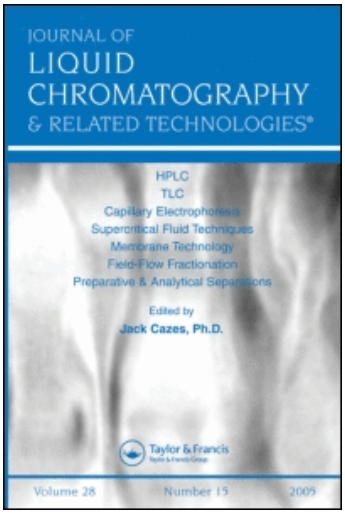

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Some Retention Characteristics of Dipeptides in Reversed-Phase High Performance Liquid Chromatography

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SOME RETENTION CHARACTERISTICS OF DIPEPTIDES IN REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The effect of hydrophobicity of amino acid subunits on the retention of dipeptides with phosphate buffer as the eluent in RP-HPLC was studied, it has been observed that the capacity factor for dipeptides combined of amino acid subunits with hydrophobicity smaller than that of Ala is smaller than 0.4, and these dipeptides had better be separated by reversed-phase ion-pair liquid chromatography or ion chromatography. Otherwise, the dipeptides combined of amino acid subunits with hydrophobicity larger than that of Ala can be separated by RP-HPLC. It has been observed that there is a good linear relationship between lnk' values in RP-HPLC with the same eluent and different packing materials used or the same packing material and different eluents containing low concentration of different organic modifiers used.

INTRODUCTION

In recent years, biochemistry and protein chemistry have been attracting considerable attention. Enzymolisis of proteins and solid-phase synthesis of peptides produce a lot of peptide mixtures containing impurities[1,2]. The separation and recovery of small quantities of a peptide of interest from synthesized mixtures is a very important task in biochemistry. Therefore, the utilization of HPLC, especially reversed-phase HPLC, for the separation of

peptide mixtures is recently increasing[3-6]. For the optimization of the separation conditions, a lot of effort has been made to predict the retention of peptides in the reversed-phase HPLC with acetonitrile/aqueous buffer or methanol/aqueous buffer as the eluent based on the quantitative structure retention relationship[7-12]. But we think that the first step for optimization of the separation conditions of peptides is the selection of right separation modes[13-15]. In this paper, the contribution of hydrophobicity of amino acid subunits on retention values of dipeptides is investigated. It is suggested that the dipeptides combined of amino acid subunits with hydrophobicity larger than that of Ala can be separated by reversed-phase HPLC. A linear relationship between lnk' values in reversed-phase HPLC with the same eluent and different packing materials used or the same packing material and different eluents containing low concentration of different organic modifiers used has been observed.

EXPERIMENTAL

1. Materials

All of the dipeptides used were purchased from Serva Feinbiochemica GmbH. (Heidelberg, Germany) and are listed in Table 2. Methanol, acetonitrile, n-propanol, KH_2PO_4 and all the other used chemical reagents are of analytical grade. Deionized and distilled water was used throughout the experiments.

2. Apparatus and Conditions

Spherisorb-ODS with particle diameter 10 μm (Phase Separation Ltd, Deeside, UK), YWG-ODS with particle diameter 10 μm (Tianjing Second Chemical Reagent Factory, Tianjing, P.R.China) and Nucleosil-ODS with particle diameter 7 μm (Macherey-Negal, Duren, Germany) were packed into the stainless steel column with 200X4.0 mm I.D., 250X4.6 mm I.D. and 150X4.0 mm I.D. respectively. The mobile phases was dilivered by a Waters Model 510 pump (Waters Assoc., Milford, MA, USA). The eluates were detected by a homemade FS-100 UV detector at 210 nm. The samples were introduced by a U6K syringe loading-sample injector. The flow

rate was set at 1.0 ml/min. The eluent pH was measured by the SA-720 pH meter (Orion Res. Inc., Chicago, IL, USA). Each sample was dissolved in the eluent. The void volume of columns was determined by the methanol peak at wavelength 210 nm. All the experimental data were processed by a NEC-APCIV personal computer.

RESULTS AND DISCUSSION

1. Contribution of Hydrophobicity of Amino Acid Subunits on the Retention of Dipeptides in RP-HPLC

In the previous papers[14,15], it has been proposed that the retention of all compounds in a sample should be adjusted in the range $0.4<k'<30$ for a suitable column system. In reversed-phase HPLC, the retention of a solute increases with its hydrophobicity and numbers of hydrophobic functional groups, and decreases with its hydrophilicity and numbers of hydrophilic groups[16].For the separation of dipeptides in reversed-phase HPLC,the retention of dipeptides increases with hydrophobicity of their amino acid subuints, and decreases with hydrophilicity of their amino acid subunit. The sequence of hydrophobicity for amino acid subunits has been determined by Jinno et al[17] and is listed in Table 1. Table 2 gives out the capacity factors of 35 peptides experimentally measured in reversed-phase HPLC with Spherisorb-ODS, YWG-ODS and Nucleosil-ODS as stationary phases and 10 mmol/l phosphate buffer (pH=2.10) as the eluent, which is the weakest eluent in reversed-phase HPLC. Only is the capacity factor of dipeptides larger than 0.4 when the weakest eluent is used in reversed-phase HPLC, the reversed phase HPLC can be recommended as a possible separation mode. It can be seen from the data in Table 2 that the contribution of each Gly to capacity factor is very low and about 0.06, and the contribution of Ala to capacity factor is about 0.2. These results imply that the dipeptides combined of amino acid subunits with hydrophobicity smaller than that of Ala (including Lys, Gln, Asp, His, Thr, Gly, Ala and Ser) have weak retention ($k'<0.4$), and can not be well separateed in the reversed-phase HPLC. One had better select

TABLE 1
The Hydrophobicity (R') of Amino Acid Subunit. The Data Are Taken From Jinno et al[17]

Amino Acid	R'	Amino Acid	R'
Trp	2.280	Phe	1.792
Leu	1.313	Ileu	1.270
Tyr	1.305	Met	0.938
Val	0.741	Arg	0.119
Lys	-0.073	His	0.033
Pro	0.395	Ala	0.024
Gln	0.024	Thr	0.024
Glu	0.119	Gly	-0.084
Asp	-0.017	Ser	-0.084
Cys	0.170		

reversed-phase ion-pair liquid chromatography, ion chromatography to separate these dipeptides, or select a suitable derivative reagent to react with these dipeptides and separation of their products by reversed-phase HPLC. Otherwise, all the dipeptides combined of amino acid subunits with hydrophobicity larger than that of Ala or the tetra- and hept-peptides combined of amino acid subunits Ala, Ser, Gly, Asp, His, Thr, Lys and Gln have k' values larger than or near 0.4 and can be separated in reversed-phase HPLC.

From the data shown in Table 1, the hydrophobicity of Gly and Ser is the lowest in all of the amino acid subunits, but still make a slightly positive contribution to retention in reversed-phase HPLC with acidic phosphate buffer as eluent. The capacity factor of dipeptide Gly-Cys shown in Table 2 is near 0.4, therefore, it can also be deduced that the peptides containing an amino acid subunit with hydrophobicity larger than that of Cys have the capacity factor larger than 0.4, and can be separated in reversed-phase HPLC. In order

TABLE 2

Capacity Factors of Peptides in Reversed-Phase HPLC with Different Packing Materials as Stationary Phases and KH_2PO_4 10 mmol/l Phosphate Buffer (pH 2.10) as Eluent. Columns I, II and III Were Packed with Spherisorb-ODS (10 μm), YWG-ODS (10 μm) and Nucleosil-ODS (7 μm) Respectively.

Peptide	Column I	Column II	Column III
Gly-Gly	0.100	0.082	0.057
Gly-Gly-Gly	0.156	0.156	0.128
Gly-Gly-Gly-Gly	0.211	0.202	0.185
Gly-Gly-Gly-Gly-Gly	0.278	0.312	0.256
Gly-Gly-Gly-Gly-Gly-Gly	0.356	0.394	0.328
Thr-Gly	0.344	0.376	0.328
Asp-Gly	0.189	0.165	0.157
Gly-His	0.356	0.119	0.100
Gly-Asp	0.111	0.037	0.043
Ser-Gly	0.122	0.073	0.057
Lys-Asp	0.189	0.119	0.096
Arg-Ser	0.222	0.202	0.171
Cys-Gly	0.422	0.413	0.300
Gly-Ser	0.089	0.027	0.028
Ala-Gly	0.222	0.293	0.228
Gly-Ala	0.278	0.396	0.328
Val-Gly	3.07	3.01	2.70
Gly-Val	4.42	4.51	3.89
Met-Gly	5.06	4.92	4.60
Gly-Met	5.49	5.62	5.13
Leu-Gly	12.91	12.03	10.37
Gly-Leu	19.23	18.54	16.28
Phe-Gly	42.17	38.38	34.16
Gly-Phe	44.40	41.83	38.91
Tyr-Gly	17.78	18.01	17.37
Gly-Tyr	18.11	18.69	19.07
Gly-Glu	0.553	0.632	0.618
Pro-Gly	1.12	1.19	1.11
Gly-Pro	1.66	1.74	1.57
Hyp-Gly	0.494	0.528	0.510
Met-Ser	2.16	2.08	2.15
Gly-Ileu	16.08	15.68	13.32
Val-Ala	2.42	3.28	2.22
Met-Thr	3.41	3.21	3.28
Met-Asp	2.91	2.87	2.99

TABLE 3

Capacity Factors of Sixteen Dipeptides in Reversed-Phase HPLC with the Same Packing Material and Different Eluents Used. The Eluents Are Organic Modifier/Phosphate Buffer (0.03/0.97) Containing 10 mmol/l KH_2PO_4 and pH 2.10; the Column Is 200X4.0 mm I.D. Packed with Nucleosil-ODS (7 μm).

Dipeptide	Methanol/Buffer (0.03/0.97)	Acetonitrile/Buffer (0.03/0.97)	n-Propanol/Buffer (0.03/0.97)
Val-Gly	1.62	0.902	0.775
Gly-Val	2.61	1.62	1.52
Met-Gly	2.61	1.51	1.25
Gly-Met	3.31	2.12	1.88
Leu-Gly	5.98	3.13	2.83
Gly-Leu	10.52	6.42	6.20
Phe-Gly	18.42	9.71	8.08
Gly-Phe	23.22	13.86	12.30
Tyr-Gly	9.45	4.37	3.89
Gly-Tyr	10.88	5.91	6.00
Met-Ser	1.30	0.775	0.696
Gly-Ileu	8.77	5.23	5.04
Val-Ser	1.38	0.775	0.686
Met-Thr	1.87	0.886	0.686
Met-Asp	1.79	1.05	0.912
Gly-Pro	0.941	0.598	0.520

to extend these obtained results to other reversed-phase HPLC system, we could make a linear regression of lnk' values in reversed-phase HPLC with different packing materials as statinoary phases and the same phosphate buffer as eluent. The obtained result of linear regression of lnk' values in Table 2 for 27 peptides is shown in Fig.1 and Fig.2. It can be seen that the intercept and slope of the obtained linear relationship are close to 0 and 1 respectively,

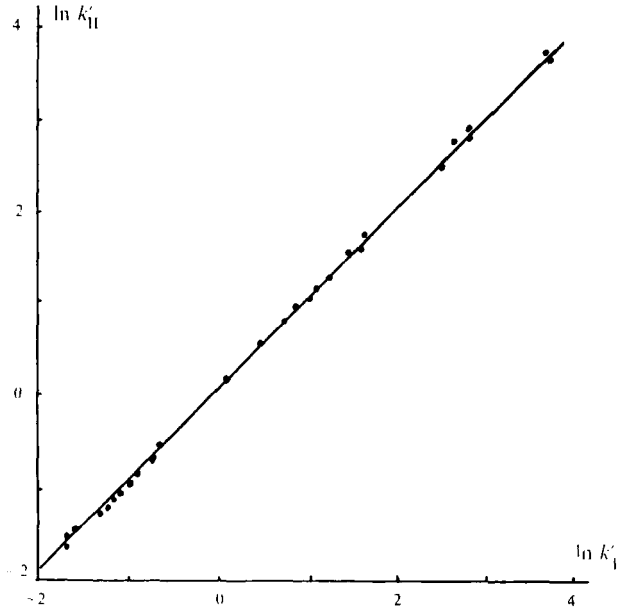

FIGURE 1 The linear regression of $\ln k'_{II}$ with YWG-ODS as packing material vs. $\ln k'_{I}$ with Spherisorb-ODS as packing material. The eluent used is 10 mmol/l KH_2PO_4 phosphate buffer (pH 2.10). $\text{Ln}k'_{II} = 0.067 + 0.959 \ln k'_{I}$, n=27, r=0.999

which means that in the most cases the dipeptide retention at different packing materials is quite close, and obtained results in Spherisorb-ODS, YWG-ODS and Nucleosil-ODS for the selection of reversed-phase HPLC according to the hydrophobicity of amino acid subunits containing in dipeptides can be extended to the most of reversed-phase HPLC system.

2. Relationship of lnk' Values at Different Organic Modifiers

In recent years, the prediction of retention of a particular peptide from its amino acid subunit has become an interesting subject[6,7-10,18-20]. But the prediction of retention from the eluent with one organic modifier to that with another organic modifier does not attract much attention yet. The capacity factors of 16 dipeptides with acetonitrile/aqueous buffer, methanol/aqueous buffer and n-propanol/aqueous buffer containing KH_2PO_4 10 mmol/l (pH=2.1) as

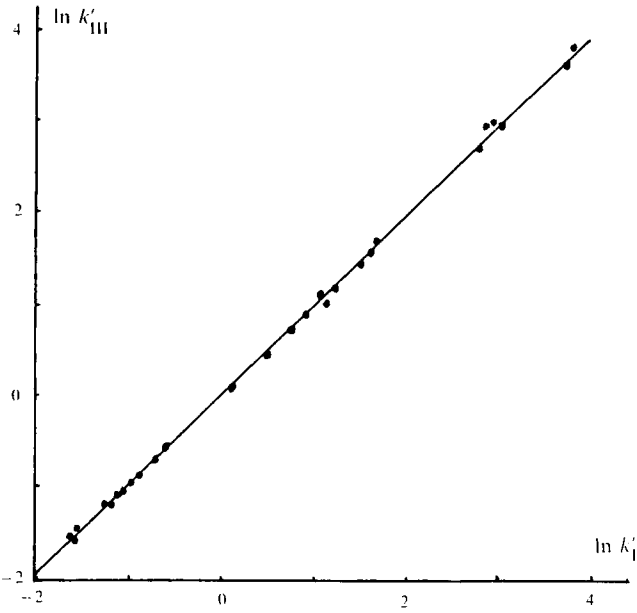

FIGURE 2 The linear regression of $\ln k'_{III}$ with Nucleosil-ODS as packing material vs. $\ln k'_{I}$ with Spherisorb-ODS as packing material. The eluent used is 10 mmol/l KH_2PO_4 phosphate buffer (pH 2.10). $\text{Ln}k'_{III} = 0.054 + 0.929\ \ln k'_{I}$, n=27, r=0.999

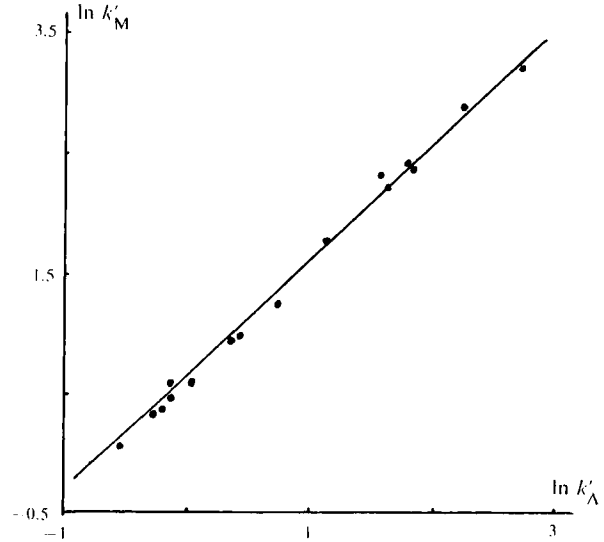

FIGURE 3 The linear regression of $\ln k'_{M}$ with methanol/phosphate buffer (0.03/0.97) containing KH_2PO_4 10 mmol/l and pH 2.10 as eluent vs. $\ln k'_{A}$ with acetonitrile/phosphate buffer (0.03/0.97) containing KH_2PO_4 10 mmol/l and pH 2.10 eluent. The column is 200X4.0 mm I.D. packed with Nucleosil-ODS (7 μm). $\text{Ln}k'_{M} = 0.556 + 1.012\ \ln k'_{A}$, n=16, r=0.996

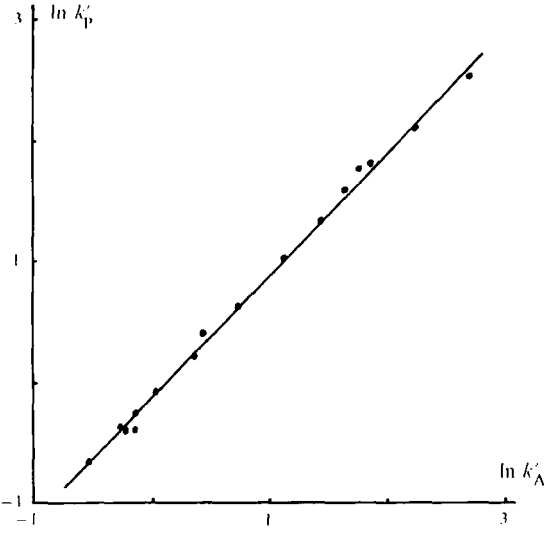

FIGURE 4 The linear regression of $\ln k'_P$ with n-propanol/phosphate buffer (0.03/0.97) containing KH_2PO_4 10 mmol/l and pH 2.10 as eluent vs. $\ln k'_A$ with acetonitrile/phosphate buffer (0.03/0.97) containing KH_2PO_4 10 mmol/l and pH 2.10 eluent. The column is 200X4.0 mm I.D. packed with Nucleosil-ODS (7 μm). $\ln k'_P = -0.140 + 1.027 \ln k'_A$, n=16, r=0.998

the eluent were measured and listed in Table 3. The result of linear regression analysis of lnk' values at different organic modifiers was shown in Fig.3 and Fig.4. It can be seen that the regression coefficient in all cases is larger than 0.99, which means that there is an effect of organic modifiers on retention, but no serious effect on the selectivity of dipeptides when the organic modifier concentration in eluent is low. Also the dipeptides in reversed-phase HPLC with low concentration of different organic modifiers in buffer as the eluents behave the similar retention mechansim.

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