981) 209.
5 H. Drossman, J. A. Lucking, A. J. Kostichka, J. D'Cunbra and L. M. Smith, *Anal. Chem.*, 62 (1990) 800.
6 G. J. M. Bruin, J. P. Chang, R. H. Kuhlman, K. Zegers, J. C. Kraak and H. J. Poppe, *J. Chromatogr.*, 471 (1989) 429.
7 S. Terabe, T. Yoshima, N. Tanaka and M. Araki, *Anal. Chem.*, 60 (1988) 1673.
8 P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, 64 (1960) 188.
9 A. W. Adamson, *Physical Chemistry of Surfaces*, Intersciences, New York, 2nd ed., 1967, Ch. 4.
10 W. P. Jencks, *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York, 1969, Ch. 4.
11 *Large's Handbook of Chemistry*, McGraw-Hill, New York, 12th ed., 1979, Section 5.
12 J. W. Jorgenson and K. D. Lukas, *Anal. Chem.*, 53 (1981) 1298.
13 H. H. Lawer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
14 T. Tsuda, C. Nakagawa, M. Sato and K. Yagi, *J. Appl. Biochem.*, 5 (1983) 330.