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Note

Prediction of retention data of protected dipeptides in normal phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) is widely used to separate complex mixtures of natural and synthetic compounds. It has been successfuly applied to the resolution and purification of synthetic products, the identification and isolation of peptides from biological materials[1,2], in quantitative analysis of drugs[3,4], in peptide sequencing[5] and to investigate racemization and by-product formation in peptide synthesis[6]. Due to the large number of peptides which can easily be separated in one chromatographic analysis, the possibility of peptide retention prediction is of interest. A general method of calculating R_F values for unprotected peptides, in paper partition chromatography was elaborated in the 1950s[7,8]. The calculations were based on Martin's theory[9] and the assumption that the total free energy of transfer of the solute can be divided into contributions from the energies required to transfer separate parts of the solute molecule.

Another example of retention data prediction in reversed-phase HPLC, this time based on statistical analysis of the retention data of over a hundred peptides, was presented a few years ago[10]. In that paper, additive retention increments for all protein amino acid residues and some terminal groups were given. These increments allow the calculation of retention data for peptides containing up to 20 amino acid residues.

Although reversed-phase chromatography is involved in the majority of applications it is not necessarily the method of choice. Many separations were done with normal phase chromatography which is particularly useful for the separation of protected peptide diastereomers. For that reason we examined the chromatographic behaviour of protected dipeptides on a LiChrosorb column.

EXPERIMENTAL

Chemicals

HPLC-grade *n*-hexane was obtained from Reachim (U.S.S.R.). Ethanol and isopropanol (POCh, Poland) were dried over molecular sieves then distilled.

Protected dipeptides were synthesized as follows. To the amino acid *p*-nitrobenzyl ester *p*-toluenesulphonate[11] (0.1 mmol) were added solutions of acetyl amino acid (1 cm^3, 0.1 *M*), triethylamine (0.25 cm^3, 0.4 *M*), 1-hydroxybenzotriazole (0.25 cm^3, 0.8 *M*) and dicyclohexylcarbodiimide (0.25 cm^3, 0.4 *M*) in methylene chloride.

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After 3 h, dicyclohexylurea was filtered off. The solution was washed with 2-cm^3 portions of 5% aqueous sodium bicarbonate, 0.5 *M* hydrochloric acid and water and dried over $MgSO_4$. Solutions of the crude product were chromatographed.

Chromatographic conditions

Chromatography was accomplished using a Kabid 5101 isocratic chromatograph equipped with a Kabid 5301 fixed wavelength detector (254 μm) and homemade column (250 × 4 mm) packed with LiChrosorb Si 60, 5 μm (E. Merck, Darmstadt, F.R.G.). An isocratic mobile phase consisting of 2% ethanol plus 2.5% isopropanol in *n*-hexane was used.

RESULTS

The chromatographic data obtained suggest the possibility of predicting the retentions of protected dipeptides. It is based on the following relationships.

The distribution coefficient in adsorption chromatography is given[12] by

$$K = k' \cdot \frac{V}{A} \tag{1}$$

where k' represents the capacity factor, V the void volume of the mobile phase and A the area of the stationary phase. As the free energy of adsorption is given by

$$\Delta G^\circ = -RT \ln K \tag{2}$$

eqn. 1 can be rewritten as follows:

$$\ln k' = \ln \frac{A}{V} - \frac{\Delta G^\circ}{RT} \tag{3}$$

On the assumption that the contributions of the amino acid residues forming a dipeptide to its free energy of adsorption are additive, one can obtain the following equations for k' and the relative retention, α, of the peptide diastereomers

$$k'_{ij}\,(\text{pred}) = \frac{k'_{io}\,k'_{oj}}{k'_{oo}} \tag{4}$$

$$\alpha_{ij}\,(\text{pred}) = \frac{\alpha_{io}\,\alpha_{oj}}{\alpha_{oo}} \tag{5}$$

where the indexes *i, j, o* represent amino acid residues.

We tested the above assumption using a randomized set of dipeptides Ac-*i*-*j*-ONBzl, where Ac is acetyl, NBzl is *p*-nitrobenzyl, *i, j* are Ala, Val, Phe, phenylglycine (Phg) and $i=j=o$ is Leu. Experimental, k' (exptl), and predicted, k' (pred), capacity factors of a series *SS*- and *RS*-dipeptides are in good agreement (Table I). The corresponding linear regression equations and correlation coefficients, r^2, are:

$$k'_{SS}\,(\text{exptl}) = 0.8636\,k'_{SS}\,(\text{pred}) + 1.53;\ r^2 = 0.9468 \tag{6}$$

TABLE I

EXPERIMENTAL AND PREDICTED CAPACITY FACTORS OF Ac-*i*-*j*-ONBzl DIPEPTIDES

Chromatographic conditions as described in the text.

Dipeptide	k'_{SS}		k'_{RS}		α	
	exptl	*pred*	*exptl*	*pred*	*exptl*	*pred*
Ac-Leu-Ala-ONBzl	8.60	–	7.38	–	0.86	–
-Val-ONBzl	3.31	–	3.75	–	1.13	–
-Leu-ONBzl	2.56	–	3.31	–	1.29	–
-Phe-ONBzl	3.45	–	4.73	–	1.37	–
-Phg-ONBzl	4.54	–	5.64	–	1.24	–
Ac-Ala-Leu-ONBzl	13.02	–	13.12	–	1.01	–
Ac-Val-	3.21	–	3.94	–	1.23	–
Ac-Phe-	2.95	–	4.63	–	1.57	–
Ac-Phg-	3.79	–	6.27	–	1.23	–
Ac-Ala-Val-ONBzl	16.92	16.83	16.37	14.86	0.97	0.88
Ac-Val-	4.56	4.15	4.86	4.46	1.07	1.08
Ac-Phe-	4.66	3.81	5.68	5.25	1.22	1.38
Ac-Pgh-	4.88	4.90	6.80	7.10	1.39	1.45
Ac-Ala-Phe-ONBzl	19.82	17.55	20.23	18.75	1.02	1.07
Ac-Val-	5.87	4.33	7.46	5.63	1.27	1.31
Ac-Phe-	5.58	3.98	8.75	6.62	1.57	1.67
Ac-Phg-	6.38	5.11	10.44	8.96	1.64	1.75
Ac-Ala-Phg-ONBzl	18.85	23.09	18.85	22.36	1.00	0.97
Ac-Val-	5.71	5.69	7.31	6.71	1.28	1.18
Ac-Phe-	6.09	5.23	9.02	7.89	1.48	1.51
Ac-Phg-	6.63	6.72	10.71	10.68	1.62	1.59

$$k'_{RS}\,(\text{exptl}) = 0.8698\ k'_{RS}\,(\text{pred}) + 1.89;\ r^2 = 0.9368 \quad (7)$$

$$\alpha\,(\text{exptl}) = 0.8363\ \alpha\,(\text{pred}) + 0.19;\ r^2 = 0.9336 \quad (8)$$

The model variance is significantly greater (confidence level 0.05) than the repeatability variance.

DISCUSSION

The correlation of k' (exptl) and k' (pred) shows that the contributions of individual amino acid residues to the free energy of adsorption of the dipeptide are not completely independent, but such a simplification appears very useful. The set of dipeptides investigated can roughly be divided into two classes: (1) those containing the Ala residue, which had k' (exptl) greater than k' (pred), and (2) those for which k' (exptl) was smaller than k' (pred). We believe this is due to the stronger adsorption of Ala-containing dipeptides to the silica surface.

Moreover, the retention mechanism in the studied chromatographic system is more complex than in partition chromatography[7] and for this reason eqns. 4 and 5 are not exactly fulfilled, but the mean errors of the prediction are not high: 12.7% for k'_{SS}, 11.7% for k'_{RS} and 5.3% for α.

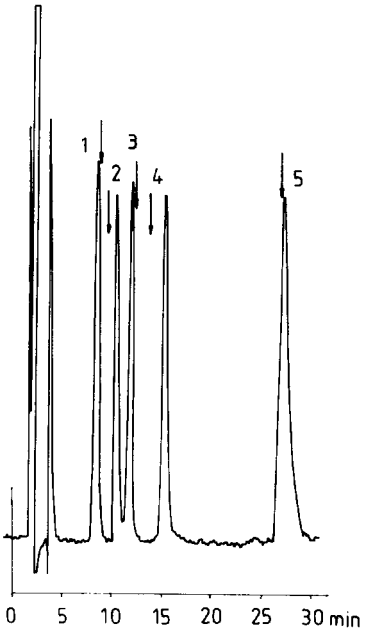

Fig. 1. Chromatogram of a mixture of protected dipeptides Ac-*i*-*j*-ONBzl. Chromatographic conditions as described in the text. Predicted retentions are indicated by the arrows. Peaks: 1 = Ac-*S*-Phg-*S*-Val-ONBzl; 2 = Ac-*S*-Phe-*S*-Phg-ONBzl; 3 = Ac-*R*-Phg-*S*-Val-ONBzl; 4 = Ac-*R*-Phe-*S*-Phg-ONBzl; 5 = Ac-*S*-Ala-*S*-Val-ONBzl.

According to Helmchen *et al.*[13], amides form two hydrogen bonds with silanol groups of silica gel. Examination of Dreiding's models of Ac-Phe-Phe-ONBzl reveals that there is little difference between the probable conformations and between the lengths of the hydrogen bonds of adsorbed *RS* and *SS* diastereomers. We noticed, however, that the hydrophobic groups in the *RS* diastereomer are assembled on the esterior of the adsorbed particle and shield the amide centre more than in the *SS* diastereomer. Hence the chromatographic separation of the diastereomers is caused not only by a difference in adsorption energy but also by a competition of the polar modifier in the mobile phase, *e.g.*, alcohol, for the amide centre. Because of the steric effects, isopropanol seems to be the best solvent modifier for the separation; the molecule of ethanol is too small and that of butanol is too large.

The described method of prediction of chromatographic data can avoid the problem of choosing proper standards for HPLC analysis of complex mixtures of peptides. Knowing the basic set of 43 k' data for dipeptides synthesized from 21 amino acids, it is possible to predict k' and α data for 400 other dipeptides. The predicted k' values can then be used to screen a chromatogram for standards and as a guide to identification (Fig. 1).

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