Journal of Chromatography, 359 (1986) 519–532
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 891

PREDICTION OF PEPTIDE RETENTION TIMES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. CORRELATION OF OBSERVED AND PREDICTED PEPTIDE RETENTION TIMES AND FACTORS INFLUENCING THE RETENTION TIMES OF PEPTIDES

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SUMMARY

We have assessed the accuracy of a set of amino acid residue retention coefficients by applying them to the prediction of the retention times of 58 peptides under linear gradient elution conditions (solvent A = 0.1% trifluoroacetic acid in water, and solvent B = 0.1% trifluoroacetic acid in acetonitrile). These coefficients were determined by examining the retention times of synthetic model peptides in reversed-phase chromatography. The high degree of correlation (0.98) between predicted and observed retention times not only indicated good predictive accuracy for our coefficients, but was also further evidence that composition is generally the major factor affecting peptide retention time. For optimum accuracy in retention time prediction on any single column, it was essential to include an internal peptide standard in each run to correct for run-to-run deviations and column aging. The resolution of five commercially available synthetic peptide standards was found to improve with increasing flow-rate and decreasing gradient steepness. Increasing temperature resulted in a decrease in peptide retention times and slightly improved resolution. Rules for retention time prediction are presented which not only enable the experimenter to correct for instrument and column (length, diameter, *n*-alkyl chain length and ligand density) specifications, but also allow the prediction of peptide retention times at any gradient steepness, flow-rate and temperature.

INTRODUCTION

We have previously reported[1] empirical sets of retention coefficients for amino acid residues at pH 2.0 and pH 7.0, obtained by measuring the effect of individual amino acid residues on the chromatographic behaviour of a model synthetic peptide in reversed-phase high-performance liquid chromatography (RP-HPLC). Although excellent separation of peptide mixtures was generally obtained by the conditions employed to determine our coefficients (linear gradient, where solvent A is 0.1%

0021-9673/86/$03.50

trifluoroacetic acid (TFA) in water and solvent B is 0.1% TFA in acetonitrile; 1% B/min, 1 ml/min, 26°C), variations in factors which influence peptide resolution and retention (gradient steepness, flow-rate, etc.) may be required to enable optimum utilisation of RP-HPLC.

In this paper we demonstrate the accuracy of our amino acid residue coefficients by applying them to a wide range of peptides and determining the correlation of observed and predicted retention times during RP-HPLC. In addition, we examine the effects of gradient steepness, flow-rate, temperature, and residue sequence specificity on peptide resolution and retention.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Reagent grade TFA was redistilled prior to use. Peptides were prepared by solid-phase synthesis in this laboratory[1] or obtained from the Institut Armand-Frappier (Laval, Quebec, Canada).

Synthetic peptide standards

A mixture of five synthetic decapeptide standards (S1–S5) was obtained from the Alberta Peptide Institute (Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada). The composition of the peptides varied as follows: peptide S2, -Gly^3-Gly^4-; peptide S3, -Ala^3-Gly^4-; peptide S4, -Val^3-Gly^4-; peptide S5, -Val^3-Val^4-. All peptides contained an N$^\alpha$-acetylated N-terminal and a C-terminal amide, except peptide S1, which was identical to peptide 3 but had a free α-amino group. The standards are also available from Pierce Chemical (Rockford, IL, U.S.A.) and Synchrom (Linden, IN, U.S.A.).

Apparatus

The HPLC instrument consisted of a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) combined with a Varian 2080 column oven and coupled to a Hewlett-Packard (Avondale, PA, U.S.A.) HP 1040A detection system, HP85B computer, HP9121 disc drive, HP2225A Thinkjet printer and HP7470A plotter. Samples were injected with a 500-μl injection loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Columns

Peptide mixtures were separated on three reversed-phase SynChropak columns (SynChrom, Linden, IN, U.S.A.): (1) RP-8 (C_8) column (250 × 4.1 mm I.D., 6.5-μm particle size, 300-Å pore size, *ca.* 7.5% carbon loading) and two RP-P (C_{18}) columns (6.5 μm, 300 Å, *ca.* 10% carbon loading): (2) 250 × 4.1 mm I.D., and (3) 250 × 10 mm I.D.

Gradient elapsed time (t_g)

Gradient elapsed time is defined in this work as the time for the gradient to reach the detector from the proportioning valve via pump, injection loop, and column[1].

RESULTS AND DISCUSSION

Accuracy of peptide retention prediction

The value of a predictive method in RP-HPLC is assessed by its accuracy in predicting the retention times of peptides not used to determine the retention coefficients. Hence, the amino acid residue coefficients, determined in our previous report[1], were applied to retention time predictions of 58 peptides (Table I). The peptides were chromatographed under the conditions used to determine our retention coefficients [linear gradient, where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile (pH 2.0); 1% B/min, 1 ml/min, 26°C]. The small average deviation of predicted values from observed retention times (1.3 min) and the high degree of correlation (Fig. 1; correlation = 0.98, calculated by linear least squares fitting) indicates that our coefficients produce good predictive accuracy for the range of peptides studied (2–16 residues). Differences in peptide retention arising from different column packings (C_8, C_{18}) or from column aging were corrected by chromatographing an internal peptide standard along with the peptides under investigation[1]. By deliberately using columns of different age, *n*-alkyl chain length, and ligand density, it was hoped to lessen any favourable bias in the comparison of predicted and observed retention times (Fig. 1). Interestingly, the only peptide, 14 residues in length, in Table I which contained two cysteine residues showed little difference in retention time between its oxidized (57a) and reduced (57b) forms. Two other peptides, 21 and 24 residues in length (not listed in Table I), containing two cysteine residues each, also showed little difference in retention time between their oxidized and reduced forms. The results from the limited range of peptides studied suggest that disulphide bridge formation and, hence, peptide folding has little effect on interaction of small peptides with the hydrophobic stationary phase. Several peptides predicted to have negative retention times were, as expected, not retained by the columns.

Effect of residue sequence

To examine the possibility of any sequence specific effects on peptide retention, a number of homologous peptide pairs (Table II) were chromatographed on SynChropak C_8 or C_{18} columns under the same conditions detailed in the previous section. All peptide pairs were eluted as single peaks, indicating that the sequence order of amino acid residues in these peptides had no effect on retention time. Peptides 35 and 44–46 are a homologous quartet and, when chromatographed in mixture, were only very slightly resolved to form a small doublet. These results indicate that, for our peptides, subtle changes in residue sequence (between peptides 18 and 19, 39 and 42, 40 and 41, 35 and 44–46) or a more complete re-arrangement of residues (peptides 20 and 22) have little or no effect on peptide retention. No doubt, under certain circumstances, sequence variations may significantly influence peptide retention, particularly in small, highly ionized peptides[2]. However, the high degree of correlation shown in Fig. 1 is not only good evidence for the accuracy of our predictive method, but, coupled with the results presented in Table II, supports the premise that composition is generally the major factor affecting peptide retention times.

TABLE I

COMPARISON OF PREDICTED AND OBSERVED PEPTIDE RETENTION TIMES

Peptide	*Sequence**	*No. of residues*	t_R *predicted (min)*	t_R *observed (min)*	*Error (min)*
1	AcKF* amide	2	3.10	3.35	−0.25
2	AcAKF* amide	3	5.03	4.45	0.58
3	AcKF*A amide	3	5.03	3.55	1.48
4	NH_2F*AA amide	3	2.13	2.92	−0.79
5	AcKF*AA amide	4	15.18	16.65	−1.47
6	AcAAKF* amide	4	6.89	6.49	0.40
7	AcAKF*A amide	4	7.07	6.04	1.03
8	AcAKF*AA amide	5	10.87	10.58	0.29
9	AcAAKF*A amide	5	8.71	8.61	0.10
10	NH_2FFGLM amide	5	19.75	20.28	−0.53
11	AcKGLGLK amide	6	11.48	14.64	−3.16
12	AcAAKF*AA amide	6	12.84	13.03	−0.19
13	NH_2AAKF*AA amide	6	11.06	12.56	−1.50
14	AcAAKF*AA OH	6	11.97	13.91	−1.94
15	NH_2AAL**F*AA amide	6	16.08	14.31	1.77
16	NH_2LSL**F*AL amide	6	25.40	26.87	−1.47
17	NH_2LSF*L**ALOEt	6	25.94	26.89	−0.95
18	AcAAAKF*AA amide	7	14.84	14.63	0.21
19	AcAAKF*AAA amide	7	14.79	14.43	0.36
20	AcKGLLLGK amide	7	24.52	23.93	0.59
21	AcKKLLLKK amide	7	21.00	20.73	0.27
22	AcKLGLGLK amide	7	23.57	21.67	1.90
23	NH_2DYMGWMDF OH	8	23.85	26.78	−2.93
24	AcTDLLAGGK amide	8	12.06	13.99	−1.93
25	NH_2TDLLAGGK amide	8	6.23	4.68	1.55
26	AcTDGLAGGK amide	8	4.11	4.57	−0.46
27	AcGAKLEAKG amide	8	6.51	6.08	0.43
28	AcTDLLGGGK amide	8	11.72	12.87	−1.15
29	pEHWSYGLRPG amide	10	19.60	19.88	−0.28
30	NH_2HKTDSFVGLM amide	10	14.47	14.91	−0.44
31	NH_2DMHDFFVGLM amide	10	30.13	29.75	0.38
32	NH_2VQAAIDYING OH	10	16.89	17.06	−0.17
33	NH_2RPKPQQFFGLM amide	11	26.57	26.03	0.54
34	NH_2DVPKSDQFVGLM amide	12	23.52	22.36	1.16
35	AcGKFKGPPLRRVR amide	12	17.03	16.16	0.87
36	NH_2GGFKRPPLRRVR amide	12	11.26	14.18	−2.92
37	AcGKFKRPPLRRVR amide	12	16.84	15.94	0.90
38	AcGKGKRPPLRRVR amide	12	8.04	10.03	−1.99
39	AcGKFGRPPLRRVR amide	12	18.39	16.02	2.37
40	AcGKFKRGPLRRVR amide	12	14.02	14.63	−0.61
41	AcGKFKRPGLRRVR amide	12	14.38	14.55	−0.17
42	AcGGFKRPPLRRVR amide	12	18.19	16.01	2.18
43	AcGKFKRPPGRRVR amide	12	7.70	11.12	−3.42
44	AcGKFKRPPLRGVR amide	12	16.28	15.73	0.55
45	AcGKFKRPPLGRVR amide	12	16.44	16.01	0.43
46	AcGKFKRPPLRRVG amide	12	16.70	15.59	1.11
47	AcGKFKRPPLRRGR amide	12	10.64	12.89	−2.25
48	AcVSKTQTSQVAPA amide	12	12.26	12.76	−0.50
49	AcVSKTETSQVAPA amide	12	12.99	13.07	−0.08
50	AcVSKTATSQVAPA amide	12	17.10	15.51	1.59

TABLE I *(continued)*

Peptide	*Sequence**	*No. of residues*	*t_R predicted (min)*	*t_R observed (min)*	*Error (min)*
51	AcASKTETSQVAPA amide	12	9.81	10.72	−0.91
52	AcDRNAEGYIDAEEL amide	13	30.11	25.67	4.44
53	AcNRNANGYIDAEEL amide	13	26.85	23.85	3.00
54	AcNRDADGYIDAEEL amide	13	28.76	24.72	4.04
55	AcDRDADGYIDAEEL amide	13	29.57	24.98	4.59
56	AcSDQEKRKQISVRGL amide	14	14.17	15.47	−1.30
57a	NH_2AGCKNFFWKTFTSC OH	14	26.57	26.03	0.54
57b	NH_2AGCKNFFWKTFTSC OH	14	26.57	25.92	0.65
58	Ac(GAKLEAKG)$_2$ amide	16	14.97	14.40	0.57

* F* denotes nitrophenylalanine; L** denotes norleucine. The contributions of the ethyl ester (peptide 17) and the pyroglutamic acid (peptide 29) were considered identical to the amide and acetylglutamic acid, respectively.

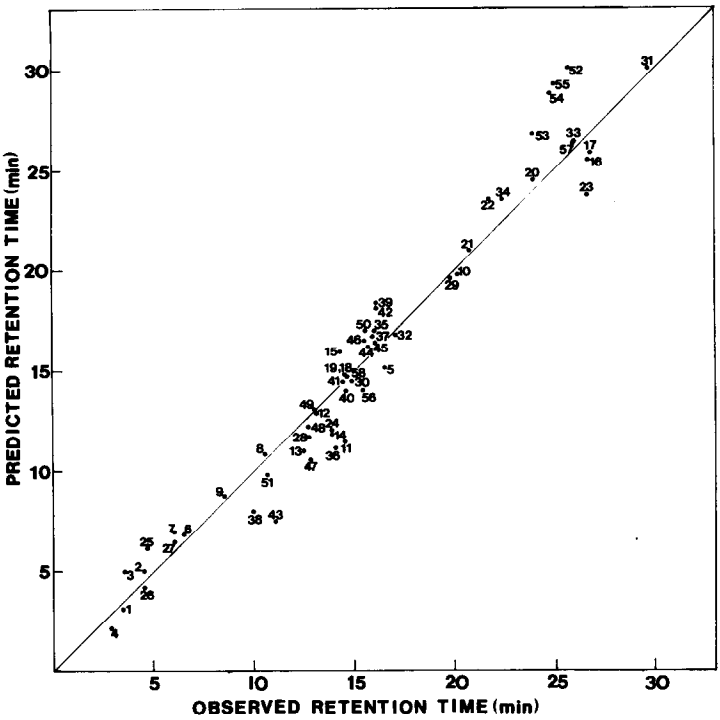

Fig. 1. Correlation of predicted and actual peptide retention times in RP-HPLC. Numbers adjacent to the data points indicate the peptides listed in Table I. The peptides were chromatographed on SynChropak C_8 or C_{18} columns (250 × 4.1 mm I.D.) under the conditions used to determine the amino acid residue retention coefficients: linear gradient (1% B/min) where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. The predicted peptide retention times were obtained by summation of retention coefficients for amino acid residues and end groups as described in ref. 1.

TABLE II

EFFECT OF SEQUENCE SPECIFICITY ON PEPTIDE RETENTION TIMES

Peptide	*Sequence**	t_R *(min)*
20	AcKGLLLGK amide	21.97
22	AcKLGLGLK amide	
18	AcAAAKF*AA amide	14.5
19	AcAAKF*AAA amide	
42	AcGGFKRPPLRRVR amide	16.0
39	AcGKFGRPPLRRVR amide	
40	AcGKFKRGPLRRVR amide	14.6
41	AcGKFKRPGLRRVR amide	
35	AcGKFKGPPLRRVR amide	21.24
44	AcGKFKRPPLRGVR amide	
45	AcGKFKRPPLGRVR amide	20.97
46	AcGKFKRPPLRRVG amide	

* F* denotes nitrophenylalanine. Brackets indicate peptides with the same amino acid composition.

Effect of gradient steepness

A mixture of five synthetic peptide HPLC standards (see Experimental) was chromatographed on a SynChropak C_8 column (250 × 4.1 mm I.D.), with linear gradients (solvent A is 0.1% TFA in water; solvent B is 0.1% TFA in acetonitrile, [pH 2.0]) of 0.5%, 1%, 2% and 4% B/min at a flow-rate of 1 ml/min and a temperature of 26°C. The retention times of the peptides were then plotted against the reciprocal of the gradient slopes[3,4] (Fig. 2). Ideally, a linear relationship should exist between peptide retention and the reciprocal of the gradient slope, with the plots for all five peptides intercepting at the gradient elapsed time, t_g (see Experimental; t_g = 7.0 min in this experiment). However, an increase in peptide partitioning as the gradient steepness decreases, resulting in larger retention times than expected, is probably causing a deviation from strict linearity. Nevertheless, Fig. 2 demonstrates that, in the gradient range used by most investigators (0.5–4% B/min), the relationship between peptide retention time and reciprocal of gradient slope may be considered linear. From the coefficients determined in our previous report[1] (linear gradient, where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile; 1% B/min, 1 ml/min, 26°C), the predicted retention time of a peptide at varying gradient rates ($\tau^{(x\%)}$) may be calculated by subtracting the gradient elapsed time at 1 ml/min (t_g) from the predicted retention time at 1% B/min (τ), multiplying by the reciprocal of the desired gradient slope ($\times$ %) and again adding t_g,

$$\tau^{(x\%)} = (\tau - t_g)\,(1/x\%) + t_g.$$

Hence, peptide retention predictions can be made for different gradient slopes, no matter what gradient slope was used to determine a particular set of coefficients.

Corrections for changes in peptide retention times at gradients other than 1% B/min cannot be made by simply chromatographing a peptide HPLC standard at the desired gradient (see below under *Rules for prediction of retention times*) and applying the subsequent value for t_s, as detailed in the next section (*Effect of flow-rate*), since variations in gradient rate affect different peptides to different extents (Fig. 2).

Effect of flow-rate

We have examined the effect of flow-rate on RP-HPLC by comparing the resolution of synthetic peptide HPLC standards (see Experimental) and four alkylphenone HPLC standards on a SynChropak C_{18} column (250 × 10 mm I.D.) with a linear gradient [solvent A is 0.1% TFA in water; solvent B is 0.1% TFA in acetonitrile, (pH 2.0)] of 1% B/min at flow-rates of 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ml/min and a temperature of 26°C. Fig. 3 demonstrates that the retention times of the alkylphenones increase more rapidly (right) with decreasing flow-rates than those of the peptides (left). The contrast can best be visualized by comparing the alkylphenone and peptide standards with similar retention times at 5 ml/min (S1 and A1, S4 and A2) and following the increasingly dissimilar profiles of the standard pairs as the flow-rate decreases. Other investigators[5–8] have suggested that, under gradient elution conditions, flow-rate changes generally have little effect on peptide or protein elution times, provided the gradient slope is kept low. Thus, under ideal conditions, subtraction of gradient elapsed time (t_g, see Experimental) from the retention times (t_R) of the peptide and alkylphenone standards and plotting this difference against flow-rate should result in straight-line plots with zero slope, *i.e.* little or no effect of

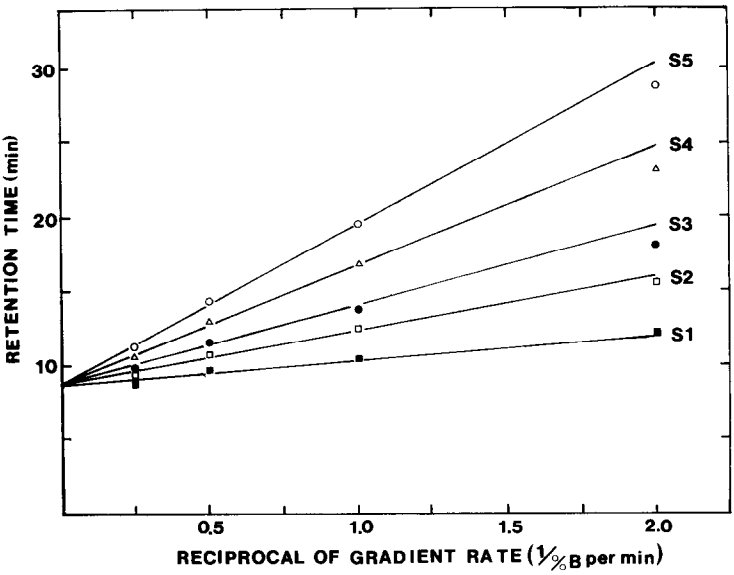

Fig. 2. Plots of retention times of five synthetic peptide HPLC standards *versus* the reciprocal of the gradient slope (1/% B per min). Conditions: column, SynChropak C_8 (250 × 4.1 mm I.D.); linear gradient (0.5%, 1%, 2% or 4% B/min), where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental.

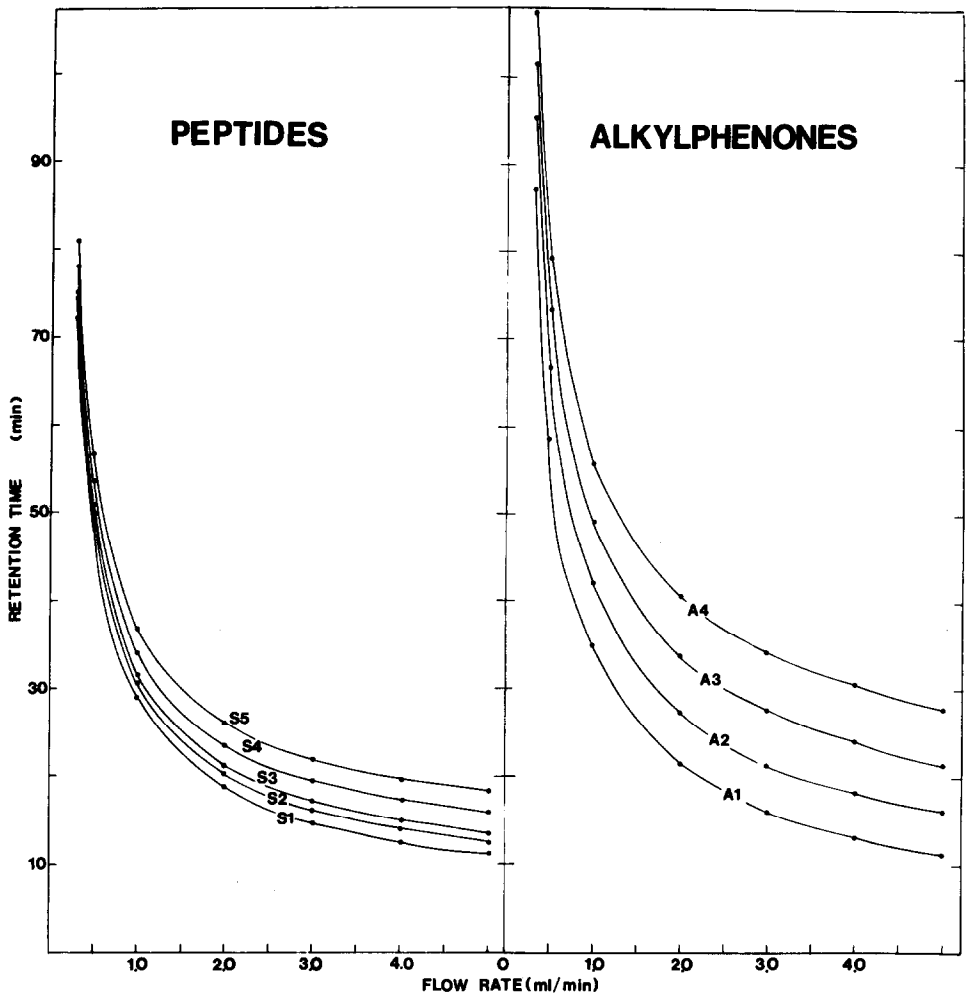

Fig. 3. Effect of flow-rate on retention time of alkylphenone and synthetic peptide HPLC standards in RP-HPLC. Conditions: column, SynChropak C_{18} (250 × 10 mm I.D.); linear gradient (1% B/min) where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental. A1–A4 denote acetophenone, propiophenone, *n*-butyrophenone, and valerophenone, respectively.

flow-rate on retention time. The relationship between ($t_R - t_g$) and flow-rate for the alkylphenone and peptide standards, derived from the results shown in Fig. 3, are demonstrated in Fig. 4. The contrast between the alkylphenone and peptide profiles is quite dramatic. Flow-rate is seen to have little effect on retention time of the peptide standards, once the value for t_g at each flow-rate is taken into account. Some increase in peptide partitioning as the flow-rate decreases may possibly be producing the slightly negative slope of the peptide plots. However, for practical purposes, this deviation from zero slope is negligible. Hence, the predicted retention time of a pep-

tide at different flow-rates may be calculated by simply correcting for the varying gradient elapsed times. Thus, from the coefficients determined in our previous report[1] [conditions: linear gradient, where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile, (pH 2.0); 1% B/min, 1 ml/min, 26°C] the predicted retention time of a peptide at varying flow-rates is obtained by subtracting the gradient elapsed time at 1 ml/min (t_g) from the predicted retention time at 1 ml/min (τ) and adding the result of dividing t_g at 1 ml/min by the desired flow-rate (y, ml/min)

$$\tau\,(y) = (\tau - t_g) + \left(\frac{t_g}{y}\right).$$

If so desired, peptide retention time corrections for flow-rates other than 1 ml/min may be made by chromatographing a peptide HPLC standard at the new flow-rate, determining t_s (see below under *Rules for prediction of retention times*), and substi-

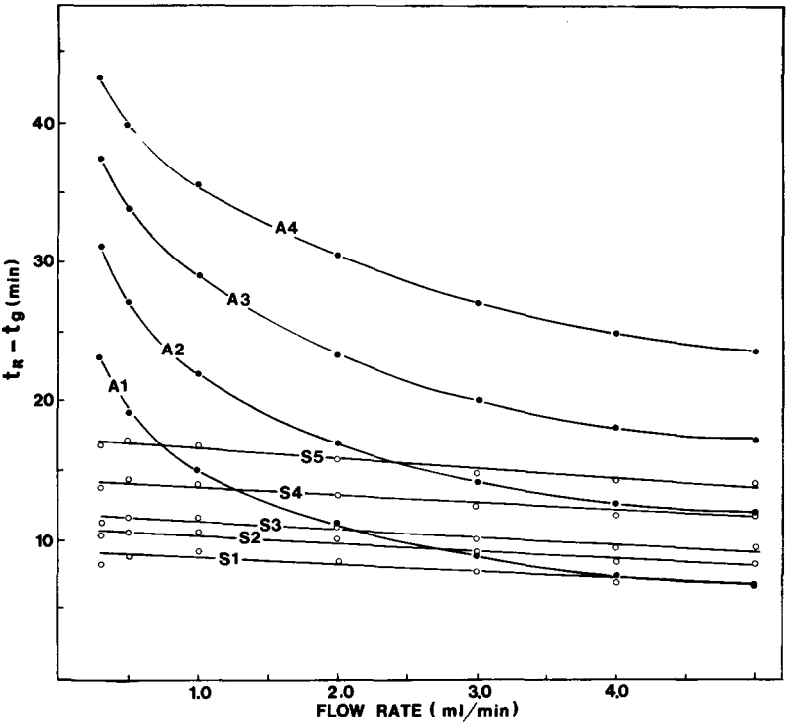

Fig. 4. Effect of subtracting gradient elapsed time (t_g) from retention time (t_R) of alkylphenone and synthetic peptide HPLC standards in RP-HPLC at different flow-rates. Conditions: column, SynChropak C_{18} (250 × 10 mm I.D.); linear gradient (1% B/min), where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental. A1–A4 denote acetophenone, propiophenone, *n*-butyrophenone and valerophenone, respectively. Measurement of gradient elapsed time was described in ref. 1.

tuting this value in the expression:

$$\tau = \Sigma R_c + t_0 + t_s$$

where ΣR_c is the sum of the retention coefficients for the amino acid residues and end groups[1] and t_0 is the time for elution of unretained compounds at the new flow-rate. In marked contrast to the peptide standards, the alkylphenones are exhibiting increasingly longer retention times (after the correction for t_g) as the flow-rate decreases (Fig. 4), this effect being particularly noticeable at lower flow-rates. Results from our previous report[1] suggested that, while peptides are separated mainly an adsorption/desorption mechanism, alkylphenones are separated mainly by a partitioning mechanism. The present study supports this view and again confirms the necessity of employing peptide rather than alkylphenone internal HPLC standards for predicting peptide retention times in RP-HPLC.

The resolution between two peaks is described quantitatively by the expression $2\Delta t/(w_1 + w_2)$, where Δt is the difference (min) between the retention times of the two retained components at their peak maxima, and w_1 and w_2 are the baseline peak widths (min). This expression was applied to the resolution of peptide standards S4 and S5 in RP-HPLC on a SynChropak C_{18} column (250 × 10 mm I.D.) with a linear gradient [solvent A is 0.1% TFA in water; solvent B is 0.1% in acetonitrile, (pH 2.0)] of 0.5%, 1%, 2% and 4% B/min, at flow-rates of 0.3–5.0 ml/min and a temperature of 26°C. Fig. 5 demonstrates increasing resolution of the two peptides with increasing flow-rate and decreasing gradient slope. Peak height was found to increase with decreasing flow-rate and increasing gradient slope. Flow-rate variations have negligible effect on peptide retention time (see above), and the distance between the S4 and S5

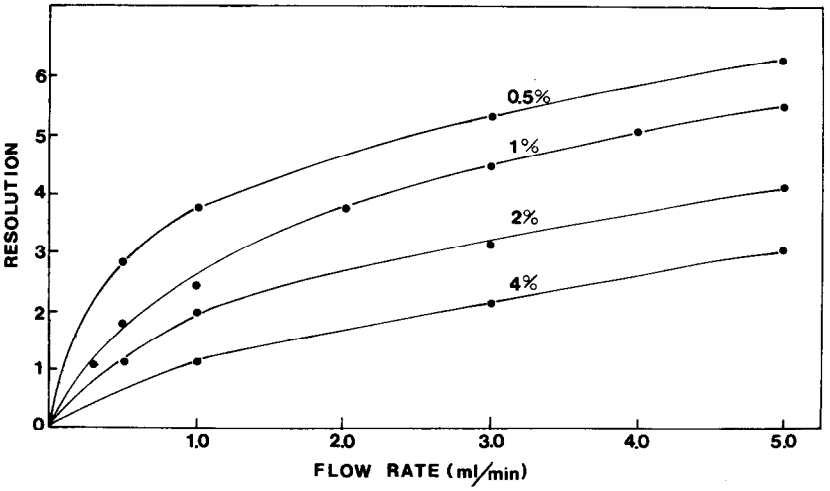

Fig. 5. Effect of flow-rate and gradient slope on resolution of two synthetic peptide HPLC standards (S4, S5) in RP-HPLC. Conditions: column, SynChropak C_{18} (250 × 10 mm I.D.); linear gradient (0.5%, 1%, 2% or 4% B/min), where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 0.3–5.0 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S4 and S5 are described under Experimental.

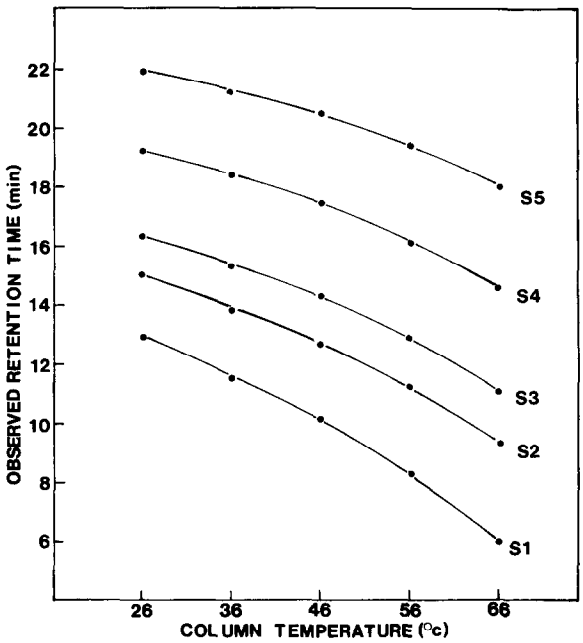

Fig. 6. Effect of temperature on retention time of five synthetic HPLC standards in RP-HPLC. Conditions: column, SynChropak C_8 (250 × 4.1 mm I.D.); linear gradient (1% B/min) where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 1 ml/min; temperature, 26°C, 36°C, 46°C, 56°C, or 66°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental.

peaks (Δt) remains essentially the same at all flow-rates. However, the tendency for the peptides to diffuse decreases as the flow-rate increases, producing smaller peak widths (w_1, w_2) and, hence, improved resolution. In contrast, improved resolution is obtained as the gradient slope decreases, since the resulting increase in Δt more than compensates for any concomitant increase in peak widths.

Effect of temperature

Previous work by other investigators[5–7,9–12] on the effect of temperature variation on RP-HPLC has generally shown a reduction in peptide or protein retention time, due to increasing solubility of the solute in the mobile phase, and improved resolution, due to a more rapid transfer of the solutes between the stationary and mobile phases, as the temperature increases. RP-HPLC of five synthetic peptide HPLC standards (see Experimental) on a SynChropak C_8 column (250 × 4.1 mm I.D.) under gradient elution conditions [linear gradient, where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile, (pH 2.0); 1% B/min, 1 ml/min] tended to confirm these previous findings by exhibiting a successive decrease in retention times and slightly improved peptide resolution as the temperature was increased from 26°C (the value used to determine our coefficients) to 66°C in 10°C increments (Fig. 6). The average change in peptide retention time with temperature was 0.13 min/°C. This may be taken into account in predicting peptide retention at temperatures other than that used to determine our retention coefficients. Adjust-

ments for changes in peptide retention times at temperatures other than 26°C may also be made by chromatographing a peptide HPLC standard at the desired temperature and applying the subsequently determined value for t_s in a fashion similar to that detailed above (see *Effect of flow-rate*). Although enhanced peptide and protein resolution may occasionally be advantageous, this improvement must be balanced against possible solute degradation at higher temperatures. Certainly, the slightly improved peptide separation obtained in the present work by raising the temperature from 26°C to 66°C (Fig. 7) does not justify the risk of peptide degradation or possible acceleration of column aging at elevated temperatures.

Rules for prediction of retention times

(I) These rules apply to linear gradients, *i.e.* starting composition of 100% A, followed by increasing concentration of B at 1%/min (where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile), a flow-rate of 1 ml/min, and a temperature of 26°C.

The predicted retention time (τ) for a peptide equals the sum of the retention coefficients (ΣR_c) for the amino acid residues and end groups[1] plus the time for

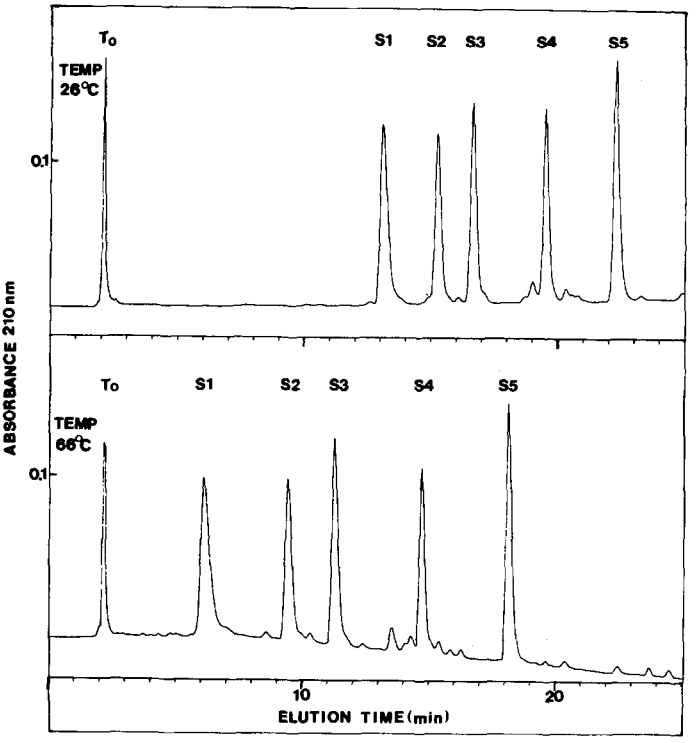

Fig. 7. Comparison of elution profiles of five synthetic peptide HPLC standards at 26°C and 66°C in RP-HPLC. Conditions: column, SynChropak C_8 (250 × 4.1 mm I.D.); linear gradient (1% B/min) where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile, (pH 2.0); flow-rate, 1 ml/ml; temperature, 26°C or 66°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental.

elution of unretained compounds (t_0) and the time correction for the peptide standard (t_s),

$$\tau = \Sigma R_c + t_0 + t_s$$

For greatest accuracy in predicting peptide retention time, it is recommended that an internal peptide standard be included in each run. Using peptide standard 4 from the Alberta Peptide Institute:

$$t_s = (t_R)^{obs}_{std} - (17.5 + t_0).$$

These corrections (t_s and t_0) allow the experimenter to use: (a) any HPLC apparatus, (b) reversed-phase columns of any length or diameter, (c) reversed-phase packings of any *n*-alkyl chain length and ligand density, (d) any temperature, (e) any flow-rate.

(II) The final step in predicting the retention time of a peptide is a correction for gradient slopes other than 1% B/min:

$$\tau^{(x\%)} = (\tau - t_g)\,(1/x\%) + t_g,$$

where t_g is the gradient elapsed time at the desired flow-rate. Note: τ must be calculated with t_s determined at 1% B/min.

The retention time of a peptide is partially dependent on its molecular weight. This molecular weight effect is relatively unimportant in small peptides, but the accuracy of peptide retention time prediction decreases significantly beyond about twenty residues. Work is in progress to extend the accuracy of retention time prediction for peptides containing up to about forty residues by introducing a molecular weight correction.

The major advantage of peptide retention predictions is that the position of a peptide(s) of interest in the elution profile of a peptide mixture will be narrowed down to a small section of the chromatogram, saving much time and effort in subsequent purification. In addition, useful information about the relative order of peptide elution of a complex mixture can be obtained through the use of these coefficients. In conjunction with detection by UV absorbance of aromatic residue-containing peptides, fluorescence detection and/or amino acid-specific colour reactions, the identification of specified peptides in a complex mixture may be greatly simplified. In conclusion, we believe that application of our hydrophobicity parameters to the prediction of peptide elution profiles in RP-HPLC could significantly increase the efficiency of an already powerful analytical and preparative method.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada and equipment grants from the Alberta Heritage Foundation for Medical Research. We are very grateful to Dr. Milton T. W. Hearn for helpful discussions during the course of this research. We thank Theo Hofmann and Brian Marsden for carrying out the synthesis, in our laboratory, of various peptides used in this study. We also thank Francisco Bellini from the Institut Armand-Frappier, Laval, Quebec, for providing several peptides.

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