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Application of capillary electrochromatography to the separation of phenylthiohydantoin-amino acids

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Abstract

The applicability of capillary electrochromatography with 1.5-μm ODS-modified non-porous particles for the separation of phenylthiohydantoin (PTH)-amino acids was investigated. The effect of the pH, organic solvent content and ion concentration of the mobile phase on the separation was investigated. On a 34-cm column, plate numbers in the order of 60 000–180 000 were obtained for the neutral and acidic PTH-amino acids. The required presence of sodium dodecyl sulfate (SDS) in the mobile phase led to broad asymmetric peaks and long retention times for the three basic PTH-amino acids (Arg, His, Lys). With a mobile phase containing a phosphate buffer with a pH of 7.2, 5 m*M* SDS, and 5% (v/v) of both acetonitrile and tetrahydrofuran, an isocratic separation in less than 8 min could be realized for all PTH-amino acids with the exception of PTH-Arg. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Electrochromatography; Mobile phase composition; Amino acids; Phenylthiohydantoin-amino acids

1. Introduction

Capillary electrochromatography (CEC) can be seen as a hybrid of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The separation mechanism in CEC is based on both the partitioning of the solutes between a mobile and stationary phase and on the electrophoretic mobility of charged solutes. This combination makes CEC a powerful technique for the separation of complex mixtures such as samples of biological origin. This type of sample often requires a highly selective and efficient separation technique. A possible application of CEC is the separation of the phenylthiohydantoin (PTH)-amino acids, resulting from the sequencing of peptides by means of the Edman degradation. The separation of PTH-amino acids is difficult because, on the one hand, closely related compounds occur (e.g., PTH-Leu and PTH-Ile) while, on the other hand, large differences in polarity occur in the same mixture. In HPLC isocratic [1–3] and gradient [4–6] elution have been applied to separate the PTH-amino acids. With both techniques overlap between solutes with similar hydrophobicity is difficult to avoid. An increase in plate numbers can be useful in those cases. From this perspective, the application of very small particles as stationary phase can increase the resolving power. In HPLC this is difficult to realize and limited to very short columns due to the extremely large pressures required to attain reasonably flow-rates [7]. In CEC, on the contrary, the electroosmotic flow (EOF) which is used as pumping mechanism is independent of the particle diameter [8,9]. This means that with CEC, in principle, very

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high plate numbers can be realized on columns packed with very small particles. Commonly 3- and 5-μm HPLC packing materials have been applied in CEC [10–13]. However, recently we have demonstrated the possibility to use 1.5-μm [14] and 1.8-μm particles [15] in CEC. On a 24-cm column packed with 1.5-μm ODS-modified non-porous particles 120 000 plates could be generated. Such a column can be suitable for separations where a high resolving power is required.

In this paper we report the results of an investigation to apply CEC for the isocratic separation of PTH-amino acids.

2. Experimental section

2.1. Apparatus

Fused-silica capillaries of 100 μm I.D.×375 μm O.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). The preparation of columns packed with 1.5-μm non-porous particles has been extensively described in our previous work [14]. The total length of the capillaries used was 42.5 cm, of which 34 cm was packed with the particles. An HPLC pump was used to flush freshly packed capillaries. All CEC experiments were performed with a HP ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector operated at 254 nm. Samples were introduced electrokinetically at the anodic side (5 kV, 2 s). In most experiments a voltage of 25 kV was applied, resulting in field strength of approximately 650 V cm^{-1} in the packed part of the capillary. The air cooling system was used to keep the temperature at 25°C in all experiments. Acetone was used as EOF marker.

2.2. Chemicals and solutions

The reversed-phase particles were 1.5-μm non-porous Chromspher-ODS that were kindly donated by Chrompack (Middelburg, The Netherlands). The PTH-amino acids were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) and tetrahydrofuran (THF) were HPLC-grade solvents. Other chemicals of analytical grade purity were obtained from various suppliers. Doubly distilled water was used in all experiments. Unless stated otherwise, the mobile phase contained 2 m*M* sodium phosphate buffer, 5 m*M* sodium dodecyl sulfate (SDS) and different volumes of an organic solvent (ACN or THF). Stock solutions of the PTH-amino acids (10 m*M*) were prepared in ACN or in a water–ACN mixture. These stock solutions were stored at −20°C and were found to be stable for several months. A stock mixture of PTH-amino acids was prepared daily. The mixture of PTH-amino acids was diluted with buffer solution prior to injection. The final concentrations of the PTH-amino acids in the sample solution were between 10^{-4} and 10^{-3} *M*. The injection volume was approximately 6 nl. All solvents and buffer solutions were filtered through a 0.45-μm HVLP Durapore filter (Millipore, Etten Leur, The Netherlands). Prior to use all solvents were degassed by ultrasonification for 20 min.

3. Results and discussion

The aim of the investigation was to demonstrate the high efficiency and speed of CEC for the separation of the 20 PTH-amino acids. The chromatographic behavior of the PTH-amino acids is strongly dependent on the type of the side group. The PTH-amino acids can be classified into four groups on the basis of their side groups (one-letter abbreviations of amino acids are used for the PTH-amino acids in the presented electrochromatograms while in the text the common three letter abbreviation are used): (**A**) hydrophilic side chain (aspargine (N), threonine (T), serine, (S), glutamine, (Q), tyrosine, (Y)); (**B**) hydrophobic side chain (glycine (G), alanine (A), proline (P), valine (V), methionine (M), iso-leucine (I), leucine (L), phenylalanine (F), tryptophan (W)); (**C**) acidic side chain (aspartic acid (D), glutamic acid (E), cysteic acid (C)); (**D**) basic side chain (histidine (H), arginine (R), lysine (K)).

Although presently considerable efforts are devoted to the development of gradient CEC, an isocratic separation is preferable, because of the simpler instrumentation and operation and better reproducibility. In order to separate the 20 PTH-amino acids isocratically on the ODS-modified particles, various parameters are available to manipulate the retention of the PTHs, such as the type and concentration of the organic modifier, the pH and the

ionic strength. The influence of these parameters was extensively studied as will be discussed below.

In our previous work with the 1.5-μm ODS-modified non-porous particles it was observed that addition of SDS to the mobile phase was necessary to attain a stable EOF and current [14]. Therefore, in the present experiments 5 m*M* SDS was added to all mobile phases used. It was noticed that with all buffers containing less than 10% (v/v) ACN, a high electroosmotic flow velocity of approximately 5 mm s^{-1} at 25 kV could be obtained. This high EOF is probably partly the result of the adsorption of SDS on the stationary phase, giving a relatively high surface charge and zeta potential. Moreover, with non-porous particles as used in this study a high EOF velocity is to be expected, due to the absence of stagnant intraparticle mobile phase as in porous packing materials.

3.1. Effect of the pH

The influence of the pH of the mobile phase on the relative velocities (observed solute velocities relative to the linear velocity of mobile phase) of the PTH-amino acids was studied using three buffer systems: borate (pH 9.2), phosphate (pH 7.2) and acetate (pH 4.8). The pH was measured in the aqueous phase before mixing with the organic solvent. In Fig. 1 the results are shown for PTH-amino acids that are representative for each group. As can be seen in the figure, the most significant influence of the pH was found for the basic PTHs, whose relative velocities increased with increasing pH. This observation is in contrast with results obtained by Huber et al. [16], who observed an increase of the retention with increasing pH for PTH-Arg. An explanation of this difference could be in the presence of SDS in the mobile phase solutions used in our experiments. For the basic PTHs three different mechanisms may exist that influence their elution behavior in CEC: (i) their positive charge at lower pH gives them an electrophoretic mobility in the same direction as the EOF; (ii) retention is possible based on hydrophobic interaction with the ODS stationary phase; and (iii) retention may be based on electrostatic interaction with negative groups on the stationary phase. For the first two processes it is expected that an increase of the mobile phase pH, decreasing the average charge on the basic compounds, will result in longer elution times: the effective cationic mobility will decrease

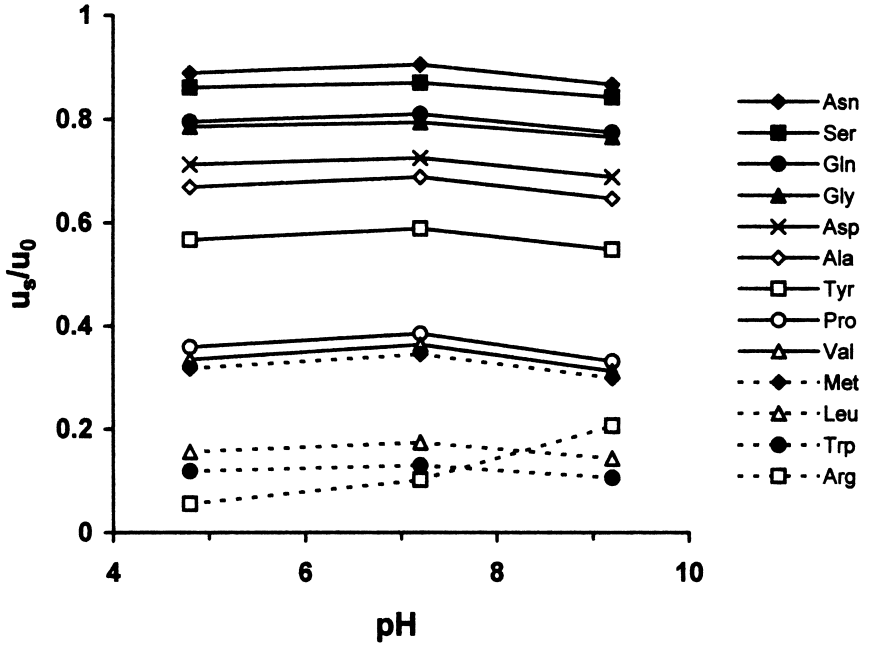

Fig. 1. Effect of the mobile phase pH on the relative velocities (compared to the velocity of the EOF) of selected PTH-amino acids. Mobile phase: 10% (v/v) ACN and 5 m*M* SDS.

and the hydrophobic interaction will increase with lower effective charge. For the third mechanism it is expected that the decreased average charge at higher pH leads to a smaller interaction with the stationary phase, and with that to shorter elution times. Apparently, the electrostatic interaction with free silanol groups and/or adsorbed SDS on the stationary phase is the most important retention mechanism for the basic PTHs in our experiments.

For the neutral and acidic compounds, the mobile phase pH had a subtle but relevant influence on their elution. At pH 9.2, Asn coeluted with Thr and Gln with Gly. At pH 4.8, three coeluting pairs of PTH-amino acids (Gln/Gly, Glu/Asp and Ala/Cys) were found. The best results were obtained at a mobile phase pH of 7.2. Therefore, in all further measurements a phosphate buffer (pH 7.2) was used.

3.2. Effect of organic solvent

In order to find the best compromise between resolution and speed, the effect of the concentration of ACN as organic modifier was investigated. The results are shown in Fig. 2. It was observed that with a high ACN content it was impossible to obtain a satisfactory separation for the hydrophilic PTH-amino acids of group A, despite the high plate number of the column. In order to attain sufficient retention for this group of solutes, low concentrations of ACN had to be used. A good resolution of the first 10, less retarded, PTHs was obtained with 10% (v/v) ACN. An electropherogram obtained under these conditions is shown in Fig. 3. However, decreasing the ACN concentration increased the retention of the other PTHs considerably and led to a long analysis time. In an attempt to decrease the retention of the hydrophobic analytes while maintaining the resolution of the hydrophilic compounds, THF was tried as an organic modifier. Fig. 4 shows a comparison of relative velocities with 10% ACN or 10% THF as organic modifier. Indeed, the retention of the hydrophobic PTHs was significantly lower with THF than with ACN. The relative velocities of the hydrophilic acidic PTHs were much less affected. With THF, PTH-Ala eluted before the three acidic side chain PTHs. The relative velocities of PTH-Lys

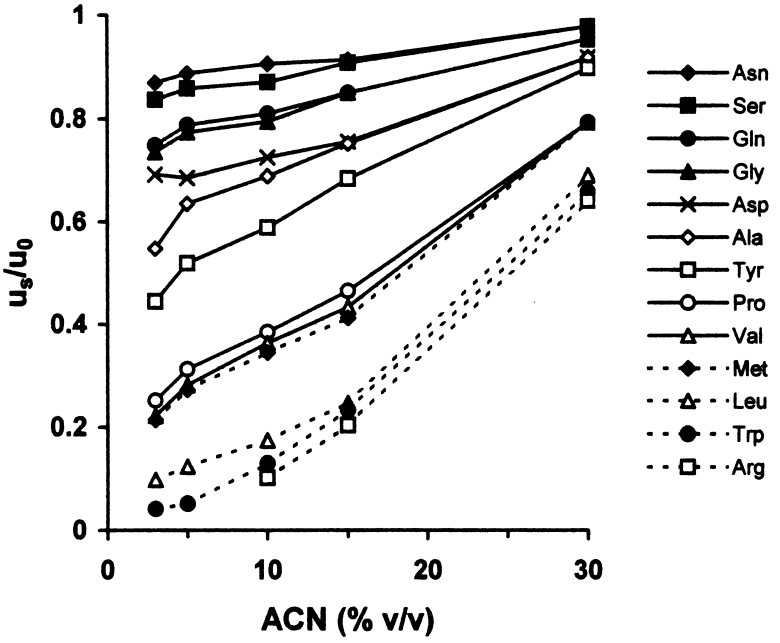

Fig. 2. Effect of the ACN concentration of the mobile phase on the relative velocities of selected PTH-amino acids. Mobile phase: 2 m*M* phosphate buffer and 5 m*M* SDS.

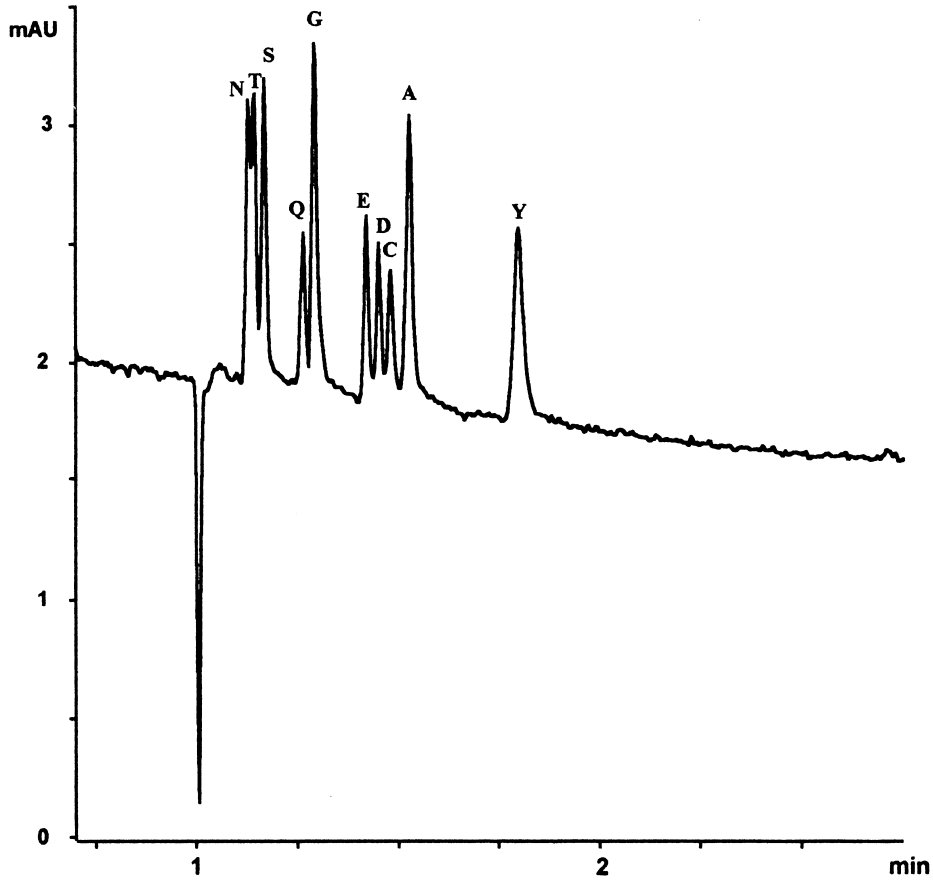

Fig. 3. Separation of the less retarded PTH-amino acids. Column: 32.5 cm×100 μm I.D., 24-cm packed bed. Mobile phase: 2 m*M* phosphate buffer (pH 7.2), 5 m*M* SDS and 10% (v/v) ACN. Applied voltage: 20 kV.

and PTH-His were also increased significantly, although the peaks of these compounds still showed severe tailing. The retention of PTH-Arg, the latest eluting compound, was not decreased.

With 10% THF it was not possible to obtain a complete separation of the hydrophilic compounds. Therefore, we tried to use a mixture of ACN and THF to retain the selectivity for the hydrophilic PTHs and speed up the separation of the hydrophobic PTHs. The relative velocities obtained with this mobile phase are presented in Fig. 4. Fig. 5 shows the separation of 19 PTHs, using 2 m*M* phosphate buffer at pH 7.2 with 5% ACN and 5% THF in the mobile phase. Under these conditions plate numbers were found for the neutral and acidic compounds ranging between 60 000 and 180 000 plates per column. The peaks of PTH-His and PTH-Lys were still broad and asymmetric, but well separated from the other peaks. The shape of the peak of PTH-Arg, with its strongly basic guanidine side chain, was even worse. Moreover, its position and shape were not repeatable.

3.3. Effect of the addition of cations to the mobile phase

As described above, a fast and efficient separation could easily be obtained for a mixture of hydrophilic, hydrophobic and acidic side chain PTH-amino acids, while the separation of the three basic side chain PTHs was problematic. In the literature it has been

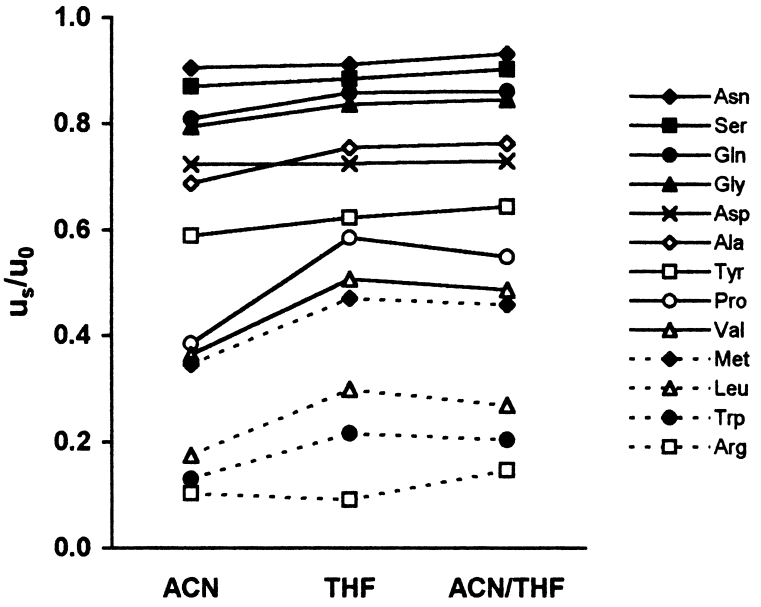

Fig. 4. Effect of the organic modifier type on the relative velocity of selected PTH-amino acids. Mobile phase: 2 m*M* phosphate buffer and 5 m*M* SDS.

mentioned frequently that the CEC separation of strongly basic compounds is not feasible with reversed-phase materials, due to strong electrostatic interactions with unprotected silanol groups on the stationary phase. For strongly basic analytes the application of strong cation-exchange stationary phases has been suggested by Smith and Evans [17] and Euerby et al. [18].

As has been discussed above, in our experiments the retention of the basic, positively charged PTHs is dominated by electrostatic interaction with the negatively charged stationary phase. This electrostatic interaction may be especially strong because of the required presence of SDS in the mobile phase; SDS adsorbs on the hydrophobic particles and creates a more negatively charged surface [14]. In this perspective the retention of the basic PTH-amino acids could possibly be decreased by increasing the buffer concentration or by replacing the cation of the buffer (Na^+) with a more competitive cation. Since increasing the buffer concentration could lead to undesired heat generation in the column, we tried to replace or replenish the buffer sodium ions by more competitive cations. The replacement of the sodium ions in the mobile phase buffer with potassium ions did not change the elution characteristics of the PTH analytes significantly. Even the electroosmotic flow velocity was not changed. The addition of 5 m*M* triethylamine (TEA) to the mobile phase significantly decreased the retention time of the basic PTHs and the related peak shapes were improved. The elution order with TEA in mobile phase was PTH-Arg, His and Lys, respectively. However, the separation for the other PTH-amino acids deteriorated when TEA was added to the mobile phase; overlapping peaks and undesirable peak shapes were observed.

The effect of the addition of magnesium ions (as $Mg(NO_3)_2 \cdot 6H_2O$) was also investigated. A 40% decrease of the electroosmotic flow velocity was observed with the addition of 5 m*M* Mg^{2+} to the mobile phase. The relative velocities of both acidic and basic PTH-amino acids were significantly changed. The acidic compounds were more strongly retarded, which could be due to the screening of the negative surface charge of the stationary phase by the Mg^{2+} ions. The relative velocities of PTH-His

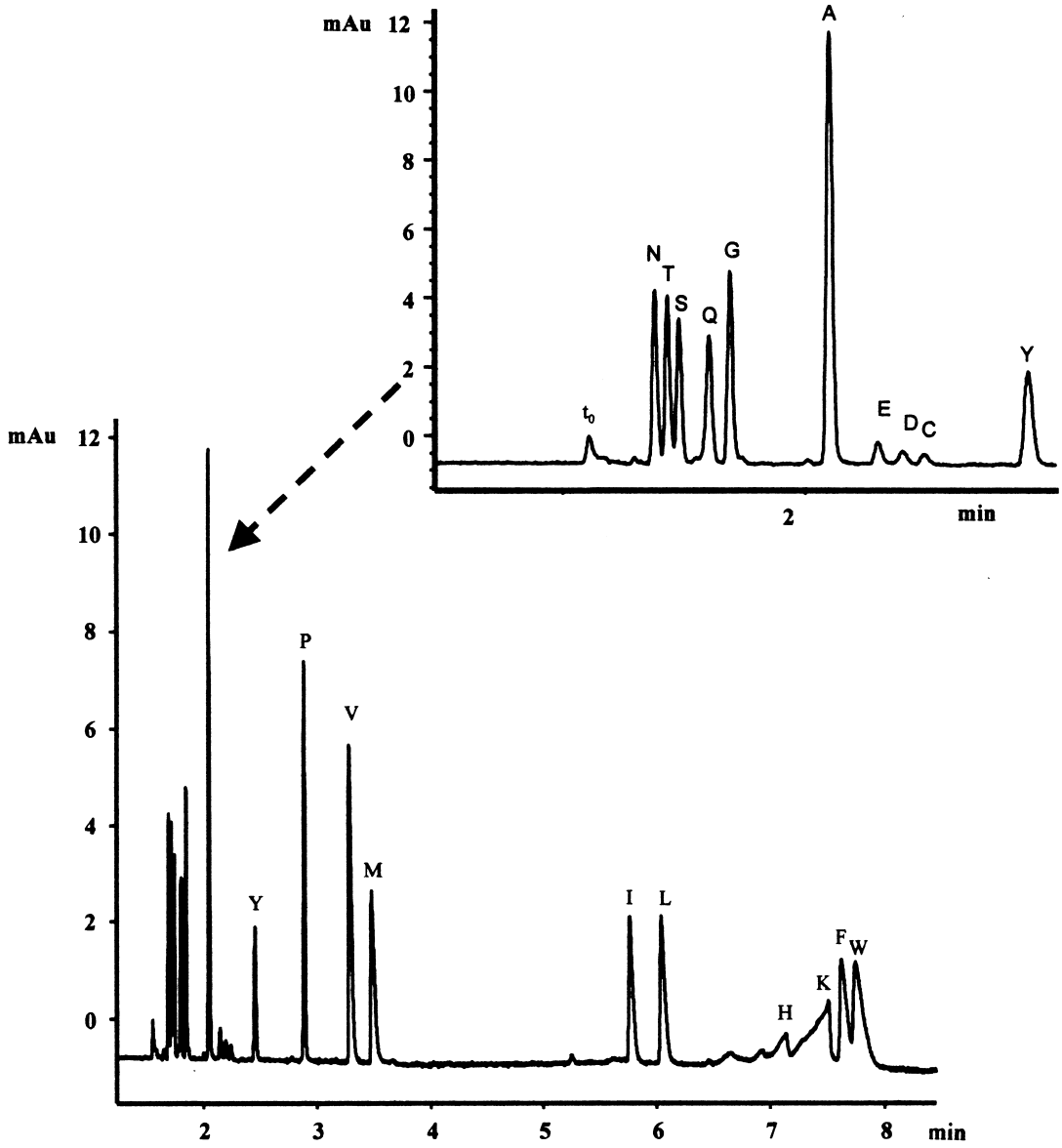

Fig. 5. Separation of 19 PTH-amino acids. Column: 42.5 cm×100 μm I.D., 34-cm packed bed. Mobile phase: 2 m*M* phosphate buffer (pH 7.2), 5 m*M* SDS, 5% (v/v) ACN and 5% (v/v) THF. Injected amounts 0.5–5 pmol. Applied voltage 25 kV. The insert shows an enlargement of the first part of the electrochromatogram.

and PTH-Lys were increased by about 60 and 50%, respectively. Apparently, magnesium ions had the desired competitive effect of the on the electrostatic interaction of these compounds with the stationary phase. However, for PTH-Arg, the most problematic compound, an increase of the elution time was observed. It had to be concluded that with our separation system it is not possible to obtain a

satisfactory chromatographic behavior of this compound.

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