Replacement of H_2O by D_2O in Capillary Zone Electrophoresis can Increase Resolution of Peptides and Proteins

Patrick Camilleri and George N. Okafo

SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Herts. AL6 9AR, UK

Water (H_2O) based buffer solutions are generally used in capillary zone electrophoresis (CZE) for the separation of peptides and proteins; the replacement of H_2O by D_2O in the supporting electrolyte (pH, pD *ca.* 7.8) contributes to the suppression of electroosmotic flow, mainly due to the higher viscosity of D_2O and can result in enhanced resolution of substrates without the use of additives.

Although capillary zone electrophoresis (CZE) is a relatively new analytical tool, compared to other well-established methods of analysis such as HPLC and GC, there has been a large number of publications in this area of separation technology during the last few years.[1–5] Several reviews have also been written about CZE.[6–8] It is now widely accepted that CZE and HPLC can be considered as complementary to one another because of the different mechanisms of separation involoved in the two methods. Thus, whereas molecules are resolved in HPLC by differences in free energy in partitioning between the mobile and stationary phases, separation in CZE occurs owing to the behaviour of molecules in an electric field. Variation in this behaviour depends on differences in shape, size and overall electrical charge.

In CZE the migration time (t) of a solute is related to the length of capillary (L), the applied voltage (V) and the sum of the electrophoretic (μ_{ep}) and the electroosmotic (μ_{eo}) mobilities, eqn. (1). The resolution (R_s) of the components in a

$$t = L^2/V\,(\mu_{ep} + \mu_{eo})^{-1} \quad (1)$$

mixture is also related to the sum of the electrophoretic and the electro- osmotic mobilities,[7] eqn. (2). In eqn. (2) D is the

$$R_s = \tfrac{1}{4}\,(V/2D)^{\frac{1}{2}}\,(l/L)^{\frac{1}{2}}\,[\Delta\mu_{ep}/(\bar{\mu}_{ep} + \mu_{eo})^{\frac{1}{2}}] \quad (2)$$

diffusion coefficient of a solute, l is the effective length of capillary from the anode to the detector, $\Delta\mu_{ep}$ is the difference in the electrophoretic mobility of two solutes and $\bar{\mu}_{ep}$ is the corresponding average electrophoretic mobility.

Both μ_{ep} and μ_{eo} are affected by changes in the electrolyte concentration, pH and viscosity. Under conditions of moderate or high pH, substrates will flow strongly towards the cathode (the detector end) independent of their charge. The control of electroosmotic mobility relative to electrokinetic mobility can enhance resolution or efficiency. Several attempts to suppress electroosmotic flow have been reported in the literature. These involve the use of additives such as ethylene glycol,[9] ionic surfactants[10] and cellulose derivatives, or the coating[11] of the silyl groups on the inside of the capillary wall. Reproducing migration times and resolution following the addition of modifiers has very often proved unsatisfactory.[12] We now report the use of deuterium oxide (D_2O) instead of water (H_2O) to control electroosmotic flow in neutral or alkaline media. D_2O has properties, such as viscosity and ionisation, that are significantly different to those of H_2O: the viscosity of D_2O at 25 °C is 1.23 times greater than that of H_2O at the same temperature; the ionisation constants of H_2O and D_2O are 1.00×10^{-14} and 1.95×10^{-15}, respectively at 25 °C. Variations in these two properties are expected to exert some control over electroosmotic flow. We have already shown[13] that the CZE of a number of closely related nucleosides and dansyl amino acid derivatives can be improved considerably by the use of D_2O- rather than H_2O-based buffer solutions. We now demonstrate the usefulness of D_2O *vs.* H_2O in CZE separations of peptides and proteins by a number of examples. In these experiments, the pD of buffer solutions was measured[14] by the addition of 0.4 units to the reading of a Radiometer PHM82 pH meter calibrated with standard pH buffers. The instrumentation used has already been detailed in ref. 13 and experimental conditions are shown in the appropriate Figures.

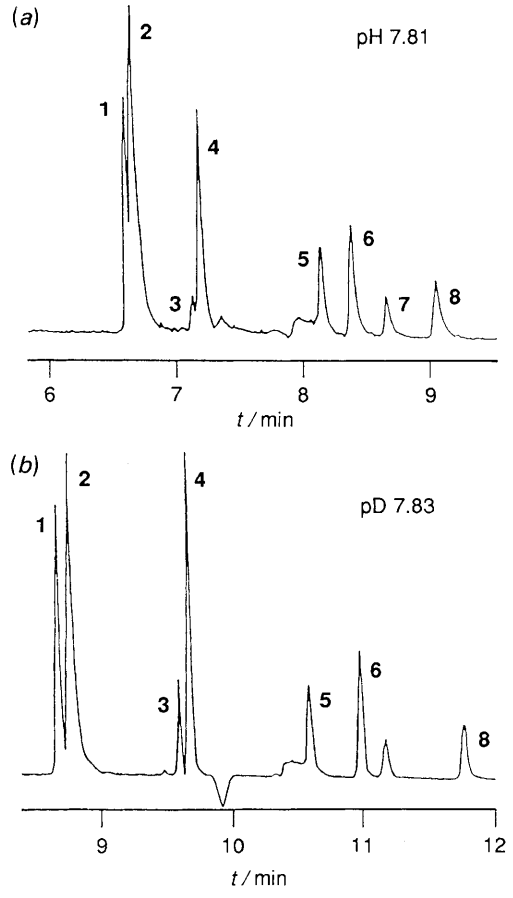

Fig. 1 Separation of bioactive peptides in (*a*) H_2O- and (*b*) D_2O-based buffer solution (L = total length of capillary; l = effective length from the anode to the detector; I.D. = internal diameter). Buffer: 20 mmol dm^{-3} NaH_2PO_4/Na_2HPO_4, pD or pH ≃ 7.8. Capillary: L = 72 cm, l = 55 cm, I.D. = 50 μm. Separation voltage: 15 kV; injection voltage: 5 kV/2s; current: <50 μA. Detection: UV, 200 nm. Temperature: ambient.

1 Ile-Ser-Bradykinin,	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
2 Bradykinin,	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
3 Unknown peptide impurity	
4 Neurotensin,	Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
5 Angiotensin I,	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
6 Angiotensin II,	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
7 [D-Ala^2, D-Leu^5]- Enkephalin,	Tyr-D-Ala-Gly-Phe-D-Leu
8 Leucine Enkephalin,	Tyr-Gly-Gly-Phe-Leu

Fig. 1 shows the separation of seven bioactive peptides and an unknown impurity **3**. In both cases samples were electrokinetically injected into the capillary from the same H_2O-based solution. As expected all the substrates show a longer migration time in D_2O compared to H_2O solution. However, the extent of 'slow-down' varies for the substrates analysed, depending on the pI values in H_2O *vs.* D_2O solution. By injection of a neutral molecule, mesityl oxide (0.1% v/v in buffer pH or pD *ca.* 7.8) the electroosmotic mobility in D_2O was found to be *ca.* 25% lower than that in water. This percentage difference is very close to the difference in the viscosities of the two solvents. Overall, this slow-down in migration has allowed almost baseline separation of bradykinin **2** from a closely related peptide **1** and neurotensin **4** from an unknown peptide impurity **3** present in the original sample of **4**.

To investigate further the usefulness of CZE in D_2O, we analysed peptides from the tryptic digest of glucagon. Mass

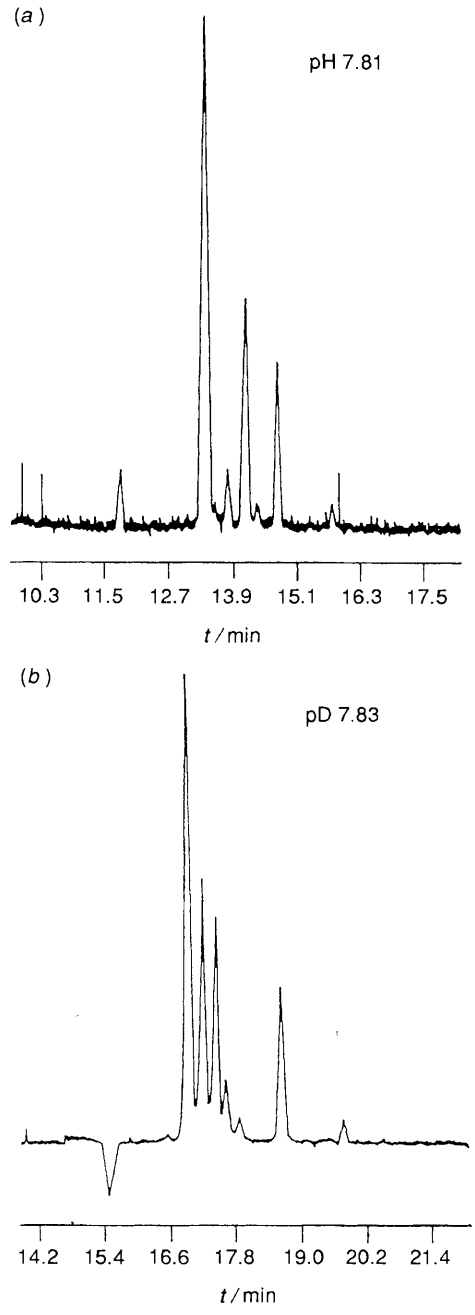

Fig. 2 Separation of peptides derived from the tryptic digest of glucagon in (*a*) H_2O- and (*b*) D_2O-based buffer solution. Buffer: 20 mmol dm^{-3} NaH_2PO_4/Na_2HPO_2 in H_2O (pH 7.81) or D_2O (pD 7.83). Capillary: L = 72 cm, l = 50 cm, I.D. = 50 μm. Separation voltage: 10 kV. Current: < 25 μA. Injection voltage: 5 kV for 3 s. Wavelength: UV, 200 nm. Temperature: ambient.

spectral analysis of this digest was shown[15] by FAB analysis to contain four main peptides. However, CZE analysis in an H_2O-based buffer solution [Fig. 2(*a*)] only shows three main peaks. In contrast, CZE in a buffer solution containing D_2O of the same acidity [Fig. 2(*b*)] shows the expected four major peaks. The negative signal before these peaks in the latter electrophoregram is due to water drawn into the capillary on electrokinetic injection and marks the point of migration of a neutral molecule only under the influence of electroosmotic flow. This negative peak is an important demarcation point as it indicates that peaks migrating before, close to or after it are overall positively charged, neutral, or negatively charged respectively. In the case of peptides obtained from the tryptic digest of glucagon none of them are expected to be overall positive in agreement with the pattern of electromigration seen in Fig. 2.

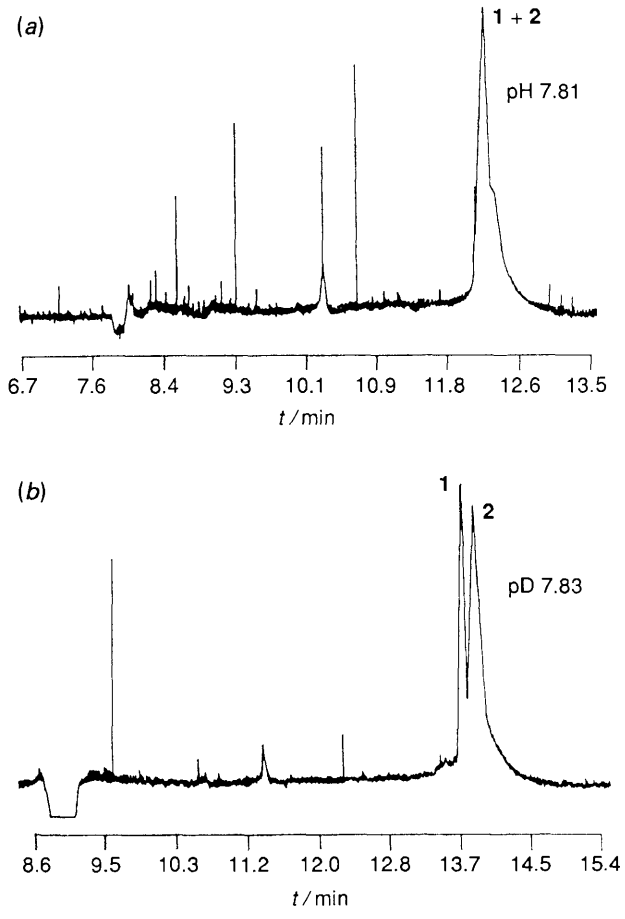

Fig. 3 Separation of bovine and canine serum albumin. (*a*) and (*b*) as in Figs. 1 and 2. Buffer: 20 mmol dm^{-3} NaH_2PO_4/Na_2HPO_4 in H_2O (pH 7.81) or D_2O (pD 7.83). Capillary: L = 72 cm, l = 55 cm, I.D. = 50 μm. Separation voltage: 15 kV. Injection voltage: 5 kV/2s. Current: < 50 μA. Detection: UV, 200 nm. Temperature: ambient. Samples: 1. BSA. 2. CSA.

Finally, Fig. 3 shows a comparison of the migration behaviour of a mixture of bovine serum albumin (BSA) and canine serum albumin (CSA) in H_2O- and D_2O-based solutions. Only a 'hint' of separation is observed in H_2O whereas the two proteins can easily be distinguished when H_2O is replaced by D_2O. This is remarkable when one expects that the two types of albumin are closely related structurally. Unfortunately, we cannot make a direct structural comparison as (to the best of our knowledge) the primary amino acid sequence of CSA has not been determined. It suffices to say that we could not separate CSA and BSA either by conventional gel electrophoresis or by HPLC using a gradient of 0.1% trifluoroacetic acid in water and acetonitrile. Resolution of the two albumins by CZE is almost certainly due to the fact that the pI values of the two albumins are slightly different in D_2O solution.

In conclusion, it has been shown that CZE in D_2O-based solutions can produce analytical data of better quality than those carried out in H_2O-based solutions. Although the migration times are longer in D_2O compared to H_2O, a significant improvement in resolution is achieved. This can be mainly explained in terms of suppression of electroosmotic flow, resulting from the higher viscosity of D_2O.

Received, 5th November 1990; Com. 0/04980H

References

1 J. W. Jorgenson and K. De Arman Lukacs, *Anal. Chem.*, 1981, **53**, 1298.
2 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 1985, **57**, 834.
3 P. Gozel, E. Gassmann, H. Michelsen and R. N. Zare, *Anal. Chem.*, 1987, **59**, 44.
4 W. Kuhr and E. S. Yeung, *Anal. Chem.*, 1988, **60**, 2642.
5 T. M. Olefirowicz and A. G. Ewing, *Anal. Chem.*, 1990, **62**, 1872.
6 M. J. Gordon, X. Huang, S. L. Pentoney and R. N. Zare, *Science*, 1988, **242**, 224.
7 W. Th. Kok and G. J. M. Bruin, *Euro. Chromatogr. News*, 1988, **2**, 22.
8 A. G. Ewing, R. A. Wallingford and T. M. Olefirowicz, *Anal. Chem.*, 1989, **61**, 292A.
9 S. Terabe, T. Yashima, N. Teraka and M. Araki, *Anal. Chem.*, 1988, **60**, 1673.
10 K. D. Altria and C. F. Simpson, *J. Pharm. Biomed. Anal.*, 1988, **6**, 801.
11 H. H. Lawer and D. McManigill, *Anal. Chem.*, 1986, **58**, 166.
12 G. J. M. Bruin, J. P. Chang, R. H. Kuhlman, K. Zegers, J. C. Kraak and H. Popple, *J. Chromatogr.*, 1989, **471**, 429.
13 P. Camilleri and G. Okafo, *J. Chromatogr.*, in the press.
14 P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, 1960, **64**, 188.
15 S. Naylor, A. F. Findeis, B. W. Gibson and D. H. Williams, *J. Am. Chem. Soc.*, 1986, **108**, 6359.