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SEPARATION OF PHENYLTHIOHYDANTOIN-AMINO ACIDS BY FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

An optimization strategy for the separation of 22 phenylthiohydantoin (PTH)-amino acids is described. The separation parameters systematically investigated were the ionic strength, pH, type of organic solvent, column temperature, gradient shape and addition of different ion-pairing agents. Two different separation systems for two different column packing materials, PepRPC™and ProRPC™, were developed. Reproducibility and detection limits are reported.

INTRODUCTION

Liquid chromatography on reversed-phase supports is the most common method for the identification of phenylthiohydantoin (PTH) amino acids obtained from the sequencing of peptides and proteins. Since the first successful separation of PTH-amino acids on a small particle size column packing by Zimmermann *et al.*[1] a vast number of publications on related systems have appeared. These include isocratic elution[2,3], flow programming[4], binary gradients[5–11], ternary gradients[12,13], combinations of different column packings[13–15] and the use of microbore columns[13,16]. The separation optimization strategies range from variation of the ionic strength and pH to more sophisticated methods based on the window-diagram technique[17] and overlapping resolution maps[15].

The present paper describes the gradient separation of 22 PTH-amino acids on two fast protein liquid chromatographic (FPLC) column packings, PepRPC™ and ProRPC™ (ref. 18). The separation has been optimized by a systematic investigation of the effects of the ionic strength, pH, type of organic solvent, gradient shape, addition of different ion-pairing agents, flow-rate and temperature.

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EXPERIMENTAL

Instrumentation

A Pharmacia automated FPLC system consisting of a LCC-500 LC-controller, two P-500 pumps, a MV-8 motor valve, a mixer, a prefilter, a MV-7 injection motor valve with a 25-μl loop, a MV-8 sample holder, a P-1 peristaltic pump and an UV-1 monitor operating at 254 nm fitted with an HR-10 flow cell was used for all the basic investigations. Chromatograms were recorded on a REC 482 dual-pen chart recorder. The retention times, peak heights and areas were measured with a LCC-500 integrator.

The high pressure chromatographic equipment consisted of a Waters Model 680 automatic gradient controller, two Model 510 pumps, a Model U6K universal injector and a Model 440 fixed wavelength UV detector measuring at 254 nm.

The column packings PepRPC and ProRPC were from Pharmacia. These are octadecyl-bonded and octyl-bonded silicas of particle size 5 μm. The pore sizes are 100 and 300 Å, respectively[18]. Glass columns (10 × 0.50 cm I.D.) and stainless-steel columns (20 × 0.46 cm I.D.) were used. The former were obtained prepacked by Pharmacia, while the latter were slurry packed by the ascending technique. The slurry was in chloroform in a stirred reservoir and the packing was performed with acetone using a Maximator LC 75 SG pump operated at 400 bar. The columns were jacketed in order to enable thermostatting by a thermostat FJ (Gebruder Haake, Berlin). The plate numbers of the ProRPC and PepRPC columns were *ca.* 13 000 and 50 000 per metre, respectively, for toluene eluted with a k' of 3.

Chemicals

Buffers were made from sodium acetate and acetic acid or sodium dihydrogenphosphate and disodium hydrogenphosphate (p.a. grade, Merck). The organic solvents used were acetonitrile (HPLC-grade, Fluka), methanol (HPLC-grade, Rathburn), ethanol (99.5% Svensk Sprit), tetrahydrofuran (p.a. grade, Merck) and 1- and 2-propanol (p.a. grade, Merck). The ion-pairing agents were sodium butane-, hexane- and octanesulphonate and sodium dodecyl sulphate (Sigma). PTH-amino acid standards were purchased from Pierce. Stock solutions with a concentration of 5 m*M* were prepared in acetonitrile or methanol and stored at −20°C. All water used was filtered through a Millipore water purification system. Table I lists the PTH-amino acids and the abbreviations used.

RESULTS AND DISCUSSION

A systematic investigation of the separation parameters was first performed on the PepRPC column. This column packing with its smaller pore size and larger calculated plate number was expected to give the better performance of the two packings.

Chromatographic optimization of the PepRPC separation system

Ionic strength. The influence of ionic strength on retention was investigated at pH 4.65, between 10 and 50 m*M* of sodium acetate buffer. As illustrated in Fig. 1 (for clarity not all the PTH-amino acids are shown), no dramatic effects were ob-

TABLE I

PHENYLTHIOHYDANTOIN-AMINO ACIDS AND THEIR ABBREVIATIONS

Name	*Abbreviation*	*Name*	*Abbreviation*
Alanine	A	Methionine	M
Cysteic acid	CA	Asparagine	N
S-Carboxymethylcysteine	CM	Proline	P
Aspartic acid	D	Glutamine	Q
Glutamic acid	E	Arginine	R
Phenylalanine	F	Serine	S
Glycine	G	Threonine	T
Histidine	H	Valine	V
Isoleucine	I	Norvaline	n-V
N-Phenylthiocarbamoyllysine	K	Tryptophan	W
Leucine	L	Tyrosine	Y
Norleucine	n-L		

served, but H and R were eluted earlier at higher ionic strength. This is the expected behaviour and is explained by the ion-exchange interaction between these positively charged amino acids and the free silanol groups on the silica. Silanol groups are present to a certain degree despite the end-capping procedure and they are known to increase in number with column degradation[19]. The same behaviour has been reported by many other authors[4,7,12].

pH. The influence of pH was investigated in the interval 3.6–7.4. As expected, only the charged PTH-amino acids show any dependence of retention time on pH (Fig. 2). With increasing pH the acidic PTH-amino acids (D and E) are eluted earlier and the basic ones (H and R) are eluted later. The pK_a values of the amino acid chains (Table II) indicate that the major factor controlling the pH dependence is the degree of ionization. Lysine is not charged in the form of PTH-phenylthiocarbamoyllysine (K). Therefore neither pH nor ionic strength variations affect the retention of K.

The results obtained above suggest a buffer of pH about 5.8 for the best resolution of the PTH-amino acids. Unfortunately, R and H are eluted as broad peaks under such conditions. The appearance of broad and asymmetric peaks for amines has been explained in terms of mixed retention mechanisms, *i.e.*, hydrophobic interaction together with ion exchange and hydrogen bonding at the silanol groups[20]. The addition of modifiers such as ion-pairing agents to the mobile phase can effectively improve the peak shape[21].

Ion pairing. Ion pairing with alkanesulphonates proved to be a very convenient way of selectively changing the retention times of the charged PTH-amino acids. The influence of ion-pairing agents is usually explained by two mechanisms. One is based on ion pairing in the mobile phase between the ion-pairing agent and the analyte of opposite charge. The increased hydrophobicity of the ion pair then leads to greater retention. The other mechanism is described as adsorption of the ion-pairing agent on the stationary phase acting like an ion exchanger. Our results suggest a combination of these mechanisms. The effect on retention of alkanesulphonates of different carbon chain lengths is illustrated in Fig. 3. The chain length determines the extent

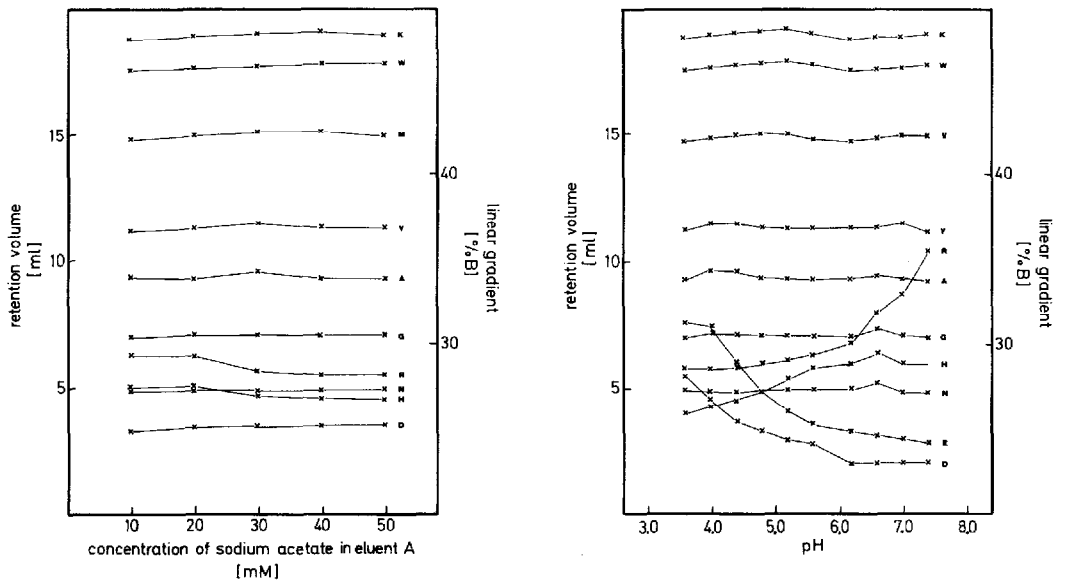


Fig. 1. The effect of ionic strength on the separation of PTH-amino acids (1 nmol of each) on a PepRPC glass column (10 × 0.5 cm I.D.). Solvents: A, sodium acetate buffer at different concentrations, pH 4.65; B, acetonitrile. Gradient: 20–50% B in 20 min with a flow-rate of 1.0 ml/min. The column temperature was 22°C and the sample volume was 25 μl.

Fig. 2. The effect of pH on retention. Solvent A: 20 mM sodium acetate buffer for pH < 5.6 and 20 mM sodium phosphate buffer for pH > 6.2. Other conditions as in Fig. 1.

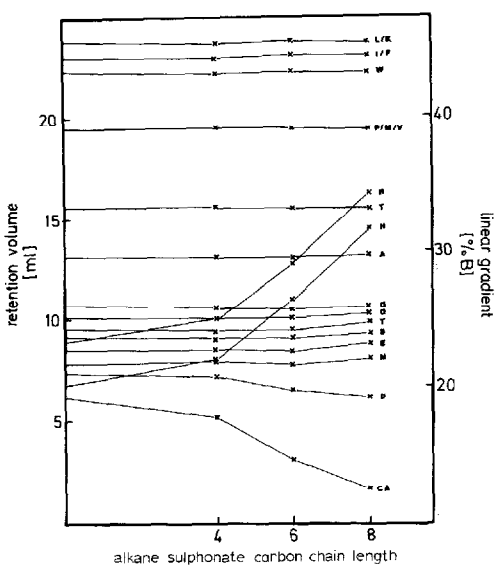
of hydrophobic interaction between the silica bonded phase and the ion-paired PTH-amino acids. Consequently, the retention of the basic PTH-amino acids H and R increases with the chain length of the alkanesulphonates. However, the opposite effect is observed for the acidic PTH-amino acids D and CA (Fig. 3). The latter behaviour is explained by a repulsive interaction between the alkanesulphonates in the stationary phase and the negatively charged PTH-amino acids. Furthermore, the basic PTH-amino acids H and R are in the form of ion pairs and are eluted as much sharper peaks. As above, this is explained by the inhibition of the ion-exchange effect involving the silanol groups.

When the type of ion-pairing agent has been chosen its concentration is a convenient way of fine-tuning the elution pattern as illustrated in Fig. 4.

TABLE II

AMINO ACID SIDE-CHAIN pK_a VALUES AT 25°C

Amino acid	*pK_a*
Aspartic acid	3.9
Glutamic acid	4.3
Histidine	6.0
Arginine	12.5



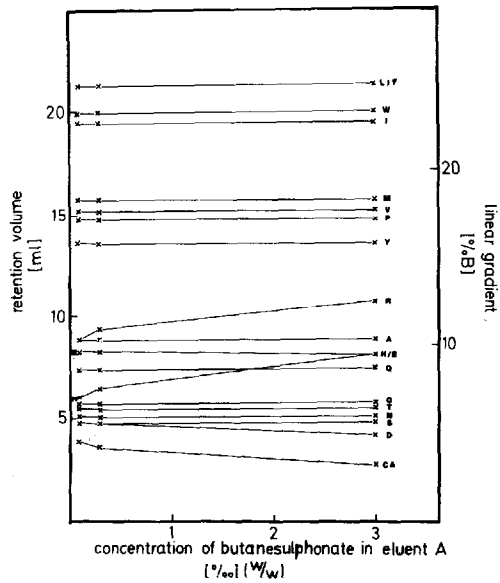


Fig. 3. The effect on retention of different ion-pairing agents. Solvent A: 20 m*M* sodium acetate buffer, pH 4.0, with the addition of 1‰ (w/w) of alkanesulphonate. Gradient: 10–55% B in 30 min. Column temperature: 40°C. Other conditions as in Fig. 1.

Fig. 4. The effect of the concentration of the ion-pairing agent. Solvent A: 20 m*M* sodium acetate buffer, pH 4.0, with different amounts of butanesulphonate. Column: ProRPC in a stainless-steel column (20 × 0.46 cm I.D.). Gradient: 0–35% B in 30 min. Other conditions as in Fig. 1.

The pH has a great influence on the ion-pairing effect as illustrated in Fig. 5. In the pH region examined (3.6–5.7) the dodecyl sulphate is negatively charged. R is fully protonated throughout this pH region and is subsequently not influenced by the pH changes. H with its lower pK_a value for the side chain (Table II) interacts less with the alkyl sulphate as the side chain becomes less positively charged at higher pH. Less ion pairing gives less hydrophobic interaction and therefore H is eluted earlier at higher pH. The same effect of weaker retention at higher pH is noted for the acidic PTH-amino acids. This is explained by the repelling effect of the adsorbed alkyl sulphate on the negatively charged D and E. This effect is more pronounced when these PTH-amino acids are more highly charged at higher pH and thus they are eluted earlier.

Organic solvent. The separation selectivity of the uncharged PTH-amino acids can effectively be optimized by changing the solvent B. Acetonitrile, methanol, ethanol, 1-propanol, 2-propanol and 1-butanol were tested for this purpose. Acetonitrile resulted in coelution of the PTH-amino acids P, M and V, whereas all the investigated alcohols resulted in baseline separation of these derivatives. On the other hand, with methanol, K and L were eluted together and with ethanol, L and F were coeluted. Butanol and 1- and 2-propanol separate all PTH-amino acids in the same order as depicted in Fig. 6 and the best selectivity was attained with the propanols. Isocratic elution at the beginning was necessary for baseline resolution of T, G and Q (Fig. 6). The addition of methanol to the buffer A was needed in order to increase the elution strength. However, if methanol is replaced by ethanol, G and Q are eluted together.

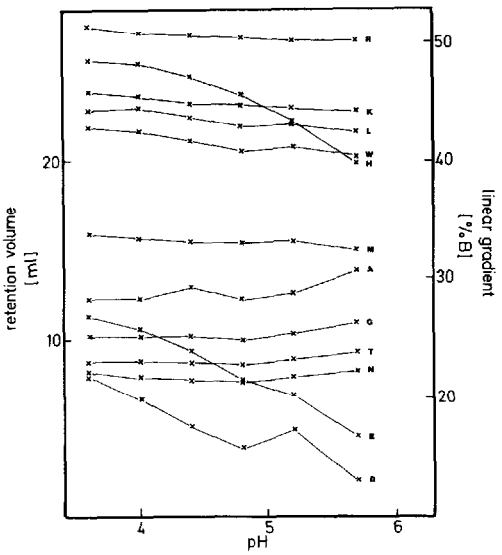


Fig. 5. The effect of pH on ion pairing. Solvent A: 20 m*M* sodium acetate buffer for pH 3.6–4.8 and 20 m*M* sodium phosphate buffer for pH 5.2–5.7; 0.3‰ (w/w) sodium dodecyl sulphate was added to the buffer. Gradient: 10–55% B in 30 min. Other conditions as in Fig. 1.

Gradient shape. The design of an optimum gradient is facilitated if the dependence of the capacity factor, k', on the composition of the solvents A and B is known. Such a plot reveals how the steepness of the gradient will influence the resolution[22].

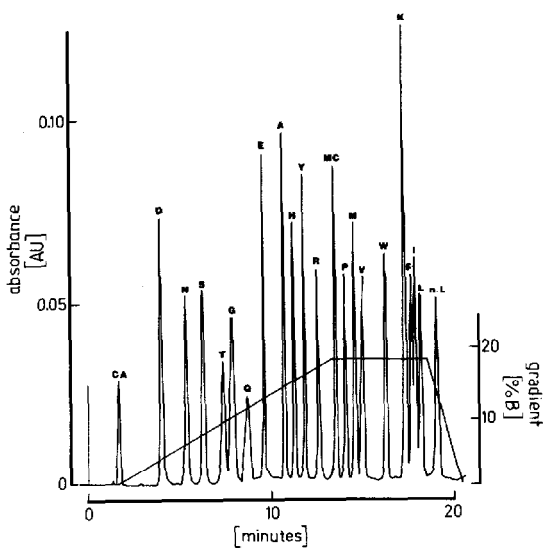


Fig. 6. The separation of 22 PTH-amino acids (1 nmol of each) on a PepRPC stainless-steel column (20 × 0.46 cm I.D.). Solvents: A, 20 m*M* sodium acetate buffer, pH 4.1, with addition of 1‰ hexanesulphonate and 20% methanol; B, 1-propanol. Flow-rate: 1.5 ml/min. The column temperature was 41°C and the sample volume was 25 µl.

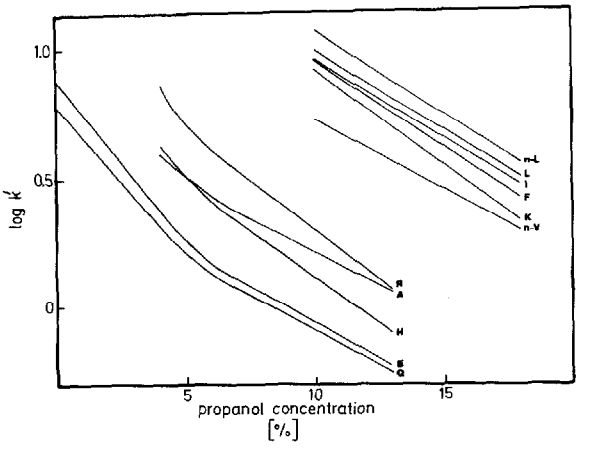


Fig. 7. The variation of log k' as a function of % B for isocratic elution of twelve PTH-amino acids. Other conditions as in Fig. 6.

The change in k' for some PTH-amino acids with increasing 1-propanol content using isocratic elution is illustrated in Fig. 7. It can be concluded that the critical resolution between I and F in Fig. 6 could be further improved by using a steeper gradient.

Column temperature. Temperature has a great influence on separation and must be controlled for precise analysis. When the temperature is increased the mobile phase viscosity decreases, resulting in a lower column pressure. In addition, the column efficiency improves and retention times show a general decrease. For the

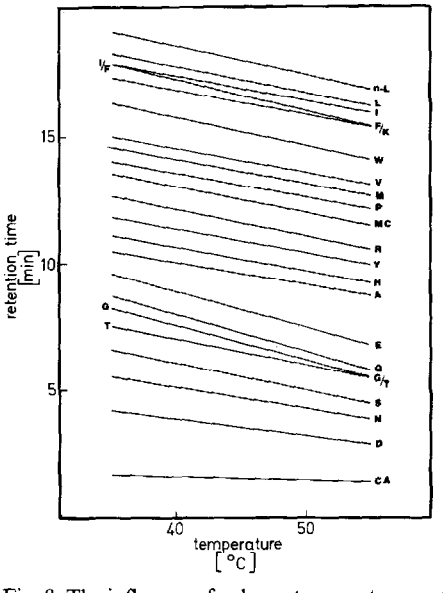


Fig. 8. The influence of column temperature on the retention times with gradient elution. Other conditions as in Fig. 6.

PTH-amino acids, the dependence of retention on temperature is illustrated in Fig. 8, where also a certain selectivity can be noted. The resolution of I and F increases at elevated temperature and F is shifted towards the position of K (Fig. 8). Moreover, the resolution between G and T decreases with increasing temperature. As a compromise, due to these temperature effects, a temperature in the interval between 41 and 49°C has to be used for the resolution of all the PTH-amino acids (Fig. 8).

Evaluation of the PepRPC system

From the basic investigations described above we designed the separation system depicted in Fig. 6. To determine the reliability of the chromatographic system for the identification of the PTH-amino acids, we have statistically evaluated the variation of the retention times (Table III) over a period of 3 weeks. The constructed confidence intervals show that the results are highly reproducible. Furthermore, neither of the confidence intervals overlap, indicating that the identification can be made with a high degree of confidence.

The peak height of the PTH-amino acids is linearly correlated to the concen-

TABLE III

VARIATION IN RETENTION TIMES OF 22 PTH-AMINO ACIDS FROM 20 INJECTIONS ON PepRPC

Chromatographic conditions as in Fig. 6. The experiments were performed during a period of 3 weeks.

PTH-amino acid	t_R *(min)*	*R.S.D. (%)*	*99% confidence interval (min)*
Cysteic acid	1.59	1.51	1.572–1.604
Aspartic acid	3.79	0.94	3.763–3.809
Asparagine	5.14	0.77	5.112–5.164
Serine	6.08	0.74	6.049–6.108
Threonine	7.10	0.74	7.063–7.131
Glycine	7.60	0.90	7.558–7.648
Glutamine	8.25	2.40	8.115–8.375
Glutamic acid	9.00	3.04	8.821–9.179
Alanine	10.24	1.75	10.120–10.354
Histidine	10.78	1.38	10.681–10.876
Tyrosine	11.37	1.13	11.285–11.453
Arginine	12.14	0.80	12.080–12.206
S-Carboxymethylcysteine	13.06	0.78	12.996–13.128
Proline	13.60	0.74	13.530–13.662
Methionine	14.11	0.65	14.052–14.172
Valine	14.54	0.63	14.481–14.600
Tryptophan	15.79	0.48	15.740–15.840
N-Phenylthiocarbamoyllysine	16.83	0.46	16.783–16.885
Phenylalanine	17.18	0.45	17.133–17.233
Isoleucine	17.39	0.46	17.337–17.441
Leucine	17.69	0.47	17.640–17.748
Norleucine	18.50	0.47	18.444–18.576

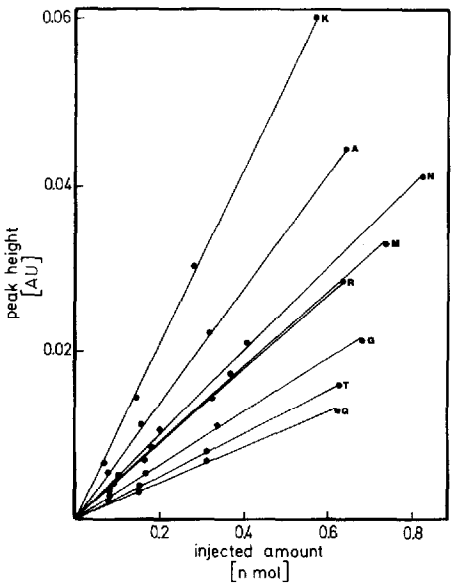


Fig. 9. Calibration curves for eight PTH-amino acids. Other conditions as in Fig. 6.

tration as depicted in Fig. 9. The sensitivity of the system makes it possible to identify and quantify PTH-amino acids down to 50 pmol without any problems.

The design of the ProRPC separation system

When optimizing the separation of PTH-amino acids on a ProRPC column advantage can be taken of the experience obtained with the PepRPC column. For example, the selective adjustment of the retention times of H and R with alkanesul-

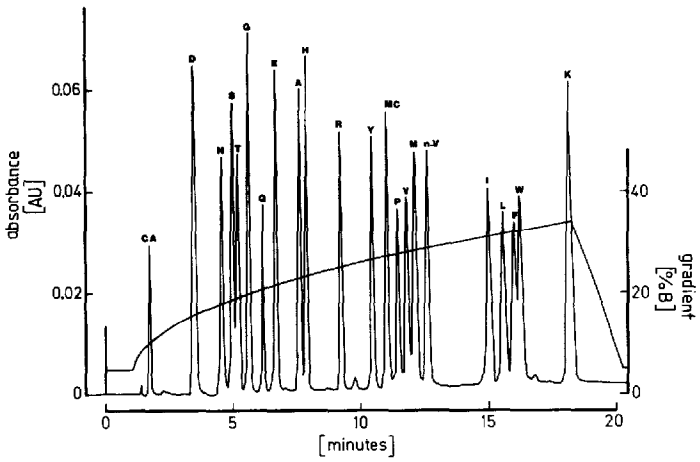


Fig. 10. The separation of 22 PTH-amino acids (1 nmol of each) on a ProRPC stainless-steel column (20 × 0.46 cm I.D.). Solvents: A, 20 m*M* sodium acetate buffer, pH 3.8, with addition of 0.1‰ of octanesulphonate; B, acetonitrile–tetrahydrofuran (93:7, v/v). Flow-rate: 2 ml/min. The column temperature was 28°C and the sample volume was 25 μl.

phonate follows the same trends as in Figs. 3 and 4. Moreover, the temperature influences the retention of F and T in the same specific way. After acetonitrile had been chosen for solvent B, the main resolution problem was the coelution of L and F. The addition of tetrahydrofuran to solvent B, adjustment of the gradient and optimization of the temperature resulted in a good resolution of these PTH-amino acids (Fig. 10). Furthermore, the addition of tetrahydrofuran improved the resolution of P, V and M. Variation of the flow-rate did not have any effect on the resolution or the retention volume. Therefore the use of a flow-rate of 2 ml/min gives as good a separation as does one of 1 ml/min, but in only half the time.

Despite the smaller plate number for this column packing (four times lower compared to PepRPC) a satisfactory separation system could be designed (Fig. 10).

CONCLUSIONS

This study demonstrates the power of alkanesulphonates as mobile phase modifiers. The peak symmetry was radically improved for the basic PTH-amino acids H and R. In addition, the retention of these PTH-amino acids can be controlled in a predictable way by the choice of the alkanesulphonate chain length, its concentration and the pH.

The effects of the sulphonates are the same for both the investigated RPC gels, which indicates the generality of the results. The retention of the non-charged PTH-amino acids is mainly controlled by the choice of organic solvent and temperature.

The separation systems described yield reproducible results and the low concentrations needed make them ideally suited for the sequence determination of peptides and proteins.

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