Journal of Chromatography, 636 (1993) 95–103
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 847

Comparison of selectivities of reversed-phase high-performance liquid chromatography, capillary zone electrophoresis and micellar capillary electrophoresis in the separation of neurohypophyseal peptides and analogues

Nick Sutcliffe and Patrick H. Corran*

Chemistry Division, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG (UK)

ABSTRACT

A number of RP-HPLC systems, including those prescribed in several official monographs, have been evaluated for separating oxytocin, the vasopressins, some clinically important analogues and two additional neurohypophyseal nonapeptides. The separation has been compared with capillary zone electrophoresis and micellar electrophoresis in four micellar systems: cationic, anionic, zwitterionic and neutral. Complete separation was achieved by both RP-HPLC and micellar CE but the importance of charge as a major parameter of separation in CE confers a distinct and useful selectivity to micellar CE based separations.

INTRODUCTION

Although some of the earliest published work on the analysis of pharmaceutical peptides and their formulations was carreid out on oxytocin, lypressin and their analogues [1–3], and although there is an extensive literature on the use of high-performance liquid chromatography (HPLC) in the assay and control of manufacture and formulation of these peptides [1–5], progress in introducing the technique into official specifications such as pharmacopoeial monographs has been disappointingly slow, and the hopes that it would replace or render unnecessary the use of biological assays have been only rather gradually realised. Nevertheless, following the initial inclusion of a test for uniformity of content of oxytocin buccal tablets [16] (subsequently omitted), the British and European Pharmacopoeias now contain HPLC-based tests for the identification of oxytocin in oxytocin injection, the identification and assay of oxytocin in a coformulation with ergometrine and for the assay and content of related peptides for desmopressin [17,18]. The United States Pharmacopoeia appears not, at present, to contain an HPLC-based test for any of these peptides. There are at least six clinically significant oxytocin or vasopressin derivatives: the native peptides themselves, the lypressin analogue felypressin, the argipressin analogue desmopressin which exhibits anti-diuretic but not pressor activity, and the prodrug terlipressin, which is hydrolysed *in vivo* to the natural lypressin (Fig. 1). In the work described below we have reviewed the reversed-phase (RP) HPLC systems recommended in pharmacopoeial monographs, and selected an effective separation of these peptides together with two closely related non-mammalian neurohypophyseal hormones isotocin (found in teleosts) and argitocin (non-mammalian vertebrates) (Fig. 1). We have also com-

* Corresponding author.

0021-9673/93/$06.00

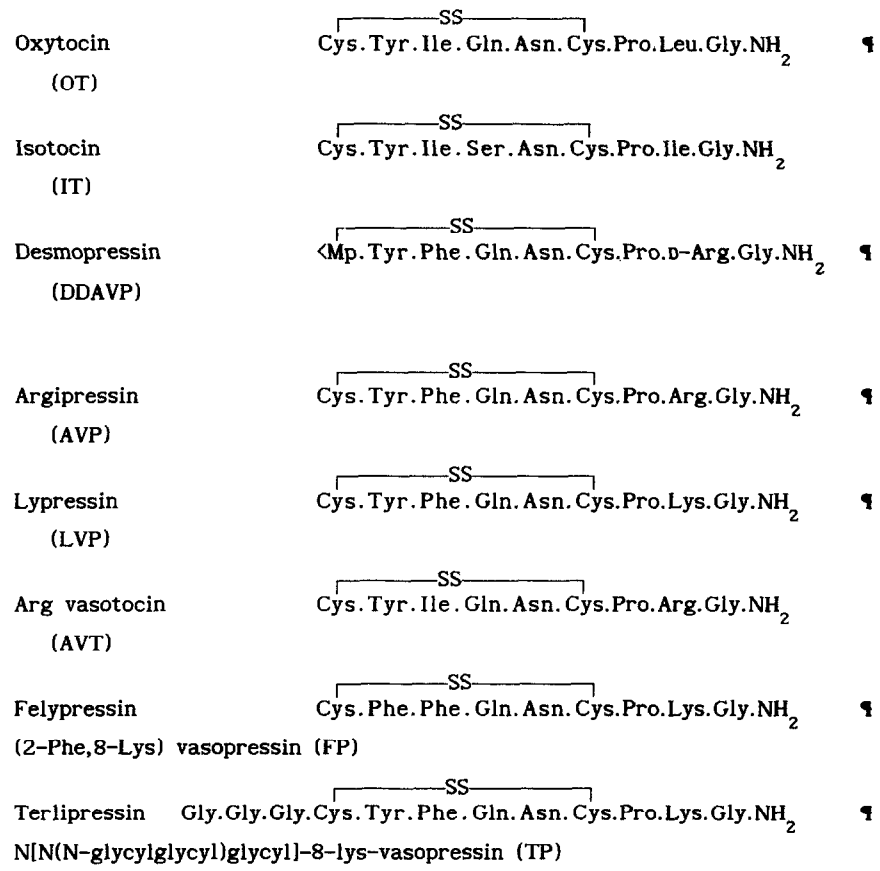

Fig. 1. Structures of oxytocin, the vasopressins and analogues. Mp = Mercaptopropionic acid. The (amidated) C-terminal is to the right.

pared the separations obtained by RP-HPLC with capillary electrophoresis (CE). Capillary zone electrophoresis (CZE) has been extensively used for separating peptides [19–27] and for tryptic mapping [24,28–32,47]. There have been fewer reports of the effects of additives on electrophoresis, but amongst those investigated have been ^2H_2O [33], cyclodextrins [34], metal ions [35], hydrophobic pairing agents [36,37] and detergents [27,34,35]. We have also therefore examined the effects of micellar detergents on separation.

From the point of view of an electrophoretic separation the peptides fall into two groups which carry a single and a double positive charge at pH <5. Oxytocin and isotocin lack the basic residue at position 8 present in vasopressin and its derivatives (either lysine or arginine), and desmopressin lacks an N-terminal amino group, and the arginine at position 8 is in the D configuration. All except felypressin possess a tyrosine at position 2, and terlipressin has a triglycine N-terminal extension (see Fig. 1). We were interested to see how the mobilities of each peptide would be affected by these structural differences, and how the separation could be modulated by he addition of charged detergents at levels above the critical micelle concentration (CMC), micellar electrokinetic chromatography (MEKC) [38], and by the addition of detergents which carry no overall charge (zwitterionic and neutral systems). In the last case the separation becomes a formal analogue

of chromatography, with the micelles as he stationary phase, and transport through the system being by electrophoretic migration.

MATERIALS AND APPARATUS

RP-HPLC was carried out on a system consisting of a Spectra-Physics SP8800 gradient pump (Spectra-Physics, Hemel Hempstead, UK), loop injector, Polychrome diode array UV monitor (Varian, Walton-on-Thames, Berskhire, UK) and Spectra-Physics SP4270 integrator. Raw data were captured via Labnet to an IBM Model 55SX computer running WINner software and reprocessed with the supplied utility DISPLAY, if necessary (Spectra-Physics). The RP-HPLC columns compared were a single or double cartridge (100 or 200 × 3 mm I.D.) of Spherisorb S50DS2 (Chrompack, Milharbour, London, UK) and a column (150 × 4.6 mm I.D.) of the poplymeric reversed-phase packing PLRP-S 300 (d_p 8 μm) (Polymer Labs., Church Stretton, UK).

CE was carried out on a Model 270HT system (Applied Biosystems, Warrington, UK). Data were collected and integrated using a PC with data acquisition card and Summit software modified for use with CE (Comus, Humberside, UK). Polymicro Technologies fused-silica capillary tubing (50 μm I.D. × 375 μm O.D.) was purchased from Composite Metal Services (Hallow, UK); 50–52-cm lengths were cut and polyimide coating removed at each end for 0.5 cm and for a detector window at about 22 cm from one end as described in the CE equipment manufacturer's manual, and prepared before use by flushing with 1 *M* NaOH for 30 min followed by water for 10 min.

Sodium dodecyl sulphate (SDS) for electrophoresis and cetyltrimethylammonium bromide (CTAB) were purchased from BDH (Poole, UK) and 3-[(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS) and Triton X100R from Sigma (Poole, UK). Sudan III was obtained from Aldrich (Gillingham, UK). Other reagents were of analytical-reagent or HPLC grade as appropriate.

Oxytocin and felypressin concentrates and lypressin acetate were obtained from Sandoz (Basle, Switzerland), argipressin, terlipressin and desmopressin acetates from Ferring Pharmaceuticals (Feltham, UK) and isotocin and arginine vasotocin from Sigma.

Methods

HPLC. The pharmacopoeial systems examined were (a) 0.1 *M* NaH_2PO_4 (pH approximately 4.5)–CH_3CN (oxytocin injection [17,18]), (b) 0.067 *M* phosphate pH 7–CH_3CN (desmopressin [17,18]) and (c) 0.2% (v/v) H_3PO_4–CH_3CN (ergometrine and oxytocin injection [17]). In addition we examined the system (d) 0.05% (v/v) H_3PO_4 + 0.5% $(NH_4)_2SO_4$ (w/v) (pH approximately 2.75)–CH_3CN. In all cases the aqueous buffer was used as mobile phase component A, B was a mixture of A–CH_3CN (50:50) and a gradient was necessary. All solvents were filtered through a 0.2-μm filter before use, and solvent reservoirs were sparged with helium and maintained with a low flow of helium throughout chromatography.

CE. Capillaries were flushed before each run for 2 min with 0.1 *M* NaOH followed by the appropriate running buffer (for 4 or 5 min). Sampling was by vacuum for 1 to 5 s depending on capillary length, and for identification of individual components a marker injection cycle was included to inject a small amount of a mixture of pure components. Benzyl alcohol was used to estimate endosmosis in free electrophoretic systems and methanol in charged micellar systems. Micellar mobilities of charged micelles were estimated with Sudan III [38]. Electropherograms were monitored at 200 nm. All buffers were passed through a 0.2-μm filter before use. Electrophoresis was carried out towards the anode for the CTAB systems and towards the cathode for all others. Free solution electrophoresis was carried out in buffers 0.1 *M* in phosphate made up by mixing 0.1 *M* solutions of H_3PO_4, NaH_2PO_4 and Na_2HPO_4 as appropriate to give pH values between 2 and 7.5. MEKC was carried out in 0.02 *M* Tris pH 8.0 with 5–25 m*M* CTAB or 5–200 m*M* SDS, or in 0.1 *M* phosphate pH 2.5 with 10–100 m*M* CHAPS or 0.02–5% (w/v) Triton X100R. All pH values were determined at ambient temperature (20–25°C during the period of the study).

RESULTS

RP-HPLC

Mobile phase c resulted in poor peak shapes on the silica-based columns. Systems a and d both gave complete separations, with the separation being slightly better with d (Fig. 2a). The polymeric col-

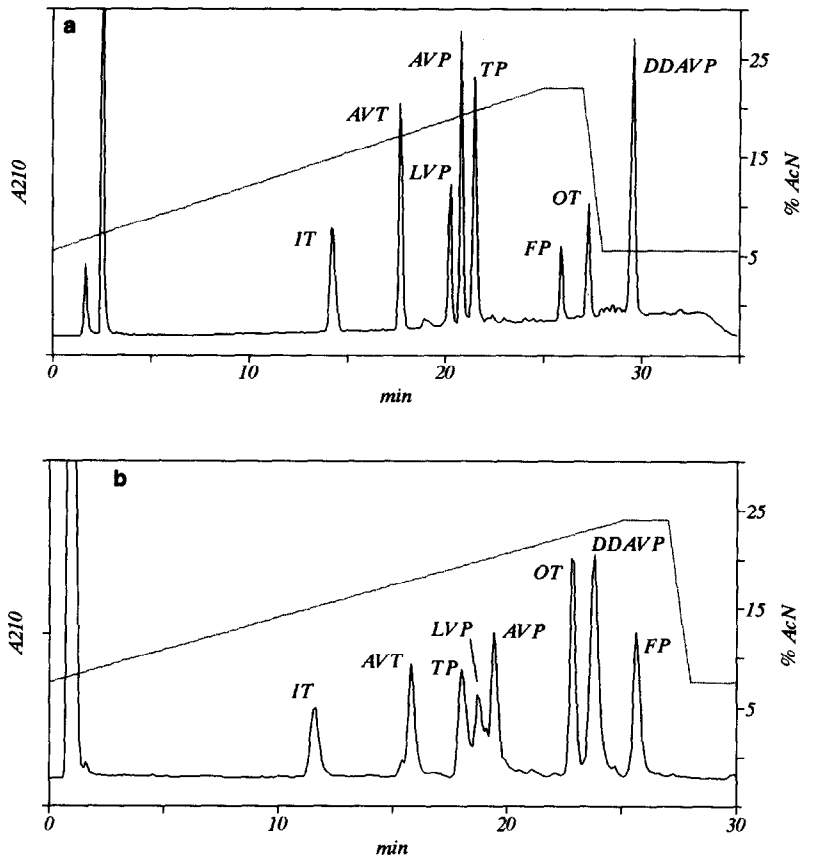

Fig. 2. Separation of peptides by RP-HPLC. (a) Mobile phase system d. Conditions: column 200 × 3 mm Spherisorb S50DS2, 0.5 ml/min, 0.05% (v/v) H_3PO_4 + 0.5% (w/v) $(NH_4)_2SO_4$–acetonitrile (AcN). (b) Mobile phase system b. Conditions: column 100 × 3 mm Spherisorb S50DS2, 0.5 ml/min, 0.07 *M* phosphate pH 7.0–acetonitrile. Abbreviations as in Fig. 1.

umn was unable to resolve felypressin and oxytocin for these systems. Mobile phase b resolved all the peptides as well, but the order of elution was different (Fig. 2b), and peak shapes were inferior to systems a and d. The group terlipressin/lypressin/argipressin was only partially resolved (Note that the column in Fig. 2b was half the length of that in Fig. 2a).

CE

CZE. As expected, the peptides formed two clear sets at pH 2.5 (Fig. 3), with some degree of separation within the doubly and singly-charged groups. Amongst the doubly charged peptides, terlipressin was clearly separated from the rest, and felypressin was almost completely resolved from the combined peak containing argipressin, arginine vasotocin and lypressin. In the case of the three singly charged peptides, all three were partly resolved,

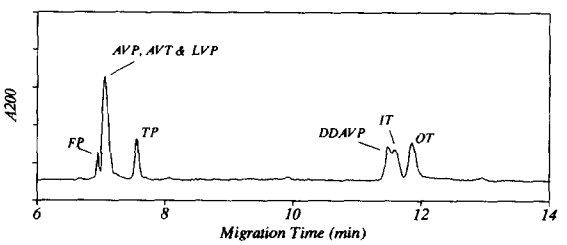

Fig. 3. CZE of peptides at pH 2.5. Conditions: 30°C, 15 kV, 0.1 *M* sodium phosphate. Abbreviations as in Fig. 1.

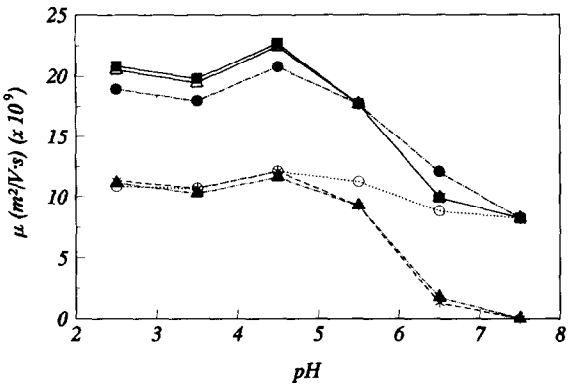

Fig. 4. Dependence of free solution mobility (μ) on pH. Conditions: 30°C, 15 kV, 0.1 *M* phosphate buffers (see Methods section). Symbols: □ = AVP and LVP; △ = AVT; ● = TP; ■ = FP; ○ = DDAVP; ✳ = IT; ▲ = OT. (Abbreviations as in Fig. 1).

with the complete resolution between oxytocin and isotocin particularly noteworthy (Fig. 3). The dependence of mobility on pH showed the expected change associated with the titration of an N-terminal amino group with a pK_a of about 6.5 for all except desmopressin (which has no N-terminal amino group) and terlipressin (which has a higher pK_a more characteristic of an N-terminal amino group) (Fig. 4).

Micellar electrophoresis. Baseline disturbance with CTAB was so severe that the CTAB system could not be used at low wavelength ($\leqslant$220 nm). Since felypressin has poor adsorption properties at 280 nm (containing no tyrosine) no further work on this micellar system was carried out. The interaction with SDS micelles seemed to be dominated by electrostatic effects between the cationic and neutral peptides and the anionic micelles, and the peptides exhibited poor peak shapes, with little evidence of separation except at the highest concentration (200 m*M*) where a partial resolution became apparent again (Fig. 5). In the case of the electrically neutral detergents, triton X100 had little effect on relative mobility (Fig. 6) except in the case of desmopressin, which was selectively retarded as the Triton concentration was increased, migrating between isotocin and oxytocin at 0.05% Triton X100R (Fig. 7) and after oxytocin at 0.5% and above. We found higher concentrations of Triton X100R difficult to work with, and there was disappointingly little effect on the co-migrating group AVT/LVP/AVT. CHAPS was the only detergent which allowed complete electrophoretic separation of all peptides (Fig. 8). Addition of 10 m*M* CHAPS (close to the nominal CMC of 8 m*M*) brought little improvement in separation, but as the concentration was raised felypressin and desmopressin were relatively delayed within their respective groups, and argipressin was resolved from lypressin. The optimum balance between the improved separation of the vasopressins and clear resolution of felypressin between arginine vasotocin and lypressin was at about 50–60 m*M* CHAPS (Figs. 8 and 9).

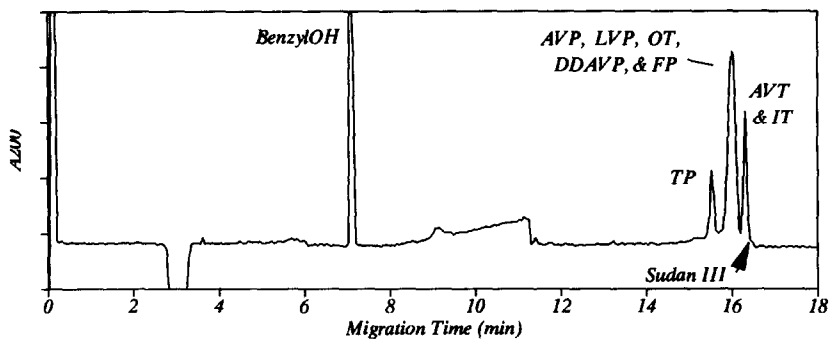

Fig. 5. MEKC of peptides in SDS. Conditions: 30°C, 15 kV, 200 M*M* SDS in 20 m*M* Tris pH 8.0. Abbreviations as in Fig. 1. The migration position of sudan III is indicated.

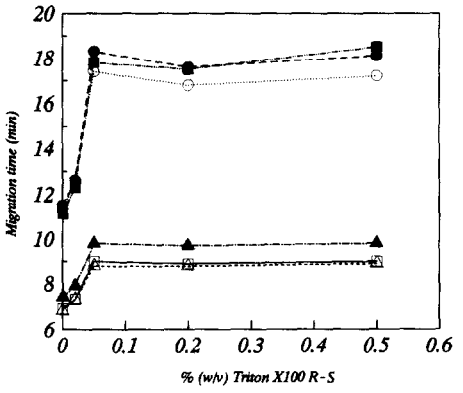

Fig. 6. Dependence of migration time on Triton X100R-s concentration. Conditions: 30°C, 15 kV, 0.1 *M* phosphate pH 2.5 with specified concentration of Triton X100R. Symbols: □ = AVT, AVP and LVP; △ = FP; ▲ = TP; ● = OT; ○ = OT; ■ = DDAVP. (Abbreviations as in Fig. 1).

DISCUSSION

RP-HPLC

The failure of HPLC system c to provide satisfactory peak shapes was not entirely unexpected, since we have found in the past that, when using mobile phases containing little or no salt for RP-HPLC, the quality of the separation obtained is very dependent on the nature of the chromatographic packing. We have therefore preferred to use system d (which is simply system a with $(NH_4)_2SO_4$ added) when carrying out the assay of oxytocin in oxytocin and ergometrine injection. In the case of system a separation was satisfactory, but peaks were slightly sharper and resolution better in system d (Fig. 2a), which we therefore favour. Although the separation at pH 7 (system b, Fig. 2b) was inferior, the differ-

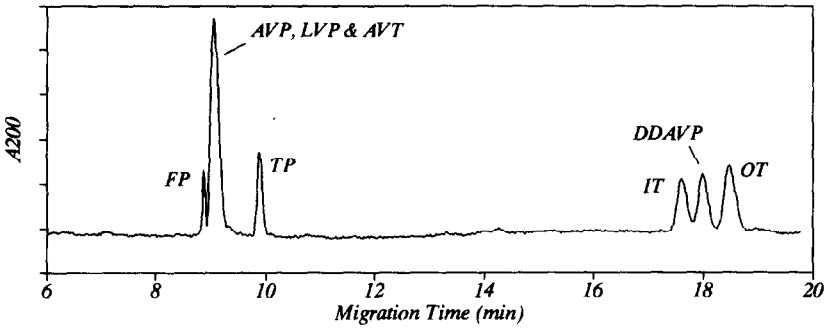

Fig. 7. Separation of peptides in presence of Triton X100R. Conditions: 30°C, 15 kV, 0.05% (w/v) Triton X100R in 0.1 *M* phosphate pH 2.5. Abbreviations as in Fig. 1.

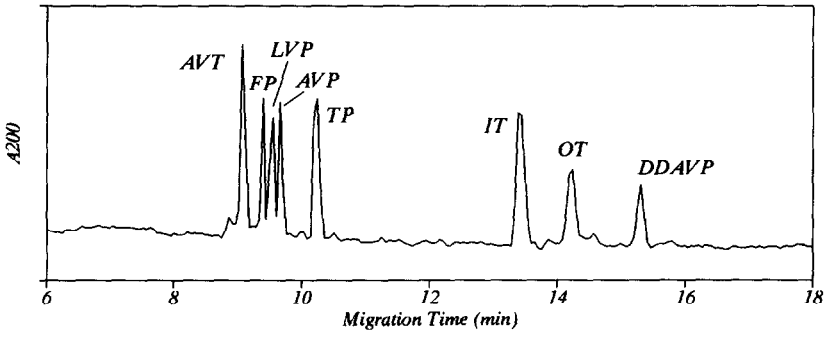

Fig. 8. Separation of peptides in presence of CHAPS. Conditions: 30°C, 15 kV, 60 m*M* CHAPS in 0.1 *M* phosphate pH 2.5. Abbreviations as in Fig. 1.

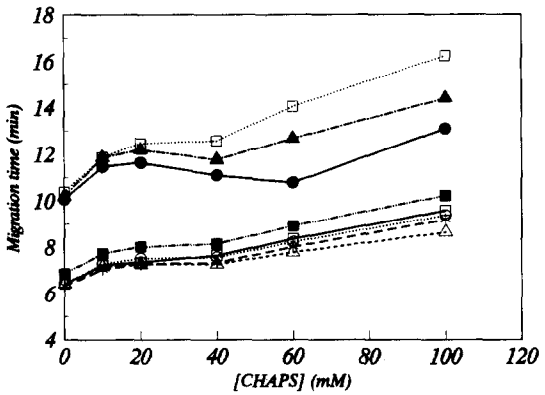

Fig. 9. Dependence of migration times on concentration of CHAPS. Conditions: 30°C, 15 kV, specified concentration of CHAPS added to 0.1 *M* phosphate pH 2.5. Symbols: △ = AVT; ✳ = FP; ○ = LVP; □—□ = AVP; ■ = TP; ● = IT; ▲ = OT; □·····□ = DDAVP. (Abbreviations as in Fig. 1).

ence in elution order may be valuable for specific purposes. One reason for this difference is the suppression of ionisation of the α-amino group, since oxytocin and felypressin are delayed relative to desmopressin at pH 7, and this may be the reason for the relative shift in order of terlipressin, since the triglycine N-terminal extension should not show the abnormally low pK_a of the N-terminal cystine in the vasopressins (see Fig. 4). Another factor is the increased interaction of amino and guanido groups with unreacted surface silanols on the silica, which are largely unionised at pH 2 and largely ionised at pH 7: such interaction would be likely to affect the vasopressin derivatives, with an extra basic group, more than oxytocin and isotocin. It is interesting to compare the actual elution orders with those predicted by several methods (Table I) which in principle might be used to identify the different analogues. Six of these are based on regression analysis of HPLC retentions [39–42], while two are based on fragmental hydrophobicity calculations [43–44]. There is poor correlation between the elution orders observed and those predicted, those for isotocin being particularly poor. It is true that these figures were derived for peptides which were not conformationally constrained as is the case with the disulphide bridged ring structures of the hypophyseal peptides. It is also clear that local structural effects within the peptides may also strongly influence interaction with RP packings [45]. Nevertheless it is disappointing that only half predict the relative elution order of argipressin and lypressin, and prediction methods appear to be of limited assistance in identifying closely related peptides such as these.

Capillary electrophoresis

Calculation of theoretical titration curves for the peptides suggested that the greatest charge discrimination would occur in the range pH 7–10. However in practice the high endosmotic flow above pH 6 resulted in low separation times and poor resolu-

TABLE I

PREDICTED ORDER OF RP-HPLC RETENTION

Peptide[a]	Actual elution order[b]	Predicted elution order						
		[39] pH 2	[39] pH 7	[40]	[41] pH 2, pH 7	[42]	[43]	[44]
IT	1	5	7	3	6	2	7	6
AVT	2	1	1	1	3	1	1	1
LVP	3	2	4	4	2	4	3	1
AVP	4	4	3	2	4	5	2	4
TP	5	3	2	6	1	3	4	1
FP	6	6	5	5	5	7	5	5
OT	7	7	6	7	7	6	6	6

[a] Abbreviations as in Fig. 1.
[b] System d (Fig. 2a).

tion, and it appeared that the control of endosmosis with buffer additives or suitably treated capillaries would be necessary to exploit these expected differences. The best CZE separation was obtained at pH 2.5. The general dependence of mobilities on pH agreed with theoretical expectations, with desmopressin showing no significant change in mobility over the pH range examined and terlipressin showing evidence of a significantly hgiher α-amino pK_a than the others. It is interesting that felypressin has a significantly lower mobility at pH 2.5 than lypressin: the difference between the two amounts to a single oxygen atom (the difference between phenylalanine in felypressin and tyrosine in lypressin). The difference in mobility can hardly be due to a charge difference, since pH 2.5 is too far from any significant pK_a, and must be due to some subtle effect on solvation or, perhaps, conformation [19]. Similar factors must be at work to explain the difference in mobility between oxytocin and isotocin, which differ by two neutral substitutions (Gln/Ser and Leu/Ile): though isotocin is about 4% lower in mass the difference in mobility is almost 10%, but the glutamine residue clearly provides greater opportunity for interaction with solvent than does serine. The difference in mobility between lypressin and terlipressin (approximately 20%) appears to agree well with the mass difference of almost 15%, since for peptides of equal charge mobility is approximately inversely proportional to the 2/3 power of the molecular mass [22,23].

With the micellar systems, the unsuitability of micelles of opposite charge for separating peptides by MEKC has been noted previously [35]. We have had success with CTAB in separating other cationic peptides which exhibit UV adsorption at 280 nm [46], and it is possible that micellar systems using alternative cationic detergents may be easier to use at low wavelengths (for instance dodecyltrimethylammonium or hexadecyltrimethylammonium [35]), and equally successful. However within the constraints of this study the most successful micellar additive was CHAPS, which was successful in resolving the group of doubly charged closely related derivatives argipressin, lypressin and argitocin. Within the charge groups the relative order of elution corresponded to the RP-HPLC elution order with the exception of felypressin, which is eluted from the RP-HPLC columns considerably later than lypressin, but migrates before lypressin in CE.

CONCLUSIONS

Systems suitable for identifying neurohypophyseal peptides and their derivatives by RP-HPLC and by micellar CE were developed, though within the constraints of the capillaries and electrolytes used a complete separation by CZE was not achieved. While the separations obtained were not any better or dramatically faster than RP-HPLC, the facility to exploit directly the difference in charge between the different peptides confers a distinct and useful selectivity to CE.

REFERENCES

1 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27.
2 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
3 M. E. F. Biemond, W. A. Sipman and J. Olivié, *J. Liq. Chromatogr.*, 2 (1979) 1407.
4 K. Krummen, F. Maxl and F. Nachtmann, *Pharm. Technol. Int.*, 2 (1979) 37.
5 F. Maxl and K. Krummen, *Pharm. Acta Helv.*, 53 (1978) 207.
6 D. H. Calam, *J. Chromatogr.*, 167 (1978) 91.
7 F. Nachtmann, *J. Chromatogr.*, 391 (1979) 391.
8 P. H. Corran and D. H. Calam, A. Frigerio and L. Renoz, (Editors) *Recent Developments in Chromatography and Electrophoresis; Proc. 9th Int. Symp. on Chromatography and Electrophoresis, Riva del Garda, May, 1978*, Elsevier, Amsterdam, 1979, p. 341.
9 F. Nachtmann, K. Krummen, F. Maxl and E. Riemer, in K. Florey (Editor), *Analytical Profiles of Drug Substances*, Vol. 10, Academic Press, New York, 1981, pp. 563–599.
10 R. A. Pask-Hughes, P. H. Corran and D. H. Calam, *J. Chromatogr.*, 214 (1981) 307.
11 R. A. Pask-Hughes, R. E. Hartley and R. E. Gaines Das, *J. Biol. Stand.*, 11 (1983) 13.
12 M. Andre, *J. Chromatogr.*, 351 (1986) 341.
13 M. Ohta, H. Fukuda, T. Kimura and A. Tanaka, *J. Chromatogr.*, 402 (1987) 392.
14 D. S. Brown and D. S. Jenke, *J. Chromatogr.*, 410 (1987) 157.
15 F. Maxl and W. Siehr, *J. Pharm. Biomed. Anal.*, 7 (1989) 211.
16 *British Pharmacopoeia 1980*, HMSO, London, 1980.
17 *British Pharmacopoeia Addendum 1992*, HMSO, London, 1992.
18 *European Pharmacopoeia 2nd Edition, Fasc. 15*, Maisonneuve, Saint-Raffine, 1991.
19 P. D. Grossman, K. J. Wilson, G. Petrie and H. H. Lauer, *Anal. Biochem.*, 713 (1988) 265.
20 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
21 J. C. Grossman, J. C. Colburn and H. H. Lauer, *Anal. Biochem.*, 179 (1989) 28.
22 Z. Deyl and V. Rohlicek, *J. Chromatogr.*, 480 (1989) 371.
23 E. C. Rickard, M. M. Strohl and R. G. Nielsen, *Anal. Biochem.*, 197 (1991) 197.
24 K. A. Cobb and M. Novotny, *Anal. Chem.*, 64 (1992) 879.
25 J. P. Advis, L. Hernandez and N. A. Guzman, *Peptide Res.*, 2 (1989) 389.

26 G. McLaughlin, R. Palmieri and K. Anderson, in J. J. Villafranca (Editor), *Techniques in Protein Chemistry II*, Academic Pess, San Diego, CA, 1991, p. 3.
27 T. A. A. M. van de Goor, P. S. L. Janssen, J. W. van Nispen, M. J. M. van Zeeland and F. M. Everaerts, *J. Chromatogr.*, 545 (1991) 379.
28 R. G. Nielsen, R. M. Riggin and E. C. Rickard, *J. Chromatogr.*, 480 (1989) 393.
29 P. Camilleri, G. N. Okafo, C. Southan and R. Brown, *Anal. Biochem.*, 198 (1991) 36.
30 M. Castagnola, L. Cassianbo, R. Rabino and D. V. Rosetti, *J. Chromatogr.*, 572 (1991) 51.
31 W. Nashabeh and Z. E. R. Rassi, *J. Chromatogr.*, 536 (1991) 31.
32 M. J.-F. Suter, B. B. DaGue, W. T. Moore, S.-N. Lin and R. M. Caprioli, *J. Chromatogr.*, 553 (1991) 101.
33 G. N. Okafo, and P. Camilleri, *J. Chromatogr.*, 547 (1991) 551.
34 J. Liu, K. A. Cobb and M. Novotny, *J. Chromatogr.*, 519 (1990) 189.
35 H. J. Issaq, G. M. Janini, I. Z. Atamna, G. M. Muschik and J. Lukzo, *J. Liq. Chromatogr.*, 15 (1992) 1129.
36 Y. Walbroehl and J. W. Jorgenson, *Anal. Chem.*, 58 (1986) 479.
37 S. E. Moring and J. A. Nolan, *Appl. Biosystems Res. News*, 2 (1990) 6.
38 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
39 J. L. Meek and Z. L. Rosetti, *J. Chromatogr.*, 211 (1981) 15.
40 K. J. Wilson, A. Honegger, R. P. Stotzel and G. J. Hughes, *Biochem. J.*, 199 (1981) 31.
41 D. Guo, C. Mant, A. Taneja, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 499.
42 C. A. Browne H. P. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
43 I. Molnár and C. Horváth, *J. Chromatogr.*, 142 (1977) 623.
44 V. Pliška, M. Schmidt and J.-L. Fauchère, *J. Chromatogr.*, 216 (1981) 79.
45 R. A. Houghten and S. T. DeGraw, *J. Chromatogr.*, 386 (1987) 223.
46 P. H. Corran and N. Sutcliffe, *J. Chromatogr.*, 636 (1993) 87.
47 J. Frenz, S.-L. Wu and W. S. Hancock, *J. Chromatogr.*, 480 (1989) 379.