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Journal of Chromatography A, 853 (1999) 263–275

JOURNAL OF CHROMATOGRAPHY A

# Separation of selected peptides by capillary electroendoosmotic chromatography using 3 μm reversed-phase bonded silica and mixed-mode phases

K. Walhagen[a,*], K.K. Unger[a], A.M. Olsson[b], M.T.W. Hearn[c]

[a]*Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg-Universität, Duesbergweg 10–14, D-55128 Mainz, Germany*

[b]*Ferring AB, Pharmaceutical and Analytical R&D, P.O. Box 30047, S-200 61 Malmö, Sweden*

[c]*Centre for Bioprocess Technology, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia*

**Abstract**

The retention behaviour and selectivity of selected basic, neutral and acidic peptides have been studied by capillary electroendoosmotic chromatography (CEC) with Hypersil $C_8$, $C_{18}$, Hypersil mixed-mode, and Spherisorb $C_{18}$/SCX columns, 250 (335) mm×100 μm, packed with 3 μm particles, and eluted with mobile phases composed of acetonitrile–triethylamine–phosphoric acid (TEAP) at pH 3.0 using a Hewlett-Packard Model $HP^{3D}$CE capillary electrophoresis system. The selected peptides were desmopressin (D), two analogues (A and B) of desmopressin, oxytocin (O) and carbetocin (C). The peptides eluted either before or after the electroendoosmotic flow (EOF) marker, depending on the concentration of acetonitrile used and the buffer ionic strength. The retention and selectivity of these peptides under CEC conditions were compared to their behaviour in free zone capillary electrophoresis (CZE), where the separation mode was based on the electrophoretic migration of the analytes due to their charge and Stokes radius properties. In addition, their retention behaviour in RP-HPLC was also examined. As a result, it can be concluded that the elution process of this group of synthetic peptides in CEC with a TEAP buffer at pH 3.0 is mediated by a combination of both electrophoretic migration processes and retention mechanisms involving hydrophobic as well as silanophilic interactions. This CEC method when operated with these 3 μm reversed-phase and mixed-mode sorbents with peptides is thus a hybrid of two well-known analytical methods, namely CZE and RP-HPLC. However, the retention behaviour and selectivity of the selected peptides differs significantly in the CEC mode compared to the RP-HPLC or CZE modes. Therefore this CEC method with these peptides represents an orthogonal analytical separation procedure that is complimentary to both of these alternative techniques. 

*Keywords:* Electrochromatography; Stationary phases, LC; Mobile phase composition; Peptides

## 1. Introduction

After its introduction in 1981 capillary electroendoosmotic chromatography (CEC) has been an area of intensive research and development [1–19]. The major focus was on the packing of the fused-silica capillary, the column efficiency, the origin and control of electroendoosmotic flow (EOF) and the retention and selectivity of analytes using *n*-octadecyl bonded silicas, such as 3 μm Hypersil ODS (Hypersil, Runcorn, UK).

One of the most promising potential areas to

*Corresponding author.

0021-9673/99/$ – see front matter 
PII: S0021-9673(99)00545-2

development of CEC lies in the separation of charged and polar analytes, e.g., peptides. The selectivity for neutral compounds in reversed-phase high-performance liquid chromatography (RP-HPLC) corresponds well to that observed in CEC. The most noticeable effects are associated with the efficiency of CEC columns that increase the overall selectivity of the neutral compounds. However, with charged analytes the impact of both multimodal chromatographic retention phenomena and electrophoretic migration effects is apparent. For example, the carboxylic functional groups of the peptides are protonated at low pH values and therefore use of buffers of low pH is desirable. Since peptides, depending on their amino acid sequence, can be acidic, basic or neutral, selection of the relevant buffer to ensure that an appropriate charge state is achieved forms an essential part of an optimisation protocol for successful resolution. When low pH buffers are used the EOF decreases tremendously [20], mainly because some of the silanol groups become protonated. One way to retain high EOF with buffers of low pH is to use columns packed with ion-exchange or mixed-mode phases [21]. Nevertheless, basic peptides can exhibit severe tailing due to silanophilic interaction with the ionised silanol groups and the positively charged functional groups of the peptide. This is a well known factor in the RP-HPLC separation of peptides [22] and can be solved by end-capping the free silanol groups in the packing material. However, end-capped $C_8$- or $C_{18}$-bonded silica does not represent an acceptable alternative in CEC due to the significant decrease in EOF. The tailing of the peptides in RP-HPLC and CEC can also be suppressed by adding a base to the eluent or using an ion-pairing reagent [22,23]. Gillot et al. [24] demonstrated the effect of adding a competing base such as triethylamine or triethanolamine in CEC to improve the peak symmetry of pharmaceutical compounds. Similar work was also performed by Lurie et al. [25] who instead added hexylamine to the eluent. As an alternative approach to overcome peak tailing and to enhance resolution, chemically modified etched capillaries have been proposed for the CEC separation of biopolymers [9,19,26].

In the present investigation, the retention behaviour and selectivity of five basic, neutral and acidic peptides have been studied in the CEC mode with the Hypersil $C_8$ and $C_{18}$ columns, Hypersil mixed-mode column and also Spherisorb $C_{18}$/SCX column. The selected peptides were desmopressin (D), desmopressin analogue A (A) which includes Arg and one amino acid with a carboxylic group, desmopressin analogue B (B) which includes Arg and two amino acids with carboxylic groups, oxytocin (O) and carbetocin (C) as shown in Fig. 1. Some of these peptides are potent pharmaceutical substances. Desmopressin is a synthetic structural analogue of the antidiuretic hormone arginine vasopressin and is used for treatment of, e.g., nocturen enuresis. Oxytocin is a natural hormone and it is used for, e.g., the induction or enhancement of labour at the time of parturition, and finally carbetocin is an oxytocin-analogue specific for modulation of uterine contraction. Experimental data obtained with this peptide set by CEC, capillary zone electrophoresis (CZE) and RP-HPLC procedures have thus enabled the molecular basis of the differences in the retention behaviour and selectivity observed for synthetic peptides in

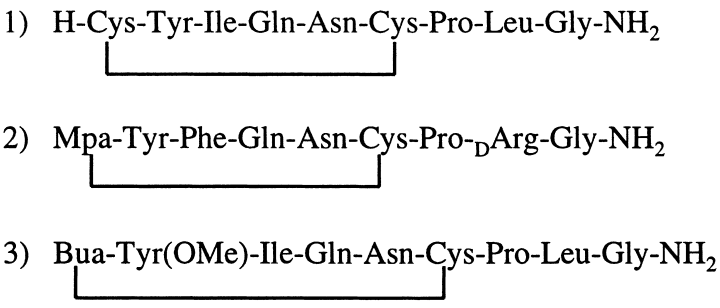


Fig. 1. Structures of the synthetic peptides (1) oxytocin (O) (2) desmopressin (D); Mpa is merkaptopropionic acid (3) carbetocin (C); Bua is butyric acid.

these different orthogonal modes of separation to be further elaborated.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile of HPLC ultra pure gradient grade was purchased from J.T. Baker (Deventer, The Netherlands). Triethylamine minimum (99%) was purchased from Sigma (Deisenhofen, Germany). Orthophosphoric acid 85% (v/v) was obtained from Fluka (Deisenhofen, Germany). Tris(hydroxymethyl)aminomethane (Tris; analytical-reagent grade), hydrochloric acid 32%, (analytical-reagent grade), and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Deionised water was obtained from Milli-Q water purification system (Millipore, Eschborn, Germany). The eluents were prepared by first adjusting the buffer to the desired pH value and then mixing with the appropriate amount of acetonitrile. The triethylamine buffer was adjusted with phosphoric acid to pH 3.0 (TEAP). The ionic strength was kept constant in the eluent when the concentration of acetonitrile was varied. Usually 10 ml of eluent was prepared in following way: *X* ml of acetonitrile was added to 2 ml of 25 m*M* TEAP at pH 3.0, and (10–2–*X*) ml of water then added. Buffers of different molarity 12, 25 and 38 m*M* of TEAP at pH 3.0 were prepared in a similar manner. The Tris buffer was prepared by adding 8 ml of acetonitrile to 2 ml of 25 m*M* Tris, pH 8.0. The eluents were filtered through a 0.2-μm PTFE filter, Nalgene, Labotec (Wiesbaden, Germany). Eluents were prepared in the same manner for the CEC, CZE and RP-HPLC studies.

### 2.2. Samples

Uracil (U) (minimum 99%) was purchased from Sigma and used as the EOF marker. The following research peptides were supplied by Ferring and Euro Diagnostica (Malmö, Sweden): desmopressin (D), desmopressin analogue A (A), desmopressin analogue B (B), oxytocin (O) and carbetocin (C). All samples were dissolved as stock solutions in water (1 mg/ml) and then an aliquot of each peptide was mixed with the appropriate eluent before analysis. The samples were injected electrokinetically at 5 kV for 4 s unless otherwise mentioned.

### 2.3. Instrumentation

A Hewlett-Packard Model HP$^{3D}$CE capillary electrophoresis system (Waldbronn, Germany) was used both for the CEC and CZE experiments. UV detection was performed at 214 nm and both the inlet and outlet capillary ends were pressurised at 10 bar during analysis. The temperature of the cassette was set to 20°C. The RP-HPLC analyses were performed on a Hewlett-Packard HP1050 system with a variable-wavelength detector set at 214 nm with 10 μl of the samples injected by an autosampler.

### 2.4. Columns and capillaries

A HPLC column (150×4.6 mm) packed with Hypersil $C_8$, 5 μm, was purchased from ChromTech, (Hägersten, Sweden). The CEC columns, 250 (335) mm×100 μm, packed with Hypersil $C_8$ and Hypersil $C_{18}$ were supplied by Hewlett-Packard. The packing material in all CEC columns had a nominal particle diameter of 3 μm; the total length of the columns was 335 mm and the packed length was 250 mm. New CEC columns were conditioned in acetonitrile–25 m*M* Tris, pH 8.0 (80:20) according to the standard procedure recommended by Hewlett-Packard. The reproducibility of the $C_8$ and $C_{18}$ CEC columns was found to be maintained throughout 500–1000 injections. However, it can be noted that the use of acetonitrile in the eluents caused swelling and weakening of the polyimide coating of the capillary [27]. The inlet end of the column was frequently checked and the polyimide was burned off if it was found to be partially covering the opening of the capillary. Hypersil mixed-mode columns, 3 μm, 250 (335) mm×100 μm, were supplied by Hypersil (Runcorn, UK). Spherisorb columns packed with mixed-mode $C_{18}$/SCX phase, 3 μm, 250 (335) mm×100 μm, were purchased from InnovaTech (Hertfordshire, UK). CZE analyses were performed with polyimide-coated fused-silica tubing of 100 μm I.D., purchased from CS Chromatographie Service (Langerwehe, Germany). The fused-silica tubing was cut into capillaries of 335 mm total length and a

detection window was made at 250 mm. The capillaries for CZE were first conditioned with 0.1 *M* sodium hydroxide for 10 min and then rinsed with water and finally conditioned with the eluent.

## 3. Results and discussion

### 3.1. System suitability test

In order to monitor the performance of the various CEC columns, in these investigations a simple neutral test mixture was frequently analysed with the Hypersil $C_8$ and $C_{18}$ CEC columns with acetonitrile–25 m*M* Tris at pH 8.0 (80:20). For this purpose, the peak efficiency and symmetry of uracil (U) and three alkylbenzenes were employed as a system suitability test between experiments with the different peptides. The stability of these CEC columns, as well as the chemically modified packing material inside them, were found to be maintained throughout at least 500 injections. In some of the experiments, up to 1000 injections were performed with the Hypersil reversed-phase CEC columns. The stability and lifetime of the CEC column depend on the composition of the buffer and the pH, the quality of the frits, the maintenance of inlet and outlet ends free of polyimide coating debris, the quality of the samples used for injection, and finally, and from a cost and performance perspective, the care taken for the handling of the capillary column when placed into the capillary cassette or also when manipulated by the instrument.

### 3.2. Retention factor $k_{cec}$

The CEC retention factor ($k_{cec}$) calculated in this study is analogous to the capacity factor employed in RP-HPLC. Thus, $k_{cec}$ can be defined in terms of the elution time ($t_m$) of the analyte with regard to the influence of the EOF, the electrophoretic migration of the analyte as well as retention of the analyte by hydrophobic and silanophilic interactions. In this treatment, the elution time of an unretained component is given by $t_{eo}$. The value of $k_{cec}$ can then be formalised according to Eq. (1) as the ratio of the elution time of the analyte and the elution time of the unretained component. A different definition of the electrochromatographic retention factor $k'_c$ is given by Rathore and Horváth [28]. If the value of the retention factor is negative then the analyte elutes before the EOF marker.

$$k_{cec} = (t_m - t_{eo})/t_{eo} \quad (1)$$

### 3.3. Hypersil n-octylsilica

In RP-HPLC as well as in CEC, the retention factors of peptides mainly depend on the concentration of the acetonitrile, the buffer ionic strength, the pH of the eluent, the length of the *n*-alkyl chain bonded to the silica and also on the charge density and intrinsic hydrophobicity of the peptide analyte. When the peptides elute before the EOF marker, uracil, the predominant transport process involved as these analyte migrate through the column is due to electrophoretic processes, but the selectivity of the peptides at high concentrations of acetonitrile emphasise still the importance of hydrophobic and silanophilic interactions. The influence of two different buffer ionic strengths, 25 m*M* TEAP and 38 m*M* TEAP at pH 3.0, with the Hypersil $C_8$ columns is illustrated in Fig. 2. As evident from these data, the interactive behaviour of carbetocin is not affected by the increase of buffer ionic strength from 25 m*M* to 38 m*M* TEAP at pH 3.0. In contrast, the silanophilic interaction of desmopressin is reduced at 38 m*M* compared to 25 m*M* TEAP as shown in an overlaid plot in Fig. 3. These charged peptides elute before the EOF marker with 30% acetonitrile and 38 m*M* TEAP at pH 3.0 but with 25 m*M* TEAP as the buffer, these peptides elute after the EOF marker. Oxytocin and desmopressin were similarly analysed using a Hypersil $C_8$ CEC column at three different buffer ionic concentrations. From theoretical considerations, the impact of buffer ionic strength is expected to be significant for charged peptides, since increasing buffer concentrations are expected to result in reduced silanophilic interactions as shown by the chromatograms in Fig. 4. From these results it can be concluded that as the molarity of the eluents was increased a lower EOF occurred coupled with weaker silanophilic interaction with the analytes. If the buffer concentration is kept constant and the content of acetonitrile was decreased then the re-

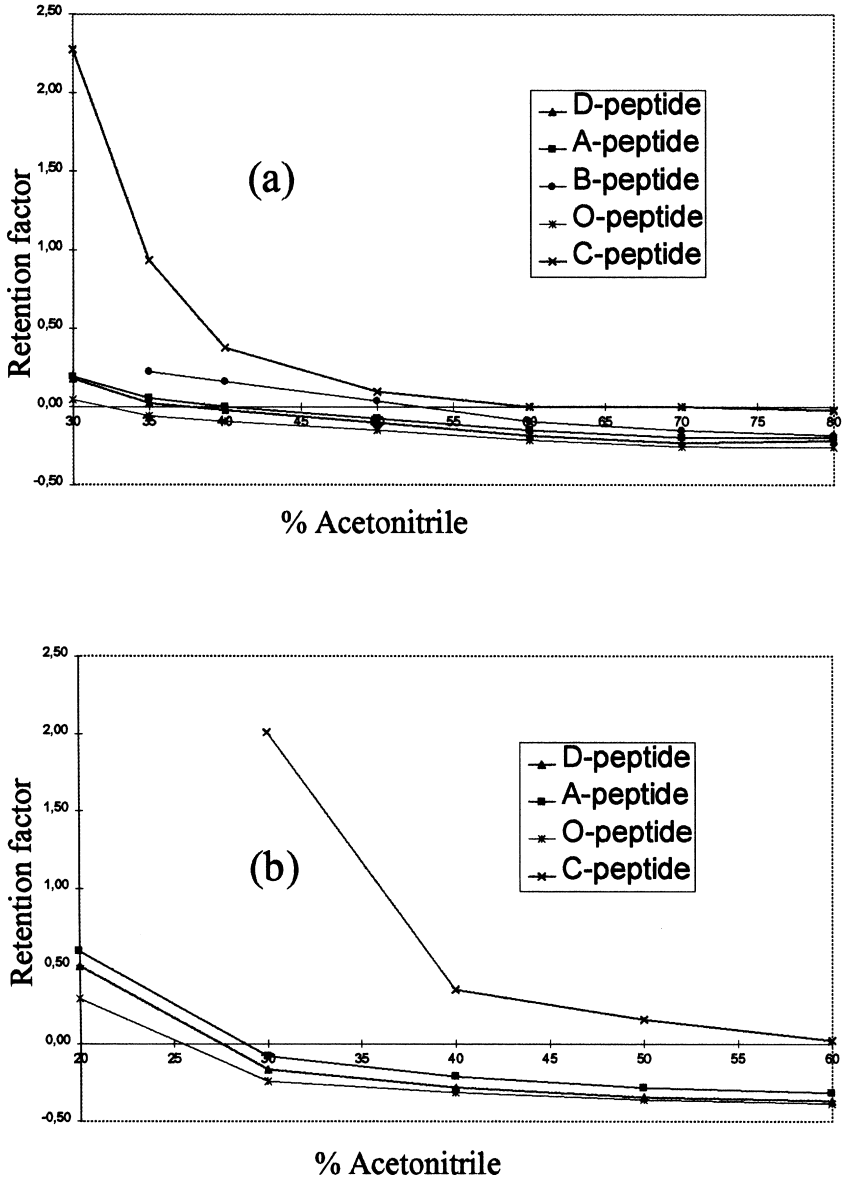


Fig. 2. The retention factors of the synthetic peptides vs. the concentration of acetonitrile in the buffer. Hypersil $C_8$ CEC column, 20 kV, with the eluents prepared from $X$ ml acetonitrile added to 2 ml 25 m$M$ TEAP or 38 m$M$ TEAP at pH 3.0, and $(10-2-X)$ ml water added to a total volume of 10 ml. (a) 25 m$M$ TEAP and (b) 38 m$M$ TEAP.

tention of the peptides were increased as shown in Fig. 5. Under these conditions, the retention factors ($k_{cec}$) of desmopressin and oxytocin change from negative to positive values with the peak shapes of the peptides progressively becoming broader as they elute later. In particular, oxytocin and desmopressin both exhibited significant peak tailing in the presence of 20% acetonitrile, indicating that the silanophilic interaction with the basic peptides is now more pronounced.

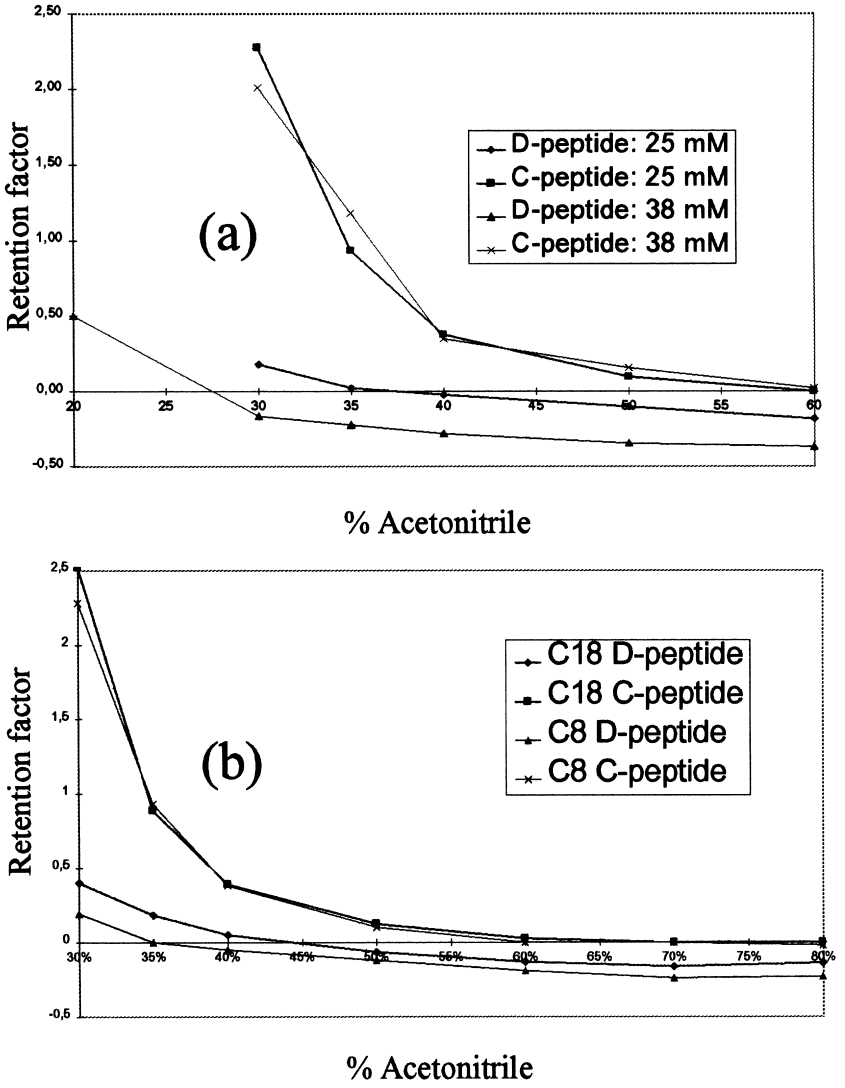


Fig. 3. An overlay of the plots of the retention factors of desmopressin (D) and carbetocin (C) vs. the concentration of acetonitrile. Eluents: *X* ml acetonitrile added to 2 ml 25 m*M* TEAP or 38 m*M* TEAP at pH 3.0, and (10−2−*X*) ml water added to a total volume of 10 ml. (a) Hypersil $C_8$ CEC column at 25 m*M* and 38 m*M* TEAP, respectively; (b) Hypersil $C_8$ and $C_{18}$ CEC columns, respectively, at 25 m*M* TEAP.

### *3.4. Hypersil n-octadecylsilica*

The retention and selectivity of the peptides have also been studied with Hypersil $C_{18}$ CEC columns, with the same eluents and conditions as used for the Hypersil $C_8$ CEC columns. The main difference between the two stationary phases is the length of the *n*-alkyl chain, which will of course contribute to a more pronounced hydrophobic interaction with the peptides. The retention factors ($k_{cec}$) of the peptides versus the percentage of acetonitrile were determined at 25 m*M* TEAP at pH 3.0 for the Hypersil $C_{18}$ column and the results are shown in Fig. 6. A comparison of the $k_{cec}$ values for desmopressin and carbetocin separated with Hypersil $C_8$ and $C_{18}$ CEC columns, respectively, is shown in the overlay plot in Fig. 3. The influence of the variation in the *n*-alkyl chain length of the two reversed-phase packing

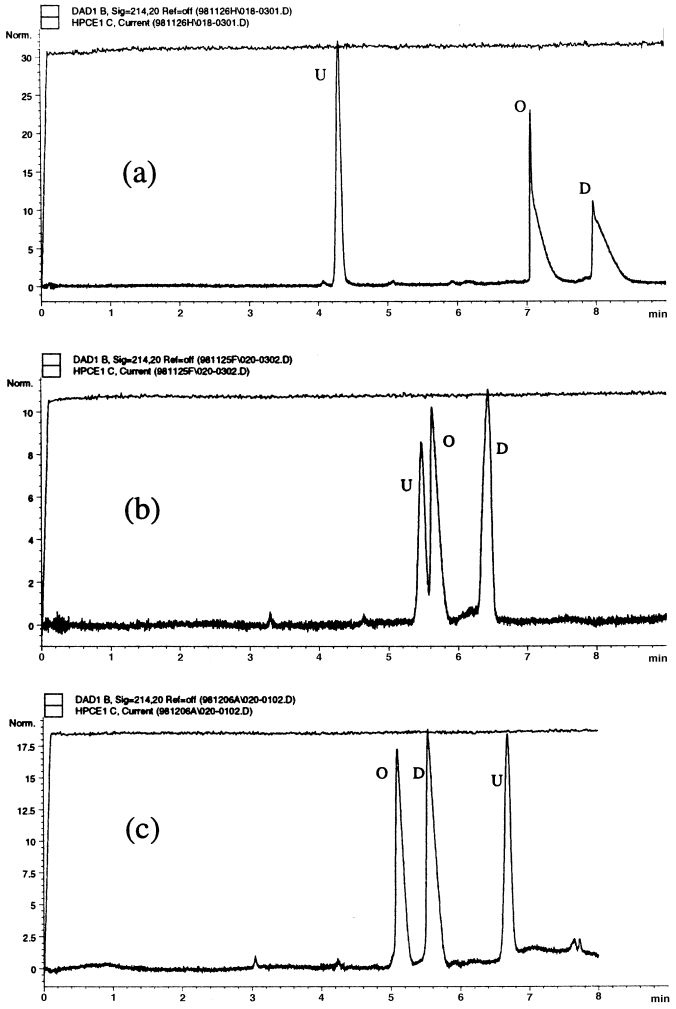


Fig. 4. Elution profiles for oxytocin (O) and desmopressin (D) separated with a Hypersil $C_8$ CEC column at three different buffer ionic strengths, EOF marker uracil (U), 20 kV; the eluents were: 3 ml acetonitrile, 2 ml 12 m*M*, 25 m*M* TEAP or 38 m*M* TEAP at pH 3.0, 5 ml water. (a) 12 m*M* TEAP; (b) 25 m*M* TEAP; and (c) 38 m*M* TEAP.

materials on the CEC behaviour of carbetocin was not significant, whereas desmopressin was more retained with $C_{18}$- compared to the $C_8$-silica material. To obtain negative values of the retention factors of the peptides in the CEC mode, higher concentration of acetonitrile were needed with the $C_{18}$- compared to the $C_8$-bonded silica phase for the same relative retention factors to be achieved. The analytical separations of the selected peptides with a $C_{18}$ CEC column at different concentration of acetonitrile and at a constant ionic strength of 38 m*M* TEAP are shown in Fig. 7. Importantly, the EOF decreases as the acetonitrile content decreases when the buffer ionic strength was kept constant. The method was not optimised but nevertheless a baseline separation of these peptides were obtained at 40% acetonitrile. The theoretical plate numbers for the synthetic peptides eluting before the EOF marker were about 160 000 plates/m and about 120 000 plates/m for the later eluting neutral peptide.

### *3.5. Hypersil mixed-mode and Spherisorb $C_{18}$/SCX*

Two different mixed-mode phases, Spherisorb

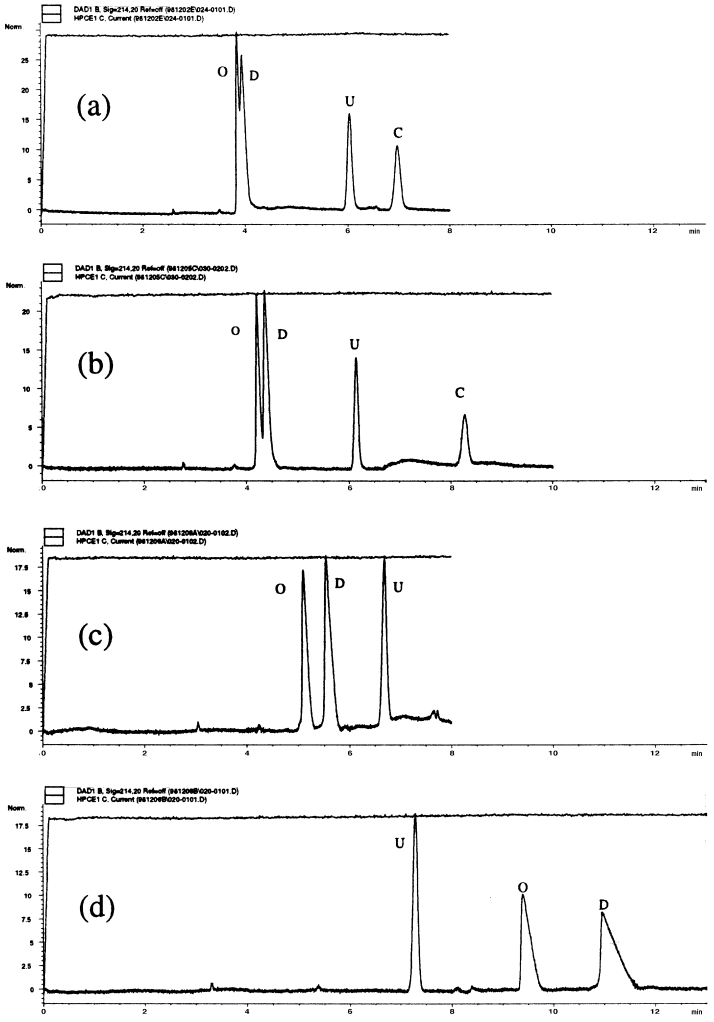


Fig. 5. Elution profile for oxytocin (O), carbetocin (C) and desmopressin (D) separated with a Hypersil $C_8$ CEC column at different concentrations of acetonitrile, EOF marker uracil (U), 25 kV, eluents: $X$ ml acetonitrile added to 2 ml 38 m$M$ TEAP at pH 3.0, and $(10-2-X)$ ml water added to a final volume of 10 ml. (a) 50% acetonitrile; (b) 40% acetonitrile; (c) 30% acetonitrile; and (d) 20% acetonitrile.

$C_{18}$/SCX and Hypersil mixed-mode, were also investigated with the synthetic peptide samples. It was interesting to observe from these preliminary studies the different elution order of desmopressin, desmopressin analogue A and oxytocin for the two mixed-mode packing materials. This behaviour was probably due to different charge density of the surface of the mixed-mode packing materials. The benefit of using this type of RP/SCX mixed-mode phases in the CEC mode are the contribution of a high EOF even at low pH because of the ionised sulphonate groups. With these sorbents, the peptides interact and are retained by the hydrophobic stationary phase but also by ionic interactions with the sulphonate groups. The values of the retention factors for the separated peptides on the mixed-mode CEC columns were thus positive at high solvent concentrations such as 60% acetonitrile. The chromatograms of these separation are shown in Fig. 8. The linear EOF velocity was about the 1.5 mm/s for

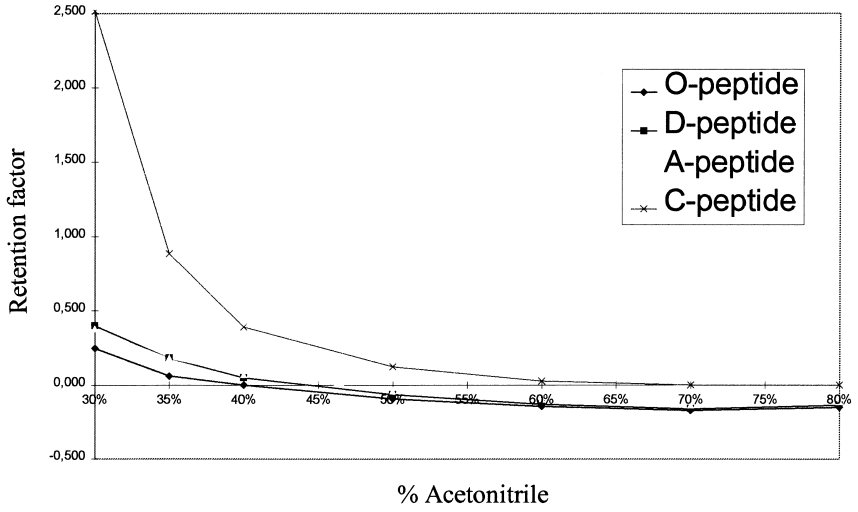


Fig. 6. The retention factors of the synthetic peptides vs. the concentration of acetonitrile with a Hypersil $C_{18}$ CEC column, 20 kV, eluents: $X$ ml acetonitrile added to 2 ml 25 m$M$ TEAP at pH 3.0, and $(10-2-X)$ ml water added to a final volume of 10 ml.

the mixed-mode phases at 60% acetonitrile and 38 m$M$ TEAP at pH 3. In comparison the linear velocity value was only 0.7 mm/s for the $C_8$- and $C_{18}$-bonded Hypersil CEC columns under similar buffer condition. An unpredictable focusing effect of the basic peptides giving raise to millions of plates/m have been observed on the mixed-mode phases. The focusing effect could sometimes be persistent for ten injections and then vanish with the eleventh injection.

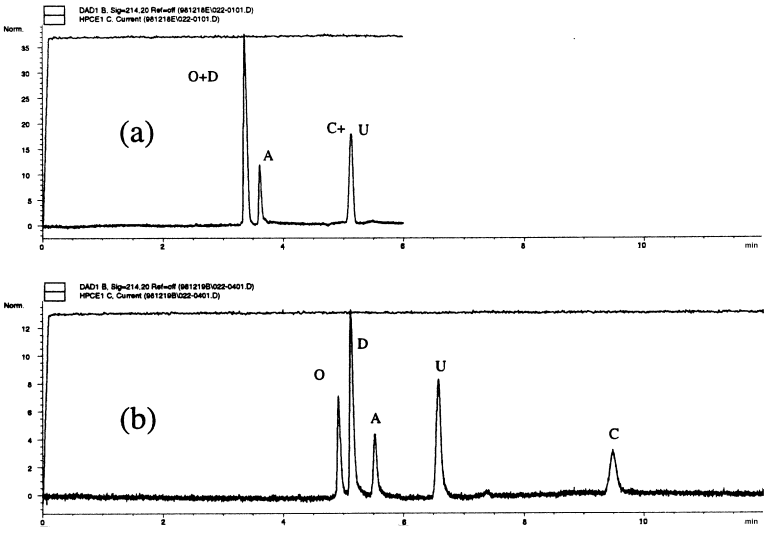


Fig. 7. Separation of the synthetic peptides with a Hypersil $C_{18}$ CEC column at different concentrations of acetonitrile, EOF marker uracil (U), 25 kV, eluents: $X$ ml acetonitrile was added to 2 ml 38 m$M$ TEAP at pH 3.0, and $(10-2-X)$ ml water added to a final volume of 10 ml. (a) 70% acetonitrile; and (b) 40% acetonitrile.

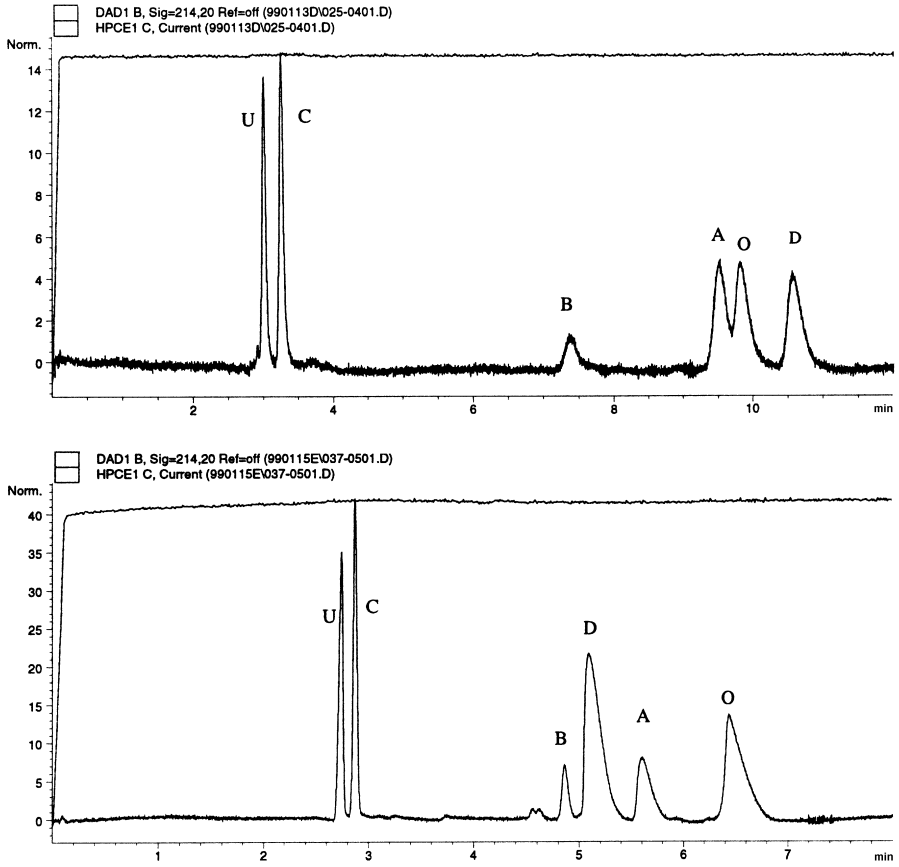


Fig. 8. Separation of the synthetic peptides with the CEC mixed-mode phases at 25 kV, using the eluent: 6 ml acetonitrile, 2 ml 38 m*M* TEAP at pH 3.0, 2 ml water, EOF marker uracil (U). (a) Spherisorb $C_{18}$/SCX; and (b) Hypersil mixed-mode.

### *3.6. CEC versus CZE of oxytocin and desmopressin*

To examine whether the magnitude of the retention factors for the peptides eluting before the EOF marker was predominantly due to chromatographic mass transport effects or if the elution behaviour was mainly caused by electrophoretic migration processes with these analytes, CZE was performed in addition to the CEC experiments. Although peptide analytes can normally be separated with aqueous buffers under such CZE conditions [3], oxytocin and desmopressin were analysed by CZE at 25 m*M* TEAP, pH 3.0 in the presence of an acetonitrile concentration altered from 30 to 80% (v/v). The values of the electromigrational retention factors of the peptides were negative at all acetonitrile concentration and their value decreased as the content of acetonitrile decreased. The retention factors for oxytocin and desmopressin versus the concentration of acetonitrile are shown in Fig. 9 for both the CEC and the CZE experiments. The conclusion that can be drawn from this comparison of the CEC versus CZE behaviour of these peptides is that electrophoretic migration as well as chromatographic interaction mechanisms contribute to the retention and selectivity of these peptides separated by CEC. Moreover, it is noteworthy that the relative $\alpha$-factors associated with the intrinsic selectivities of these orthogonal techniques show trends consistent with the molecular basis of the separation process per se. As a consequence, the opportunity arises from these investigations to explore physico-chemical and biophysical parameters associated with the molecular

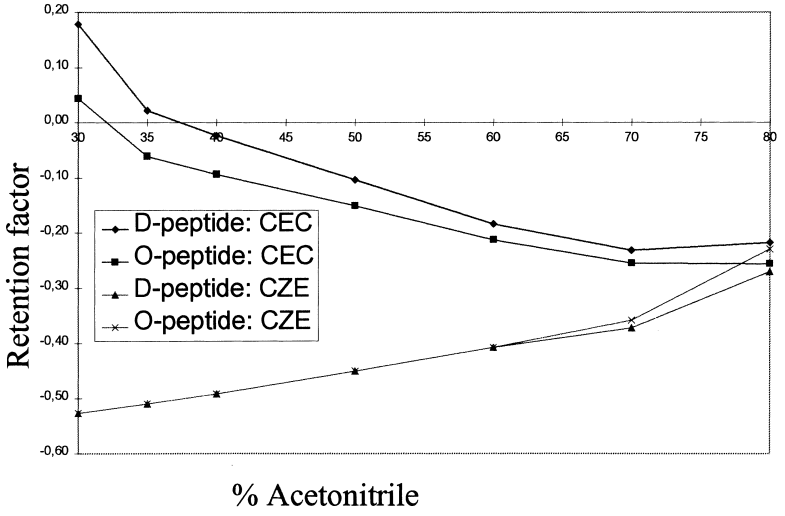


Fig. 9. Overlay of the plots of the retention factors of desmopressin (D) and oxytocin (O) vs. the concentration of acetonitrile, using the CEC Hypersil $C_8$ and CZE-opened fused-silica capillary systems operated at 20 kV, with eluents containing *X* ml acetonitrile added to 2 ml 25 m*M* TEAP at pH 3.0, and $(10-2-X)$ ml water added to a final volume of 10 ml.

properties of peptides in the CEC mode in terms of the underlying thermodynamic and extra-thermodynamic relationships in a manner analogous to that employed with RP-HPLC systems [29,30]. Further examination of this facet of CEC separations of peptides will be reported in subsequent manuscripts.

### *3.7. Comparison of RP-HPLC, CZE and CEC*

In order to provide further comparison with these analytical separation methods based on CEC and CZE electromigratory processes, the peptides have also been analysed under reversed-phase chromatographic conditions with Hypersil $C_8$, 5 μm, 150 mm×4.6 mm, HPLC columns, with the same eluent composition (60% acetonitrile, 25 m*M* TEAP, pH 3.0) at a flow-rate of 1 ml/min. Under these RP-HPLC conditions, the more hydrophobic peptide, carbetocin, eluted before desmopressin and oxytocin which co-eluted. The explanation for the reversed elution order at 60% acetonitrile must be due to the fact that the peptides are retained mainly by silanophilic interaction effects, which will predominantly influence the chromatographic properties of charged peptide analytes. However, in the CZE mode, the two charged peptides co-migrate whilst the neutral peptide eluting near to the EOF marker. When the same peptides were separated by CEC the selectivity between oxytocin and desmopressin were increased whilst the carbetocin was more effectively retained by hydrophobic interaction effects as shown in Fig. 10. When the acetonitrile content in the buffer was further decreased the uncharged peptide was more retained and the oxytocin and desmopressin were baseline separated in the CEC mode. However, in CZE mode there was no improvement of the separation of these peptides as the content of acetonitrile in the buffer was decreased.

## 4. Conclusions

The above experimental data have confirmed that the retention mechanism of these charged synthetic peptides in CEC is a hybrid of electrophoretic migration processes and the interplay of hydrophobic as well as silanophilic interactions. The retention and selectivity of the selected peptides differs significantly between these RP-HPLC, CZE and CEC modes. The selectivity results for the peptides with mixed-mode CEC columns provides a further extension of the selectivity opportunities and warrants further elucidated. CEC procedures thus represent an orthogonal analytical separation method to CZE and

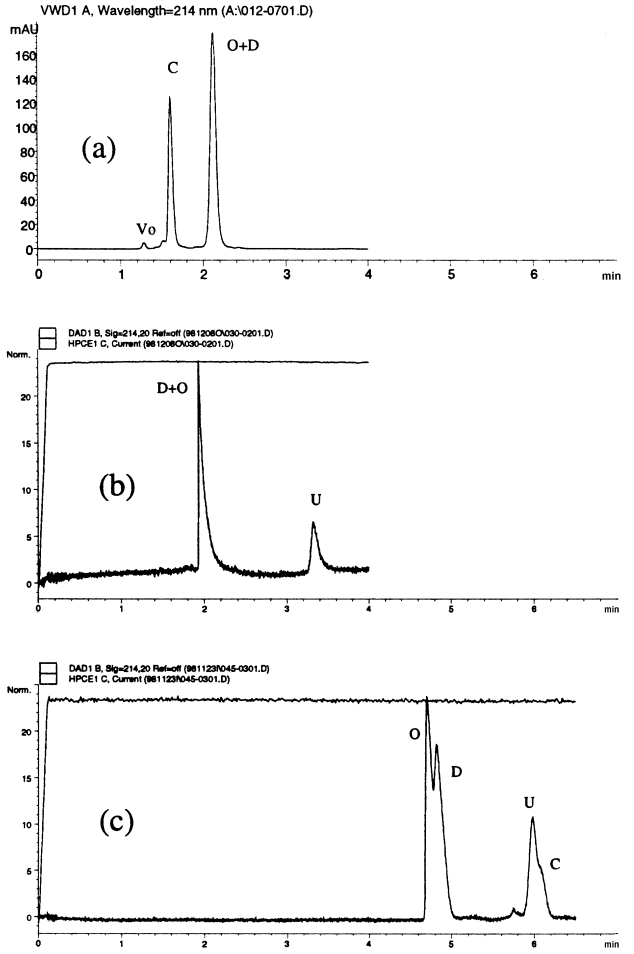


Fig. 10. Separation of oxytocin (O), desmopressin (D) and carbetocin (C) by the three different analytical separation methods, using the eluent: 6 ml acetonitrile, 2 ml 25 m*M* TEAP at pH 3.0, 2 ml water. (a) HPLC; Hypersil $C_8$, (b) CZE; opened fused-silica capillary, 25 kV, and (c) CEC; Hypersil $C_8$, 20 kV.

HPLC, respectively, and thus should with additional development have considerable potential in the analysis of synthetic peptides.

## Acknowledgements

This work was supported by Ferring AB, Sweden. We would like to thank Drs. G.P. Rozing and M.M. Dittmann at Hewlett-Packard, Waldbronn, Germany, for their excellent co-operation and for supplying us with Hypersil $C_8$ and $C_{18}$ CEC columns. We also would like to thank Dr. P. Ross at Hypersil, Runcorn, UK for excellent co-operation and supplying us with Hypersil mixed-mode columns.

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