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RETENTION BEHAVIOUR OF PARACELSIN PEPTIDES ON REVERSED-PHASE SILICAS WITH VARYING *n*-ALKYL CHAIN LENGTH AND LIGAND DENSITY

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SUMMARY

As part of further investigations on the characterization of the ligand-induced conformational stabilization of peptides, two series of *n*-alkyldimethylsilyl bonded silicas have been prepared. In series A the *n*-alkyl chain length, *n*, of the bonded phase was varied between 1 and 20 carbon atoms at a constant ligand density. In series B the ligand density, α_{exp}, was gradually changed from 0 to 4.1 μmol/m^2 on a C_1, C_4, C_6, C_8 and C_{18} bonded phase. The retention behaviour of four peptides of the paracelsin family were examined under isocratic conditions, using a ternary mobile phase of water–methanol–acetonitrile (22:39:39, v/v/v). Plots of k' *versus* n showed pronounced maxima between $n = 2$ and 4 carbon atoms, followed by a decrease by a factor of 3 at $n = 5$, whereas above 5 carbon atoms only a slight increase in k' was observed.

The selectivity behaviour of the paracelsins A–D can be mainly rationalized by interaction of the amphiphathic polypeptide $3.6_{13}(\alpha)$-helix with the hydrophobic ligand and protrusion of the key amino acid residues at positions 6 and 9 in the sequence. However, from experiments with a polystyrene stationary phase it is evident that hydrophobic interactions and different partition coefficients also contribute to the resolution of the paracelsin peptides. Furthermore, Van 't Hoff plots confirm significant free energy changes associated with retention. These observations provide the basis for evaluating the enthalpic and entropic changes associated with peptide interactions with *n*-alkyl silicas.

INTRODUCTION

The wide use of reversed-phase (RP) silica packings in high-performance liquid chromatographic (HPLC) separations of peptides and proteins has stimulated in-

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tensive studies on the stationary phase properties and on the properties of the peptide itself and their effects on retention and selectivity[1–3]. Numerous studies have shown[4] that the position of an amino acid within the peptide sequence and the neighbouring groups influence retention. For example, it is well known[4] that a series of peptides containing the same amino acids but with different sequence can be separated.

The influence of the mobile phase on the retention of peptides and proteins under reversed-phase conditions has been broadly investigated[1,2,5,6]. However, there is a lack of understanding of the molecular mechanism(s) of interaction between the hydrocarbonaceous surface representing the stationary phase and the peptides and proteins as solutes.

While the dependence of the retention and selectivity of polar and non-polar small size solutes on reversed-phase chain length and on ligand density has been the subject of several studies[7–16], no systematic investigations of the influence of these stationary phase parameters on the retention of peptides and proteins have been made. In a recent paper[7] it was shown that solute retention in reversed-phase chromatography (RPC) as a function of either *n*-alkyl chain length or ligand density both follows a linear relationship up to characteristic critical values whereby the dependencies progressively diverge and reach asymptotic limiting values. The critical chain length and the critical ligand density are dependent on the solute size for polar low-molecular-weight compounds: the larger the solute, the higher is the critical chain length, but the lower is the critical ligand density. These phenomena can be understood on the basis of a restricted intercalation of the solutes into the stationary phase.

However, peptides and proteins exhibit secondary and tertiary structures and can therefore behave differently. For large molecules, *e.g.*, proteins, the retention was found to be fairly independent of the chain length of an alkyl-bonded silica[17].

This paper aims to investigate the influence of ligand density, *n*-alkyl chain length and column temperature on the retention of peptides of the paracelsin family in RPC. These studies thus provide further evidence for the critical chain length concept, and demonstrate that enthalpy-driven processes play an important role in the retention of amphiphatic peptides on hydrocarbonaceous stationary phases.

EXPERIMENTAL

Reagents and procedures

A series of *n*-alkyldimethylchlorosilanes [X-Si$(CH_3)_2C_nH_{2n+1}$] with an *n*-alkyl chain length, *n*, varying from 1 to 20 carbon atoms were prepared by hydrosilylation of *n*-alkenes with dimethylchlorosilane by using hexachloroplatinic acid as catalyst. The physical properties of these silanes are listed in a recent paper[7]. The starting silica was LiChrospher Si-100-10 (Batch No. F 667512), a gift of Merck (Darmstadt, F.R.G.) with a specific surface area of a_s(BET) = 388 ± 5 m^2/g, a mean pore diameter of p_d = 10 nm and a mean particle diameter of d_p = 10 μm.

The silanization of the silicas was performed in dry dimethylformamide (50 ml) at a temperature of 426 K under gentle stirring, applying the prepared chlorosilane (30 mmol) and 2,6-lutidine (36 mmol) as catalyst. The silica samples (0.1 mol) were previously dried at 423 K under vacuum at $P \leqslant 30$ Pa for 16 h. The reversed-phase silica formed was washed with different solvents in succession and then dried at 353 K under vacuum overnight. The procedure has been described in detail elsewhere[7].

The ligand density (α_{exp}) of bonded *n*-alkyldimethylsilyl groups was determined by means of elemental analysis from the carbon content of the modified sample[7].

Chromatographic measurements

The reversed-phase silicas were packed into columns, 250 × 4.6 mm I.D. by means of the slurry packing technique. In addition, a polystyrene column PRP-1, 125 × 4 mm I.D., was used (Hamilton, Darmstadt, F.R.G.). The chromatographic measurements were performed on an LKB (Pharmacia-LKB Biotechnology, Freiburg, F.R.G.) liquid chromatograph with a Model 2150 HPLC pump and a Model 2151 variable-wavelength monitor. The chromatograms were recorded on a Shimadzu (Shimadzu, Duisburg, F.R.G.) Model CR-3A integrator. All columns were thermostated at a temperature of 301 K using an LKB 2155 column oven. In order to realize low column temperatures for the investigation of the influence of temperature on retention a Colora (Lorch, F.R.G.) WK 30 A cryostate filled with ethanol was employed.

The peptides used in this study were a mixture of the paracelsins A–D, dissolved in methanol at a concentration of 0.1 mg ml^{-1}. The mobile phase was water–methanol–acetonitrile (22:39:39). Methanol and acetonitrile were of chromatography grade, supplied by Merck and the water used was destilled and then additionally purified by means of Milli-Q water system (Millipore/Waters, Bedford, MA, U.S.A.). The injection loop had a volume of 20 µl, the flow-rate was 1 ml/min and the detection wavelength was 206 nm.

Properties of paracelsin

Paracelsin[18–20], a membrane-active polypeptide, is an antibiotic of the peptaibol class[21], excreted by the mould *Trichoderma reesi*. It exhibits a pronounced microheterogeneity, *i.e.*, it is composed of four peptides differing only in two positions (positions 6 and 9)[18–20]. The primary structures of the paracelsins A–D are given in Fig. 1, where the differences are indicated. The N-termini of these peptides are acetylated, and the C-termini linked in a peptide bond with phenylalaninol (Pheol). Furthermore, it is noteworthy that the paracelsins exhibit a high proportion of the uncommon α-aminoisobutyric acid (Aib). By means of circular dichroism and ^{13}C NMR spectroscopy Brückner *et al.*[18] have demonstrated the helical structure of the paracelsins. The presence of a mainly 3.6_{13} (α)-helical solute structure of paracelsin, and not a 3_{10}-helix, as found in certain Aib-peptides[22], is the most likely conformation since X-ray crystallographic and NMR spectroscopic investigations of the paracelsin analogues alamethicin[23] and trichorzianine[24] have shown that they have α-helical conformations.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ac-	Aib-	Ala-	Aib-	Ala-	Aib-	**Ala**-	Gln-	Aib-	**Val**-	Aib-	Gly-	Aib-	Aib-	Pro-	Val-	Aib-	Aib-	Gln-	Gln-	Pheol (A)
Ac-	Aib-	Ala-	Aib-	Ala-	Aib-	**Ala**-	Gln-	Aib-	**Leu**-	Aib-	Gly-	Aib-	Aib-	Pro-	Val-	Aib-	Aib-	Gln-	Gln-	Pheol (B)
Ac-	Aib-	Ala-	Aib-	Ala-	Aib-	**Aib**-	Gln-	Aib-	**Val**-	Aib-	Gly-	Aib-	Aib-	Pro-	Val-	Aib-	Aib-	Gln-	Gln-	Pheol (C)
Ac-	Aib-	Ala-	Aib-	Ala-	Aib-	**Aib**-	Gln-	Aib-	**Leu**-	Aib-	Gly-	Aib-	Aib-	Pro-	Val-	Aib-	Aib-	Gln-	Gln-	Pheol (D)

Fig. 1. Primary structure of the paracelsin peptides A–D.

RESULTS AND DISCUSSION

Retention of paracelsin

Despite the small differences between the four paracelsins (PCs), separation of all four components could be achieved under isocratic conditions[25]. A chromatogram of the paracelsin peptides on an octyldimethylsilyl bonded silica showing the characteristic elution profile is presented in Fig. 2.

In order to rationalize that the paracelsin peptides are separated in spite of their small molecular differences the following facts have to be taken into account. It is known from numerous examples that reversed phases are able to separate peptides according to their hydrophobicities and differences in their conformations. The most surprising effect in the case of the paracelsins is that an unexpectedly good separation of the microheterogenous peptides occurs. This is manifested in the k' values and resolution factors. However, no significant differences in the α-helical conformations of components A–D are to be expected. Since PC-A and PC-B, as well as PC-C and PC-D, differ from each other just by the insertion of one methylene group in an amino acid side chain (exchange of Val against Leu in position 6, *cf.* Fig. 1) the question arises whether the chromatographic separation is attributable solely to minor differences in hydrophobicities or not.

The differenees in the numbers of methine, methylene, and methyl groups of the amino acids exchanged in the decisive positions 6 and 9 shown in Table I.

From the temperature-difference of the paracelsin peptides (*cf.* Fig. 3) it is obvious that the enthalpy values increase by 0.5–0.6 kJ/mol in the series PC-A to PC-D. These enthalpy differences are sufficient for the separation of paracelsin peptides, but the data describe a macroscopic phenomenon rather than providing a separation mechanism. Furthermore, in analogy to the structurally related trichotoxins, which partly have been separated by counter-current distribution[19], it is reasonable to assume that also the paracelsins differ in their partition coefficients. These coefficients also reflect the differences in the hydrophobicities of the peptides. The conclusions are supported by the ability of a polystyrene stationary phase to separate PC-A and PC-B from PC-C and PC-D, whereby the latter coelute (*cf.* Fig. 4). However, the above findings hardly explain the maxima of k' values of paracelsins found

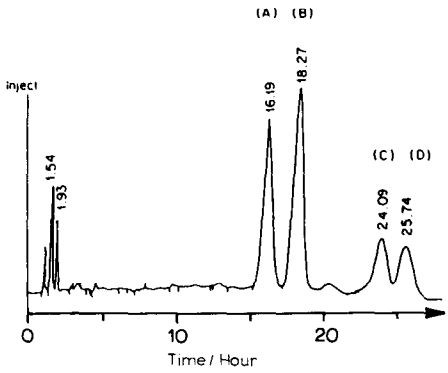

Fig. 2. Chromatogram of four paracelsin peptides on a LiChrospher Si-100 RP-8 column. 5 μm, 250 × 4.6 mm I.D. Eluent: water–methanol–acetonitrile (22:39:39, v/v/v).

TABLE I

NUMBERS OF CH, CH_2 AND CH_3 GROUPS OF AMINO ACIDS IN POSITIONS 6 AND 9 OF PARACELSIN A–D

Paracelsin compound	*Number of groups*		
	CH	*CH_2*	*CH_3*
A	1	0	3
B	1	1	3
C	1	0	4
D	1	1	4

for the C_4 phase, and to a lower extent, for the C_2 stationary phase (*cf.* Fig. 5). In the case of paracelsins it is therefore reasonable to assume a certain degree of conformational interaction between the stationary phases and the paracelsins, in particular with the alkyl side-chains of the peptide-bonded amino acids.

This is supported by recent results of Pfleiderer *et al.*[26] who performed cross polarization magic angle spinning (CP-MAS) NMR measurements on reversed-phase silicas with varying *n*-alkyl chain length. It was found that in particular *n*-butyl bonded silica (C_4) showed a minimum in mobility (or maximum in rigidity) compared to other *n*-alkyl bonded silicas. As a result of the restricted conformational degrees of freedom of the C_4 phase it is reasonable to assume an optimal interaction of this stationary phase with the alkyl side chains of peptides. With regard to paracelsins, in particular when restricting to the amino acid exchange positions, this interaction preferently takes place, with the isopropyl and isobutyl chains of Val and Leu, respectively, in position 9, and the methyl and geminal dimethyl group of Ala and Aib, respectively, in position 6 (*cf.* Fig. 1).

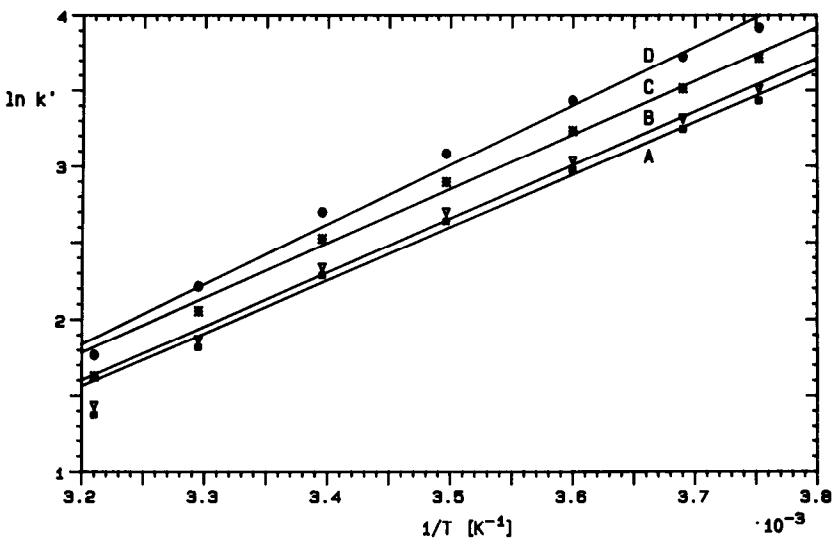

Fig. 3. Van 't Hoff plot of the separation of paracelsin A–D on a LiChrospher Si-100-10 RP-18 column.

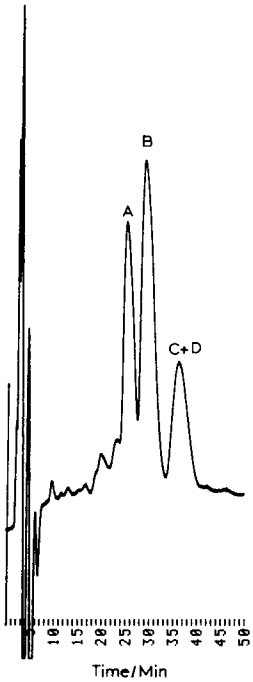

Fig. 4. Chromatogram of the natural mixture of paracelsins (A–D) on a polystyrene stationary phase (PRP-1). Column, 150 × 4.1 mm I.D. Eluent: water–methanol–acetonitrile (35:30:35, v/v/v); flow-rate: 0.4 ml/min; absorbance: 215 nm.

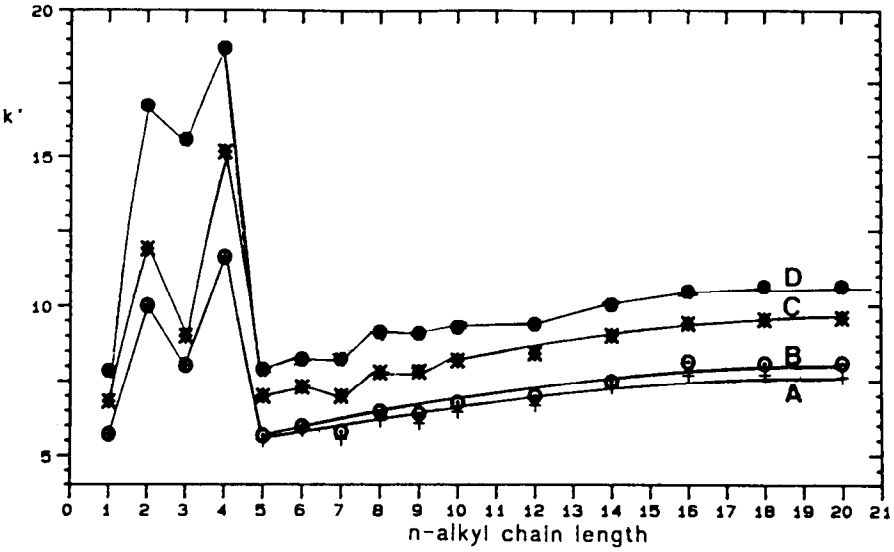

Fig. 5. Capacity factors, k', of paracelsin as a function of n-alkyl chain length of reversed-phase silicas at constant ligand density in water–methanol–acetonitrile (22:39:39, v/v/v).

Finally the question remains open whether the α-helical solute structures of the individual paracelsins contribute to their chromatographic separation. It should be emphasized that the α-helical conformations of paracelsins are not induced (but might be enforced) by interaction with the stationary phase since circular dichroitic measurements proved a high degree of helical conformation even in aqueous solvents such as water–methanol (30:70, v/v). Fig. 6 represents the axial α-helical wheel projection of paracelsins showing also the amino acid exchange positions. As can be seen these amino acids are located at the hydrophobic side of the helix. Since it is reasonable to assume that the hydrophobic regions (positions 1–17, *cf.* Fig. 1) of the amphipathic helices of paracelsins are directed towards the reversed phase, optimal side chain recognition should occur for the amino acids exchanged. The conformational interaction of the hydrophobic part of the amphipathic paracelsin helix and the C_4 stationary phase might contribute therefore to the highest k' values found for this phase and also to the unexpectedly good separation of the microheterogeneous paracelsins peptides by RP-HPLC.

Dependence of the retention of paracelsin on n-alkyl chain length and ligand density

In a series of experiments, the retention behaviour of the paracelsins on reversed-phase silicas with an *n*-alkyl chain length ranging from C_1 to C_{20} was investigated while the ligand density was kept constant ($\alpha_{exp} = 3.5 \pm 0.2$ μmol/m^2). The results are presented in Fig. 5. In an other series with trimethyl(C_1)-, *n*-butyl(C_4)-, *n*-hexyl(C_6)-, *n*-octyl(C_8)- and *n*-octadecyl(C_{18})-bonded reversed.phase silica, the ligand density was varied between 0 and 4.1 μmol/m^2. Fig. 7 shows the dependence of the capacity factor of paracelsins on the ligand density of the reversed-phase silica.

Testing small solutes (anilines and bencoic acid esters)[7] we found that the capacity factor did not markedly increase above a certain *n*-alkyl chain length, called the critical chain length, L_{crit}. This result is in agreement with earlier findings of Berendsen and De Galan[12,13]. Surprisingly, the plot showing the dependence of paracelsin retention on the *n*-alkyl chain length of the stationary phase (Fig. 5) exhibits two maxima, corresponding to an *n*-alkyl chain length between 2 and 4 carbon atoms.

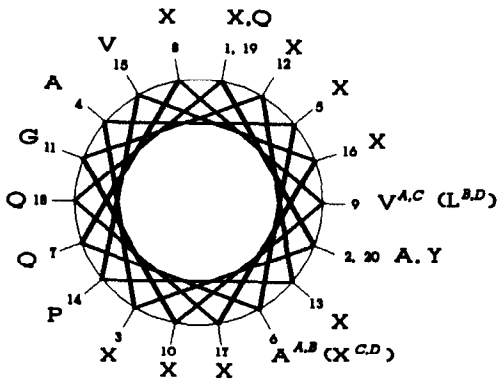

Fig. 6. Axial projection of the helical wheels of paracelsins A–D showing amino acid exchange in position 6 and 9 (superscripts of amino acids refer to paracelsin peptides A, B, C and D). One-letter nomenclature of amino acids: A = Ala, G = Gly, L = Leu, P = Pro, Q = Gln, V = Val, X = Aib, Y = Pheol (phenyl-alaninol).

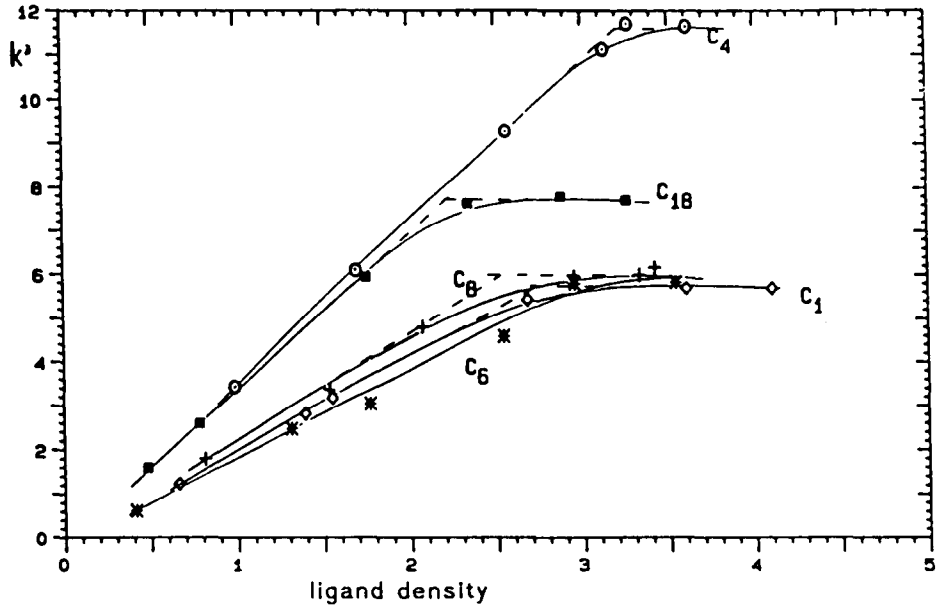

Fig. 7. Capacity factors, k', of paracelsin A as a function of ligand density of reversed-phase silicas at constant *n*-alkyl chain length in water–methanol–acetonitrile (22:39:39, v/v/v).

From 4 carbon atoms on, the capacity factors are three times lower but increase slowly and linearly up to a plateau, where the retention remains constant. The critical chain length found for paracelsin was about 14 carbon atoms.

The phenomenon of the critical chain length has been explained previously by Berendsen and De Galan[12,13] by means of the compulsory absorption model, where the solute is squeezed into spaces created between the bonded *n*-alkyl chains. This is the reason why the critical chain length increases with increasing size of the solute molecule.

Considering the plot in Fig. 7, it is evident that the capacity factors linearly increase with increasing ligand density of the reversed-phase silica up to a plateau, where the retention remains constant. This observation is in agreement with our recent results[7]. By interpolation of the straight lines in the plot of k' against the ligand density, a critical ligand density, α_{crit}, between 2.3 and 3.2 μmol/m^2 (depending on the *n*-alkyl chain length of the stationary phase) is found for paracelsin. These values are in the same range as those obtained for anilines and esters[7]. From the latter data the conclusion can be drawn that the retention of paracelsin is solely caused by hydrophobic effects and is therefore not influenced by residual silanol groups of the stationary phase. On the other hand, retention above a distinct ligand density is limited by the greatly reduced ability of the solute to penetrate into the stationary phase[3]. *n*-Alkyl groups that are packed together very closely loose the possibility of lateral evasion and this explains the critical ligand density.

Dependence of temperature on paracelsin retention

The significance of the influence of temperature on RPC is often underestimat-

ed[27,28]. Gilpin and Gangoda[29], Morel and Serpinet[30] and others have observed a distinct phase transition, depending on temperature, ligand density and *n*-alkyl chain length.

Fig. 3 shows the dependence of the separation of paracelsin peptides on temperature in the range 265–315 K. It is evident that in this range the dependence of retention on temperature is linear. Hancock *et al.*[31] and Hearn *et al.*[32] have shown, that a linear dependence means that the helical conformational structure is not significantly altered within the temperature range chosen.

In general, for chromatography of small molecules it has been observed that the retention time decreases with an increase in temperature[29,33]. This temperature effect is opposite to that observed for many biopolymers, for which an increase often results in strengthened hydrophobic interactions[31,34]. Such an increase is usually attributed to an entropy-driven process. Hearn *et al.*[32] and Hancock *et al.*[31] have observed that the retention of different peptides decreases with increasing temperature. The same trend is found for paracelsin: the Van 't Hoff plot exhibits a positive slope, corresponding to a decrease of retention with increasing temperature.

Hearn *et al.*[32] and Hancock and co-workers[31,35] have postulated when an amphipathic peptide adopts a nearly 100% helical structure during RPC, maximal hydrophobic interactions result between the stationary phase and the non-polar face of the amphipathic peptide. As shown previously, paracelsin exhibits a helical structure and even in aqueous organic solvents this conformation seems to be maintained during the chromatographic separation.

By means of eqn. 1, relating the capacity factor, k', the phase ratio, Φ, the absolute temperature, T, and the gas constant, R, it is possible to calculate the standard free enthalpy, ΔG^0, of the interaction between the solute and the stationary phase from the slope of the Van 't Hoff plot (Fig. 3):

$$\ln k' = \ln \Phi - \Delta G^0/RT \tag{1}$$

The following enthalpy values were found for the corresponding paracelsin peptides: paracelsin A, $\Delta G^0 = -30.8$ kJ/mol; paracelsin B, $\Delta G^0 = -31.3$ kJ/mol; paracelsin C, $\Delta G^0 = -31.8$ kJ/mol; paracelsin D, $\Delta G^0 = -32.4$ kJ/mol. Eqn. 1 can be expressed according to the Gibbs equation as follows:

$$\ln k' = -\Delta H^0/RT + \Delta S^0/R + \ln \Phi \tag{2}$$

Assuming that the entropy remains constant, the free standard enthalpy values are identical with the standard enthalpies.

The enthalpy values for paracelsins listed above are in the same range as those of other peptides undergoing conformational changes at the stationary phase surface (*ca.* -36 kJ/mol)[31,32]. Because the value of Φ is not available, the entropy cannot be calculated by means of eqn. 2. Comparing Fig. 3 and the Van 't Hoff plots of other peptides[31,32] it is evident that the intercepts for the paracelsin peptides in terms of entropy contributions have values in the same range. For example, Hancock *et al.*[31] showed that the association of the peptide LAP with the stationary phase is enthalpy-driven, in contrast to the association of insulin, which was found to be entropy-driven. By comparison with these and other results[32], the separation of paracelsin is

obviously enthalpy-driven. Finally, it should be pointed out that a difference in the enthalpy of *ca.* 0.5 kJ/mol is sufficient for a complete separation of the four paracelsin peptides.

CONCLUSION

This investigation with paracelsin peptides has demonstrated that solute retention in RPC as a function of *n*-alkyl chain length ($n > 4$) and ligand density follows linear relationships and reaches limiting values. This phenomenon can be understood on the basis of a restricted intercalation of the solutes into the stationary phase.

While basic and non basic small-sized solutes exhibit nearly the same pattern (*cf.* ref. 13), peptides having a tertiary structure behave quite differently. In this study, the family of paracelsin peptides, which possess an α-helical structure, showed a pronounced maximum in retention as a function of the *n*-alkyl chain length ($\alpha_{exp} < 5$). However, the retention of paracelsin as a function of the ligand density at constant chain length increased linearly up to a critical point, similar to that observed in the case of the anilines and esters. Furthermore, an exceptionally high selectivity was achieved under isocratic elution conditions, *i.e.*, 20-mer peptides were separated that differed only by one or two methylene groups, respectively.

It appears that the *n*-alkyl chains of the reversed-phase packing, depending on their length and conformation, have the ability to distinguish peptides with amino acid side chain residues differing by only one methine, methylene or methyl group. These investigations indicate that the effect of alkyl chain length and conformation on retention and selectivity are of critical importance for solutes with a secondary structure, such as peptides.

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