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

I. DETERMINATION OF RETENTION COEFFICIENTS OF AMINO ACID RESIDUES OF MODEL SYNTHETIC PEPTIDES

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SUMMARY

We have examined the contribution of individual amino acid residues to peptide retention on reversed-phase (RP) columns by measuring their effect on retention of a model synthetic peptide: Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X is substituted by the 20 amino acids found in proteins. Consistently similar resolution of the 20 peptides on several RP columns enabled the determination of empirical sets of retention coefficients, describing the hydrophobicity of amino acid residues at pH 2.0 and pH 7.0. The much superior resolution and selectivity obtained with acetonitrile, compared to 2-propanol and methanol, confirmed its value as the best organic eluent for most practical purposes. The necessity of using peptides rather than alkylphenones as internal standards for peptide retention prediction is demonstrated and underlined by the predictive accuracy of our coefficients when applied to the resolution of a mixture of five commercially available synthetic peptide standards on several RP columns. Rules for retention time prediction for linear elution gradients, employing our hydrophobicity parameters, of peptides of known composition are presented and enable the researcher to correct for: (a) instrument variations, (b) varying length or diameter of RP columns, (c) varying *n*-alkyl chain length and ligand density of RP packings and (d) column aging.

INTRODUCTION

In the years since its introduction, reversed-phase high-performance liquid chromatography (RP-HPLC) has proved very versatile in aiding the isolation of peptides from a variety of sources. In the structure/function studies of proteins, for example, it is often necessary to isolate a few peptides from a complex synthetic and/or proteolytic peptide mixture. Many biologically active peptides, including those with hormonal, antibiotic, or toxic properties, are often found in only small quantities and may require extensive purification. The wide use of automated solid-

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phase peptide synthesis in recent years has also called for efficient isolation of peptides from various impurities.

The ability to predict the elution profiles of peptides of known composition would greatly enhance the value of RP-HPLC. Even though peptides derived from various sources differ widely in size and polarity, it is now recognized that, unless a peptide is subject to conformational restraints, its chromatographic behaviour in RP-HPLC can be correlated with its amino acid composition and, in particular, with the summated relative hydrophobic contribution of each amino acid residue. Several research groups have determined sets of coefficients for predicting retention of peptides on RP columns[1–6]. Retention values have generally been obtained by computer-calculated regression analyses of the retention times of a wide range of peptides of varied composition. We believe a more precise method for determining the contribution of individual amino acid residues would be to measure their effect on retention of a model synthetic peptide, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where position X is substituted by the 20 amino acids found in proteins. Two residues were substituted each time to amplify their effect on the peptide retention time and enable those residues with only small effects to be evaluated more accurately. This paper presents empirical sets of hydrophobic parameters for amino acid residues, at pH 2.0 and pH 7.0, obtained by comparing the retention times of our 20 model peptides on several RP columns. The validity of our parameters when applied to packings of different *n*-alkyl chain lengths and ligand densities and different column lengths and diameters is also discussed.

EXPERIMENTAL

Materials

Unless otherwise stated, chemicals and solvents were reagent grade. Diisopropylethylamine (DIEA), dichloromethane and trifluoroacetic acid (TFA) were redistilled prior to use. Picric acid was dissolved in dichloromethane and dried over magnesium sulphate. Acetonitrile (HPLC-grade) was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Double distilled water was purified by passage through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Co-poly(styrene, 1% divinylbenzene)benzhydrylamine–hydrochloride resin (0.75 mmol of NH_2/g of resin) and co-poly(styrene, 1% divinylbenzene)chloromethyl resin (*ca.* 1.0 mmol of Cl/g of resin) were purchased from Beckman (Palo Alto, CA, U.S.A.) and Pierce (Rockford, IL., U.S.A.), respectively. *tert.*-Butyloxycarbonyl (Boc) amino acids were purchased from Vega Biochemicals (Tucson, AZ, U.S.A.), Bachem Fine Chemicals (Torrance, CA, U.S.A.), Beckman, Chemical Dynamics (South Plainfield, NJ, U.S.A.) and the Protein Research Foundation (Peptide Institute, Osaka, Japan).

Synthetic peptide standards

A mixture of five synthetic decapeptide standards was obtained from the Alberta Peptide Institute. The composition of the peptides varied as follows: peptide 2, -Gly^3-Gly^4-; peptide 3, -Ala^3-Gly^4-; peptide 4, -Val^3-Gly^4-; peptide 5, -Val^3-Val^4-. All peptides contained an N^α-acetylated N-terminal and a C-terminal amide, except peptide 1, which was identical to peptide 3 but had a free α-amino group. These standards are also available from Pierce and Synchrom (Linden, IN, U.S.A.).

Apparatus

Peptide synthesis was carried out on a Beckman Model 990 peptide synthesizer. The HPLC instrument consisted of a Spectra-Physics (San Jose, CA, U.S.A.) SP8700 solvent delivery system and SP8750 organizer module, combined with a Hewlett-Packard (Avondale, PA, U.S.A.) HP 1040A detection system, HP3390A integrator, HP85 computer, HP9121 disc drive 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 seven RP columns: (1) Beckman Ultrapore RPSC C_3, 75 × 4.6 mm I.D., 5 μm particle size, 300 Å pore size, *ca.* 2.9% carbon loading (Altex, Berkeley, CA, U.S.A.); (2) SynChropak RP-4 (C_4), 250 × 4.1 mm I.D., 6.5 μm, 300 Å, *ca.* 7.5% carbon loading (SynChrom, Linden, IN, U.S.A.); (3) SynChropak RP-8 (C_8), 250 × 4.1 mm I.D., 6.5 μm, 300 Å, *ca.* 7.5% carbon loading; (4) Whatman Partisil 5 C_8, 250 × 4.6 mm I.D., 5 μm, 60 Å, *ca.* 9% carbon loading (Whatman, Clifton, NJ, U.S.A.) and three SynChropak RP-P (C_{18}) columns, (5) 6.5 μm, 300 Å, *ca.* 10% carbon loading, 250 × 4.1 mm I.D., (6) 50 × 4.1 mm I.D., and (7) 250 × 10 mm I.D.

Peptide synthesis

The peptide analogues were synthesized using standard procedures for solid-phase synthesis[7]. The co-poly(styrene, 1% divinylbenzene)benzhydrylamine–hydrochloride resin was neutralized with 5% DIEA in dichloromethane for 1 h. The protected C-terminal amino acid (Boc-lysine, 400 μmol/g of resin) was coupled to the resin with dicyclohexylcarbodiimide (DCC, 1.1 equivalents) in dichloromethane for 1 h. This resulted in a substitution of 300 to 350 μmol/g of resin, as determined by picric acid monitoring[8]. The remaining free amino groups on the resin were terminated by treatment (1 h) with acetic anhydride–toluene–pyridine (1:3:3).

To generate peptides with free α-carboxyl groups, the C-terminal lysine was esterified with the co-poly(styrene, 1% divinylbenzene)chloromethyl resin as the Boc-lysine [2-chlorobenzoxycarbonyl(2,Cl-Z)]cesium salt[9].

All α-amino groups were protected with the Boc group. The following side-chain blocking groups were used: tosyl (Arg), benzyl (Ser and Thr), benzyl ester (Asp and Glu), 2,6-dichlorobenzyl (Tyr), N^i-formyl (Trp), N^{im}-tosyl (His), 4-methoxybenzyl(Cys), 2-chlorobenzoxycarbonyl (Lys), and 4'4'-dimethoxybenzhydryl (Asn and Gln).

The Boc groups were removed at each cycle of the synthesis by treatment with TFA–dichloromethane (1:1). Following each deprotection step, the resin was neutralized with 5% DIEA in dichloromethane. The Boc-amino acid (3 equivalents) in dichloromethane was added to the peptide resin followed by a solution of DCC (3.3 equivalents) in dichloromethane (4 ml). The program used for the deprotection cycle of each amino acid consisted of: TFA–dichloromethane (1:1) (1 min), TFA–dichloromethane (1:1) (20 min), dichloromethane (5 × 1 min); the program used for the coupling cycle (double coupling for each amino acid) of most of the amino acids consisted of: 5% DIEA in dichloromethane (3 × 2 min), dichloromethane (6 × 1 min), Boc-amino acid (5 min), DCC (30 min), dichloromethane (2 × 1 min), 2-propanol (1 min), dichloromethane (6 × 1 min). The above deprotection and cou-

pling program is a modification of that previously described by Hodges *et al.*[10] and Parker and Hodges[11]. To obtain satisfactory levels of coupling, the following amino acids (N^{α}-Boc- and side chain protected) were coupled by the symmetrical anhydride technique: Gln, Asn, His, Arg, Glu, Asp and Thr. A large stock of Boc-$(Leu)_3$-$(Lys)_2$-benzhydrylamine resin was prepared. Subsequent amino acids were then coupled to 300 mg portions of this stock Boc-peptide-resin.

The program used for picrate monitoring consisted of: 5% DIEA in dichloromethane (3 × 2 min), dichloromethane (6 × 1 min), 0.1 *M* picric acid (from BDH) in dichloromethane (2 × 5 min), dichloromethane (6 × 1 min), 5% DIEA in dichloromethane (3 × 2 min, collect), dichloromethane (3 × 1 min, collect), dichloromethane (3 × 1 min). The collected DIEA and dichloromethane washes were diluted to a known volume with dichloromethane. The absorption of the solution was recorded at 362 nm. The total resin substitution (μmol) was then calculated using a DIEA-picrate molar extinction of 15 100. When monitoring was performed after deprotection, a second deprotection cycle was carried out after the monitoring cycle was complete with only a 5-min treatment of TFA–dichloromethane (1:1) instead of 20 min. Monitoring was always carried out to determine the substitution of the first amino acid on the resin, and following symmetrical anhydride couplings.

Following deprotection of the N-terminal amino acid (Gly), the α-amino group was acetylated by treatment for 30 min with acetic anhydride–toluene–pyridine (1:3:3), with the exception of peptides for which a free N-terminal α-amino group was required. The peptides were cleaved from the resin supports with hydrofluoric acid (15 ml hydrofluoric acid/g peptide resin), containing 10% anisole, at 4°C for 45 min[12]. The cleavage mixture for the methionine-containing peptide resin also contained free methionine as a scavenger. The solvents were removed under reduced pressure at 4°C. The resins were then washed with ether, and the peptides were extracted with TFA (3 × 10 ml). The combined TFA extracts were evaporated, and the residue was redissolved in water and lyophilized. The N^{i}-formyl protecting group of tryptophan, stable to hydrofluoric acid cleavage, was removed by treatment with 1 *M* ammonium hydrogen carbonate (pH 9.0) for 24 h[13]. Removal of the formyl group was monitored by disappearance of the strong 300 nm absorption.

Peptide purification

The crude peptides were purified on column 5 (see above). Solvent A was 0.1% aq. TFA and solvent B was 0.1% TFA in acetonitrile. Linear gradients varied from 0.5% to 1% B/min, with a flow-rate of 1 ml/min. Peptide analogues containing Asn, Asp, Gln, Glu, Arg, Lys, and His residues were checked for purity and correct net charge by high-voltage paper electrophoresis at pH 6.5. Aliquots (10 μl) of stock solutions of the individual, purified peptides were hydrolyzed in 100–200 μl of 6 *M* hydrochloric acid at 110°C for 24 h in evacuated, sealed tubes. The hydrolysates were subsequently analyzed on a Durrum 500 amino acid analyser to confirm peptide composition. The destruction of tryptophan by 6 *M* hydrochloric acid necessitated the use of 4 *M* aq. methanesulphonic acid, containing 0.2% 3-(2-aminoethyl)indole, as hydrolyzing agent for the Trp-containing peptide[14]. The hydrolysate was partially neutralized with an equal volume of 3.5 *M* sodium hydroxide prior to analysis.

Measurement of gradient elapsed time (t_g)

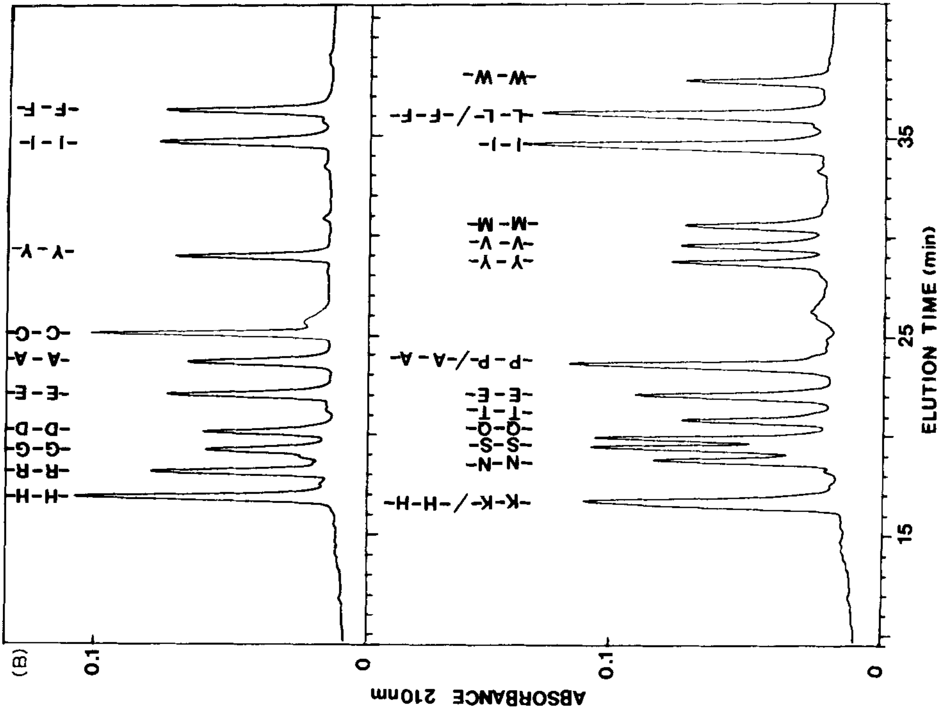
Gradient elapsed time is defined in this work as the time for the gradient to reach the detector from the proportioning valve via pump, injection loop, and column. This value was measured by using 20% acetone in solvent B (0.1% TFA in acetonitrile) of an AB gradient. The system was equilibrated with solvent A (0.1% aq. TFA) and the time was measured from the start of the gradient to the observed offscale change in absorbance at 270 nm. The solvent composition at the elution time of a peptide was subsequently calculated by subtracting t_g from the peak elution time and then multiplying by the % B/min used in the linear gradient.

RESULTS AND DISCUSSION

Retention coefficients

Although retention times are valid for only a specific chromatographic system (mobile phase, stationary phase, pH and temperature), most investigators use TFA–water to TFA–acetonitrile gradients (pH 2.0) at room temperature. The excellent resolving power and selectivity of this system was demonstrated by the resolution of representative samples of model peptides on SynChropak C_8 (Fig. 1A) and C_{18} (Fig. 1B) columns with an AB gradient which increased linearly at 1% B/min (B = 0.1% TFA in acetonitrile) from a starting solvent of 100% A (A = 0.1% aq. TFA). When a model peptide mixture was chromatographed on the C_{18} column at pH 7.0, the addition of $NaClO_4$ to the gradient buffers (A = aq. 10 m*M* $(NH_4)_2HPO_4$–0.1 *M* $NaClO_4$; B = 0.1 *M* $NaClO_4$ in 60% aq. acetonitrile) was discovered to be essential for producing the excellent resolution shown in Fig. 1C. All peptide separations, at either pH, were carried out at 26°C. Prior to use, the pH 7.0 gradient buffer A and the aqueous component of buffer B were passed through a preparative Synchropak C_{18} column to remove impurities in the buffer salts. In the absence of perchlorate, the peptides exhibited high retention times and peak broadening. RP silica gel columns may contain surface silanols which act as weak acids and are ionized above pH 3.5–4. These weak acids may interact with the basic residues of peptides chromatographed on RP columns and have an adverse effect on resolution. This interaction is absent at pH 2.0 where the addition of TFA to the mobile phase suppresses ionization of the surface silanols. At pH 7.0, the $NaClO_4$ may be suppressing ionic interaction between the peptides and packing material through ion-pair formation[1]. Despite the excellent resolution of peptide mixtures at either pH (Fig. 1), the volatility of the solvents used at pH 2.0 makes this the more desirable system for most purposes. Amino acid residue retention coefficients for pH 2.0 and pH 7.0 were determined from the separations demonstrated in Fig. 1 and are shown in Table I. The most striking changes in the retention coefficients in raising the pH from 2 to 7 are seen in the values for Glu, Asp, His, Arg and Lys. At pH 7.0, the side chains of the acidic residues (Glu, Asp) are completely ionized, making their relatively large negative shift in retention reasonable. The largest shift is seen for histidine, which loses its positive charge above pH 6–6.5. The significantly higher retention coefficients of the basic residues (Arg, Lys) are possibly due to ionic interaction with the negatively charged silanols above pH 3.5–4 (see above).

Retention coefficients for N- and C-terminal groups (Table I) were determined from the separation of a mixture of model peptides with the sequence Y-Gly-


(B)
ABSORBANCE 210nm
0.1
0
0.1
0
-H-H-
-R-R-
-G-G-
-D-D-
-E-E-
-A-A-
-C-C-
-Y-Y-
-I-I-
-F-F-
-K-K-/-H-H-
-N-N-
-S-S-
-Q-Q-
-T-T-
-E-E-
-P-P-/-A-A-
-Y-Y-
-V-V-
-M-M-
-I-I-
-L-L-/-F-F-
-W-W-
15
25
35
ELUTION TIME (min)
(A)
ABSORBANCE 210 nm
0.1
0.1
-K-K-
-N-N-
-S-S-
-Q-Q-
-T-T-
-E-E-
-A-A-
-C-C-
-Y-Y-
-V-V-
-M-M-
-I-I-
-F-F-
-W-W-
-K-K-/-H-H-
-R-R-
-G-G-
-D-D-
-T-T-
-E-E-
-A-A-/-P-P-
-Y-Y-
-V-V-
-M-M-
-I-I-
-L-L-/-F-F-
-W-W-
15
25
35
ELUTION TIME (min)

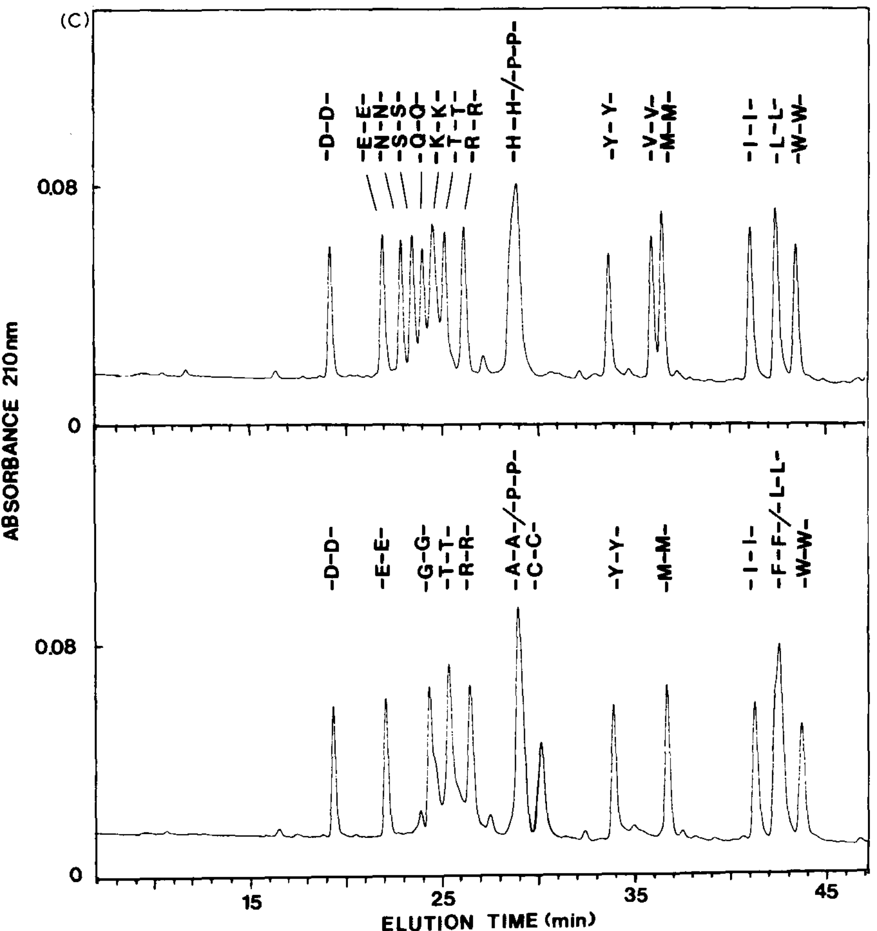


Fig. 1. RP-HPLC of synthetic peptides having the sequence Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where position X is substituted by the 20 amino acids (denoted by the standard single letter code) found in proteins. Representative elution profiles for a SynChropak RP-8 C_8 column, 250 × 4.1 mm I.D. at pH 2.0 (A) and a SynChropak RP-P C_{18} column, 250 × 4.1 mm I.D., at pH 2.0 (B) and pH 7.0 (C). Conditions: linear gradient; at pH 2.0, solvent A consisted of 0.1% aq. TFA and solvent B of 0.1% TFA in acetonitrile, 1% B/min; flow-rate, 1 ml/min, 26°C; at pH 7.0, solvent A consisted of aq. 10 m*M* $(NH_4)_2HPO_4$–0.1 *M* $NaClO_4$ buffer and solvent B consisted of 0.1 *M* $NaClO_4$ in 60% aq. acetonitrile, 1.67% B/min (equivalent to 1% acetonitrile/min); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm.

TABLE I

RETENTION COEFFICIENTS OF AMINO ACID RESIDUES

The retention coefficients (min) were determined from retention times in RP-HPLC as shown in Fig. 1. The predicted retention time for a peptide equals the sum of the retention coefficients for the amino acid residues and end groups (ΣR_c) plus t_0 (the time for elution of unretained compounds) plus t_s (the time correction for the peptide standard). All parameters are calculated for N$^{\alpha}$-acetylated and C-terminal amide peptides; only the values of the end groups shown above need be considered.

Amino acid residue	*Retention coefficient (min)*	
	pH 2.0	*pH 7.0*
Trp	8.8	9.5
Phe	8.1	9.0
Leu	8.1	9.0
Ile	7.4	8.3
Met	5.5	6.0
Val	5.0	5.7
Tyr	4.5	4.6
Cys	2.6	2.6
Pro	2.0	2.2
Ala	2.0	2.2
Glu	1.1	−1.3
Thr	0.6	+0.3
Asp	0.2	−2.6
Gln	0.0	0.0
Ser	−0.2	−0.5
Gly	−0.2	−0.2
Arg	−0.6	+0.9
Asn	−0.6	−0.8
His	−2.1	+2.2
Lys	−2.1	−0.2
α-Amino	−6.9, −3.0*	−2.4, 0*
α-COOH	−0.8	−5.2

* The charged α-amino group had a smaller effect in an N-terminal Arg residue than an N-terminal residue with an uncharged side chain.

$(Leu)_5$-$(Lys)_2$-Z, where Y = N$^{\alpha}$-acetyl (A) or α-amino (B) and Z = C$^{\alpha}$-amide (C) or α-carboxyl (D). Chromatograms of mixtures of the four peptides on different SynChropak C_{18} columns at pH 2 and 7 are demonstrated in Fig. 2. The much superior resolution of the peptides at pH 7.0, compared to pH 2.0, is immediately obvious. The effect of changing the acetyl moiety to a free α-amino group at pH 2.0 is shown by comparing peptides AC and BC or AD and BD (Fig. 2, top panel). This additional positive charge results in a large decrease in retention time for the peptide. In contrast, the effect of changing the C-terminal amide to an α-carboxyl group shows a much smaller decrease in retention time (compare peptides BC and BD or AC and AD; Fig. 2, top panel). The C-terminal α-carboxyl group would be highly protonated (COOH) under these conditions (pH 2.0) and would be expected to have only a small effect. The results at pH 7.0 are very different (Fig. 2, bottom panel). The effect of changing the acetyl moiety at the N-terminal to a free α-amino group is shown by comparing peptides AD and BD or AC and BC (Fig. 2, bottom panel). It is very

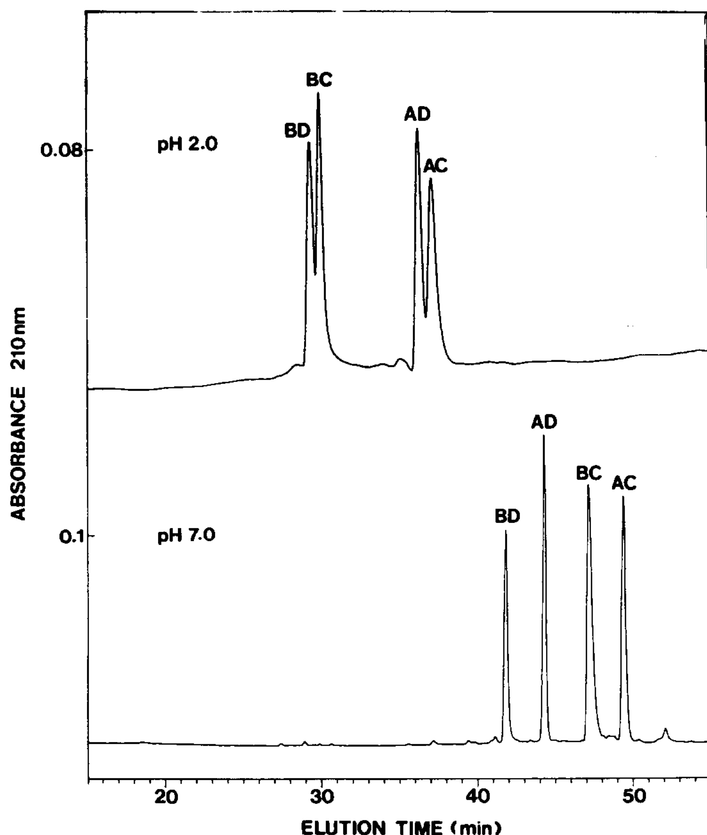


Fig. 2. RP-HPLC of four synthetic peptides with the sequence Y-Gly-(Leu)$_5$-(Lys)$_2$-Z, where Y = N$^\alpha$-acetyl (A) or α-amino (B) and Z = C$^\alpha$-amide (C) or α-carboxyl (D). Representative elution profiles for different SynChropak RP-P C_{18} columns, 250 × 4.1 mm I.D., at pH 2.0 (top) and pH 7.0 (bottom). Conditions: linear gradient; at pH 2.0, solvent A consisted of 0.1% aq. TFA and solvent B of 0.1% TFA in acetonitrile, 1% B/min; flow-rate, 1 ml/min; 26°C. At pH 7.0, solvent A consisted of aq. 10 m*M* $(NH_4)_2HPO_4$–0.1 *M* $NaClO_4$ buffer and solvent B consisted of 0.1 *M* $NaClO_4$ in 60% aq. acetonitrile, 1.67% B/min (equivalent to 1% acetonitrile/min); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm.

common for the pK_a of a peptide α-amino group to be near 7, and this partial deprotonation would explain why the effect at pH 7.0 is smaller than that at pH 2.0 (Table I). In contrast, the effect of changing the C-terminal amide to an α-carboxyl group shows a large decrease in retention time (compare AC and AD or BC and BD; Fig. 2, bottom panel). The C-terminal α-carboxyl group would be completely ionized (COO^-) under these conditions (pH 7.0) and would be expected to have a large effect on hydrophobicity of the peptide. Thus, at pH 7.0 the α-carboxyl group is fully ionized, and at pH 2.0 the α-amino group is fully protonated. These charged end groups, in contrast to blocked and uncharged end groups, can drastically affect the retention time of a peptide.

The predicted retention time of a peptide is calculated by summating the coefficients of all the amino acid residues and end groups it contains (ΣR_c) and adding

the time for the elution of unretained compounds (t_0) and the correction for the peptide standard (t_s) as described later in this report. Since the parameters were calculated for N^{α}-acetylated and C-terminal amide peptides, only the contribution of free terminal groups needs to be taken into account.

The retention coefficients for pH 7.0 have also been used to derive a new set of hydrophilicity parameters for predicting possible antigenic sites on the surface of a protein[15]. These are the first reported parameters derived from amino acid residues in synthetic peptides.

Table II compares our retention coefficients (pH 2.0), obtained from model synthetic peptides, with those reported previously by other research groups using

TABLE II

COMPARISON OF PREDICTED RETENTION COEFFICIENTS OF AMINO ACID RESIDUES FROM LITERATURE AND THOSE OBTAINED FROM SYNTHETIC PEPTIDES IN THIS STUDY

Conditions: (I) Synchropak RP-P C_{18} column (250 × 4.1 mm I.D.), gradient (A = 0.1% aq. TFA, B = 0.1% TFA in acetonitrile) at 1% B/min, flow-rate, 1 ml/min, 26°C; (II) Waters μBondapak C_{18} column (300 × 4.0 mm I.D.), gradient (A = 0.1% aq. TFA, B = 0.07% TFA in acetonitrile) at 1% B/min, flow-rate, 2 ml/min; (III) Waters μBondapak C_{18} column, gradient (A = 0.1% aq. TFA, B = 0.1% TFA in acetonitrile) at 0.33% B/min, flow-rate, 1.5 ml/min; (IV) Waters μBondapak C_{18} column, gradient [A = aq. 50 m*M* NaH_2PO_4, B = A–acetonitrile (1:1)] at 0.83% B/min, flow-rate, 1 ml/min, 18°C; (V) Bio-Rad C_{18} column (250 × 4.0 mm I.D.), gradient (A = aq. 0.1 *M* NaH_2PO_4–0.2% H_3PO_4, B = 0.1% H_3PO_4 in acetonitrile) at 0.75% B/min, flow-rate, 1 ml/min, room temperature.

Amino acid	*Retention coefficient at pH 2 relative to Leu, taken as 100*				
	I (this study)	*II (ref. 6)*	*III (ref. 5)*	*IV (ref. 3)*	*V (ref. 2)*
Trp	109	136	82	−9	157
Leu	100	100	100	100	100
Phe	100	119	96	80	131
Ile	91	104	33	184	73
Met	68	55	28	113	42
Val	62	28	18	34	48
Tyr	56	80	30	28	70
Cys	32	–	−46	−40	48
Pro	25	30	26	−13	32
Ala	25	9	37	−4	10
Glu	14	10	−36	−24	11
Thr	7	28	4	−25	−6
Asp	2	0	−15	61	−5
Gln	0	12	−2	14	−21
Gly	−2	15	−6	−26	2
Ser	−2	4	−21	21	−29
Arg	−7	0	−18	−43	−20
Asn	−7	−43	−29	−56	−31
Lys	−26	−12	−19	−17	−30
His	−26	33	−11	−85	−23
α-Amino	−85, −37*	–	21	49	9
α-COOH	−10	–	12	49	17

* The charged α-amino group had a smaller effect in an N-terminal Arg residue than in an N-terminal residue with an uncharged side chain.

computer-calculated regression analysis of the retention times of a wide range of peptides of varied composition[2,3,5,6]. Retention coefficients were normalized relative to leucine (assigned a value of 100) to allow a direct comparison. It is immediately apparent that there are large discrepancies between the two different approaches. One possible explanation is that certain residues did not appear often enough in the studies of other workers to enable an accurate determination of their contribution. Other possible explanations are unknown nearest-neighbour and chain-length-dependence effects. The effect of molecular weight on retention is relatively unimportant in small peptides and is eliminated in our model peptide approach where single amino acid substitutions were made in an eight-residue peptide. This approach offers the most accurate method of determining retention coefficients.

Effect of different packings

The elution profiles of an identical peptide mixture chromatographed on three different RP columns (SynChropak C_4, SynChropak C_8, Whatman C_8), under the same conditions used to obtain our retention coefficients (Fig. 1), were very similar (Fig. 3); the main difference was a shift in peptide retention times (Whatman C_8 > SynChropak C_4 > SynChropak C_8). The particle sizes of all three packings are similar (5–6.5 μm) and, for the small peptides used in this study (8 residues, *ca.* 900 daltons), pore diameter has little effect on resolution[16]. In general, increased ligand density results in greater retention with a given mobile phase[16–22], and this agrees with the results of the present study. The increased retention on the C_4 compared to the SynChropak C_8 column, can be explained by the increased *n*-alkyl chain ligand density (double for the C_4 column). The two C_8 columns show better overall resolution than the C_4 column (Arg/Gly, Leu/Phe/Trp separations). Interestingly, the Lys/His separation demonstrated by the new SynChropak C_8 column was lost after short usage (compare Fig. 3, middle panel, with Fig. 1A, lower panel). From these results, it is apparent that the amino acid residue coefficients, determined from RP-HPLC of all our model peptides (Table I), may be used to predict the retention time of any peptide of known composition, provided an internal standard is always run to correct for column and instrumentation variations.

Effects of different organic solvents

Of the three organic solvents most commonly used in RP-HPLC, the order of effectiveness in eluting peptides has been shown to be 2-propanol > acetonitrile > methanol[4,23,24]. This order of effectiveness is reflected in Fig. 4, which demonstrates the elution profiles of an identical model peptide mixture when chromatographed on a SynChropak C_8 column at a flow-rate of 1 ml/min and a temperature of 26°C, using a linear AB gradient (1% B/min), where A = 0.1% aq. TFA and B = 0.1% TFA in 2-propanol (top), acetonitrile (middle) or methanol (bottom). However, the much superior resolution and selectivity obtained with acetonitrile compared to that with the alcohols (with the exception of the Pro/Ala peptides) confirms its value as the best organic eluent for most practical purposes. The usefulness of the alcohols is generally limited to special cases where, with very hydrophobic peptides, a more non-polar solvent (2-propanol) or, with very hydrophilic peptides, a more polar solvent (methanol) may be advantageous.

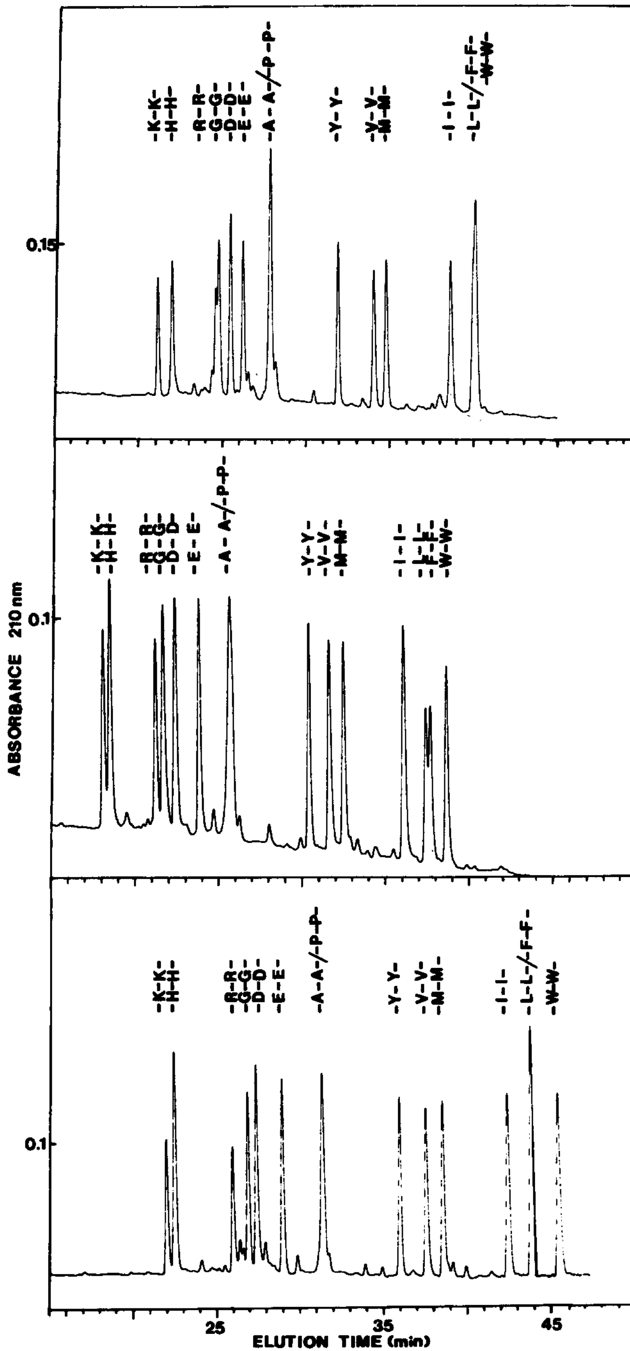


Fig. 3. Separation of an identical mixture of synthetic model peptides by RP-HPLC at pH 2.0. Top, SynChropak C_4 column (250 × 4.1 mm I.D.); middle, SynChropak C_8 column (250 × 4.1 mm I.D.); bottom, Whatman C_8 column (250 × 4.6 mm I.D.). Conditions: linear gradient (1% B/min) where A = 0.1% aq. TFA and B = 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm.

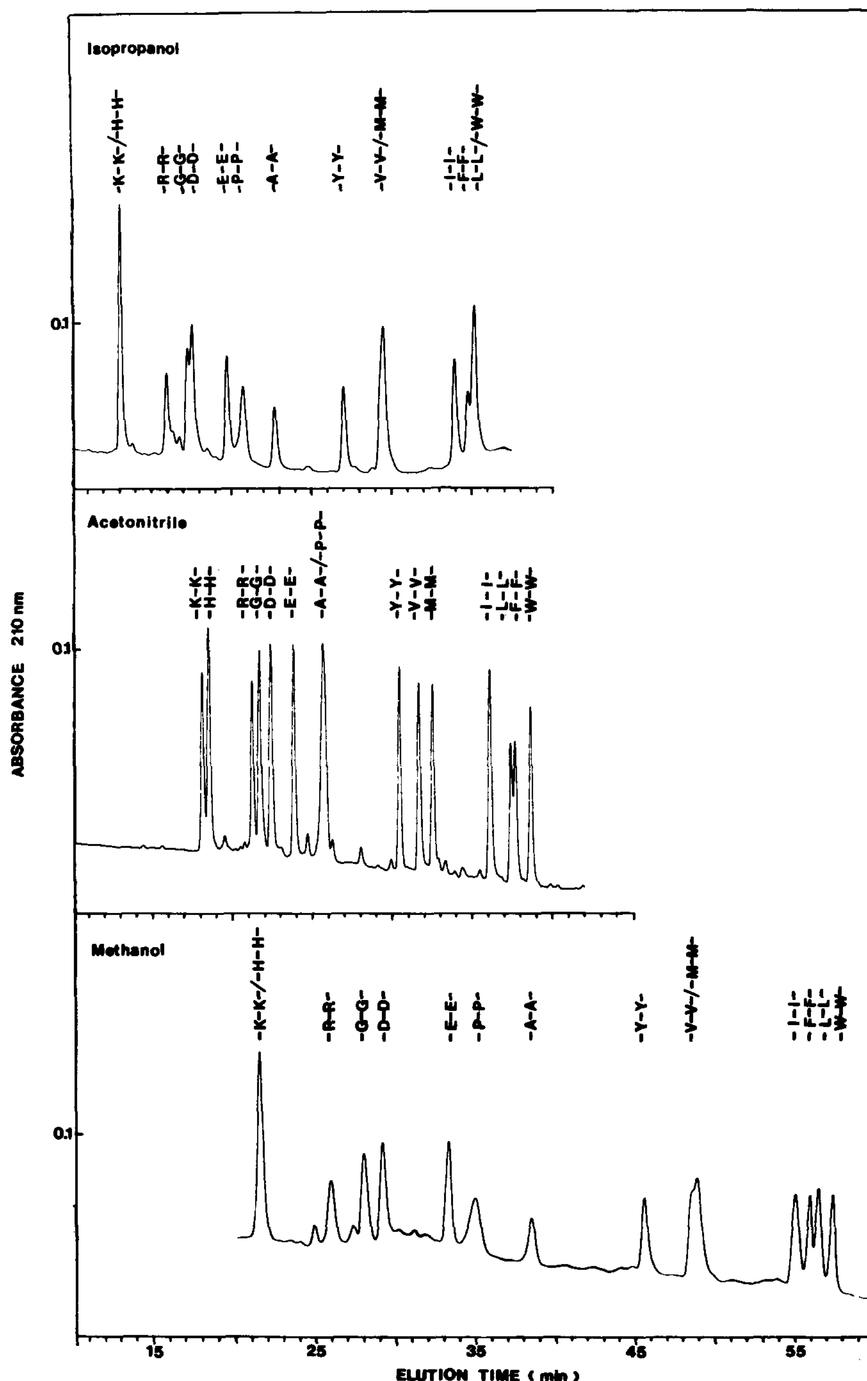


Fig. 4. Separation of an identical mixture of synthetic model peptides on a SynChropak RP C_8 column (250 × 4.1 mm I.D.) at pH 2.0. Organic solvents: top, isopropanol; middle, acetonitrile; bottom, methanol. Conditions: linear gradient (1% B/min), where A = 0.1% aq. TFA and B = 0.1% TFA in one of the above three organic solvents; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm.

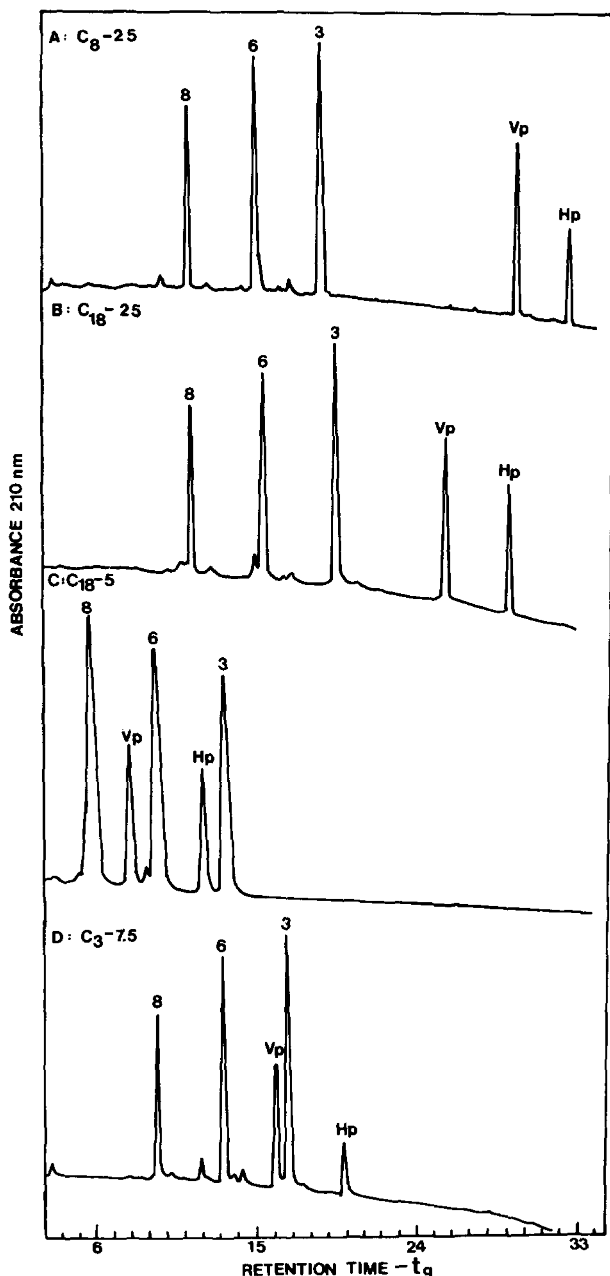


Fig. 5. Comparison of the RP-HPLC elution profiles of a mixture of three peptides and two alkylphenones on columns of different lengths with packings of different chain lengths. (A) Whatman C_8 (250 × 4.6 mm I.D.); (B) SynChropak C_{18} (250 × 4.1 mm I.D.); (C) SynChropak C_{18} (50 × 4.1 mm I.D.); (D) Beckman C_3 (75 × 4.6 mm I.D.). The column length (cm) is denoted by the number following the hyphen and the subscript denotes the *n*-alkyl chain length of the support. Position X in the synthetic peptide sequence (Fig. 1) was substituted by Leu (peptide 3), Val (peptide 6) or Ala (peptide 8). VP = valerophenone; HP = hexanophenone. Conditions: linear gradient (2% B/min) where A = 0.1% aq. TFA and B = 0.05% TFA in acetonitrile (pH 2.0); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm; t_g = gradient elapsed time (see Experimental).

Requirements for peptide standard

Differences in peptide retention may arise from a variety of factors, including instrumentation variations, column aging and variations in the ligand density or *n*-alkyl chain length of packing materials. In addition, the resolving ability of packings of identical chain length may vary from manufacture to manufacture or from batch to batch of support from the same manufacturer. As mentioned earlier, if an internal HPLC standard is used, it should be possible to predict peptide retention on any RP column by using the amino acid residue coefficients determined in this study. Fig. 5 shows the elution profiles of three synthetic peptides and two alkylphenone standards on two SynChropak C_{18} columns of different lengths, a Whatman C_8, and a Beckman Ultrapore C_3 column used under identical conditions [linear gradient, where A = 0.1% aq. TFA and B = 0.05% TFA in acetonitrile (pH 2.0); 1% B/min, 1 ml/min, 26°C]. It is apparent that the mechanism of interaction of the peptides with the hydrophobic stationary phase is different from that of the alkylphenones. The length of the columns has little effect on the resolution of the peptides or alkylphenones. However, column length has a greater effect on the retention times of the alkylphenones than on that of the peptides (compare the identical materials in two columns of different length, C_{18} 5 cm and C_{18} 25 cm). In addition, the effects of different *n*-alkyl matrices on the alkylphenones are different from those on the peptides. For example, peptide retention times on C_8 and C_{18} columns of the same length are very similar. However, the alkylphenones are bound more tightly to the C_8 material, which has about double the ligand density of the C_{18} material. These results suggest that the alkylphenones are separated mainly by a partitioning mechanism, while the peptides are separated mainly by an adsorption/desorption mechanism. Consequently, the alkylphenone HPLC standards cannot be used as standards for predicting peptide retention times on different stationary phases.

To investigate further the suitability of peptides as internal chromatographic standards, the resolution of a mixture of five synthetic standard peptides (see Experimental) was examined on several RP columns. Elution profiles of the peptide standard mixture chromatographed on four SynChropak C_{18} columns at pH 2.0 [columns 5 (two columns), 6, and 7] under the conditions used to obtain our retention coefficients (Fig. 1) are demonstrated in Fig. 6. The peptide mixture was dissolved in 0.5% aq. TFA (Fig. 6A–C) or 2% aq. TFA (Fig. 6D). Following sample injection, the absorbance peak at 210 nm, produced by the excess of TFA concentrations in the sample, represented the elution time for unretained compounds (t_0). The elution profiles are similar for all four columns, the main difference being a shift in peptide retention times. The effect of aging on the performance of a column is clearly demonstrated in Fig. 6A and B, where the peptides bind more tightly to a new column (Fig. 6A; 250 × 4.1 mm I.D.) than a similar column extensively used over a period of months (Fig. 6B). The slightly shorter peptide elution times on the 5-cm column compared to the 25-cm column (Fig. 6A) indicates that, although the standards are interacting with the RP material mainly by an adsorption/desorption mechanism, some peptide partitioning may be occurring. The similarity of the elution profiles on a preparative column (Fig. 6D; 10 mm I.D.) and an analytical column (Fig. 6A; 4.1 mm I.D.) demonstrates that the use of our retention coefficients is not invalidated by variations in column diameter. A comparison of the retention times of the standard peptide mixture on the four SynChropak C_{18} columns, a SynChropak C_8 and a

TABLE III

PREDICTED AND OBSERVED RETENTION TIMES FOR A PEPTIDE STANDARD MIXTURE ON DIFFERENT COLUMNS

Conditions: linear gradient (1% B/min) where A = 0.1% aq. TFA and B = 0.1% TFA in acetonitrile (pH 2.0); flow-rate, 1 ml/min. Peptide mixture dissolved in 0.5% aq. TFA (columns 3–6) or 2.0% aq. TFA (column 7). Detailed column descriptions are found in the Experimental section.

Peptide standard	*Column numbers (see Experimental)*											
	5*		5 ("old")**		6		7		3		4	
	Pred.	*Obs.*	*Pred.*	*Obs.*	*Pred.*	*Obs.*	*Pred.*	*Obs.*	*Pred.*	*Obs.*	*Pred.*	*Obs.*
1	19.0	20.9	12.7	13.2	13.7	15.6	29.4	30.2	14.0	14.0	16.5	16.6
2	19.8	21.4	13.5	14.7	14.5	16.2	30.2	31.7	14.8	15.9	17.3	18.9
3	22.0	22.4	15.7	16.0	16.7	17.3	32.4	32.8	17.0	17.2	19.5	20.1
4***	25.0	25.0	18.7	18.7	19.7	19.7	35.4	35.4	20.0	20.0	22.5	22.5
5	30.2	27.9	23.9	21.4	24.9	22.4	40.6	38.3	25.2	22.8	27.7	25.4

* The column temperature for all runs was 26°C.

** This column was extensively used over a period of 4 months (at least 50 runs).

*** Peptide standard 4 was used to determine t_s for each column; the t_s values were 5.2, −1.7, 1.7, 5.2, 0.4 and 1.9 for columns 5, 5 ("old"), 6, 7, 3 and 4, respectively; the t_0 values were 2.3, 2.2, 0.5, 12.7, 2.1 and 3.1 for columns 5, 5 ("old"), 6, 7, 3 and 4, respectively; ΣR_c values for peptide standards 1–5 were 11.5, 12.3, 14.5, 17.5 and 22.7, respectively.

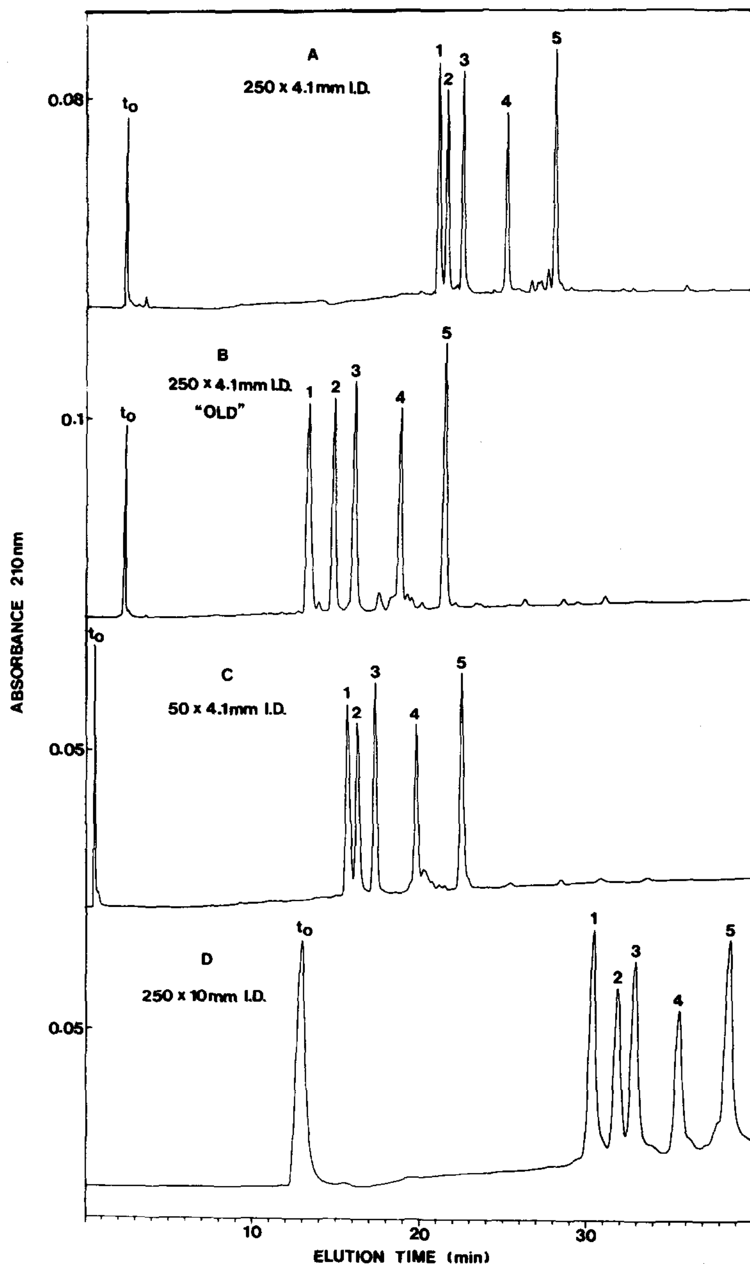


Fig. 6. Separation of a mixture of internal HPLC peptide standards on RP SynChropak C_{18} columns at pH 2.0. (A) 250 × 4.1 mm I.D.; (B) 250 × 4.1 mm I.D., used extensively over a period of 4 months (at least 50 runs); (C) 50 × 4.1 mm I.D.; (D) 250 × 10 mm I.D. Detailed column descriptions and sequence variations of the peptide standards 1–5 are found in the Experimental section. Conditions: linear gradient (1% B/min) where A = 0.1% aq. TFA and B = 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Peptide mixture dissolved in 0.5% aq. TFA (A–C) or 2% aq. TFA (D); sample volume for Fig. 6D was double that for Fig. 6A–C.

Whatman C_8 column, is shown in Table III. Several columns with various *n*-alkyl chain lengths and ligand densities from other suppliers were unable to resolve all five components of the peptide mixture completely. The difference between the observed and predicted retention time of a peptide standard (t_s), when chromatographed on a particular reversed-phase column, is used in subsequent calculations to predict retention times of peptides of known composition. Peptide standard 4 was used to determine t_s [$t_s = (t_R)_{std}^{obs} - (\Sigma R_c^{std} + t_0)$, where $(t_R)_{std}^{obs}$ = observed peptide retention time and ΣR_c = the sum of the coefficients of the amino acid residues and end groups] for the six columns and predict retention times for the other four peptide standards (Table III). The best resolution of a peptide mixture is usually achieved between 15% and 40% of the organic solvent in the gradient[24]. Although all five peptide standards were eluted in this range of acetonitrile concentrations, the use of peptide 4 was prompted by its good separation from the other peptides and the almost central position of its elution time between peptides 1 and 5. Comparison of the predicted and observed retention times of the peptides on all six columns (Table III) indicates good predictive accuracy for our retention coefficients and confirms the need for peptide standards when these parameters are used to aid the isolation of peptides of known composition. Although any peptide with a convenient retention time may be used to determine t_s, peptide standard 4 shows perfect predictive behaviour on the same column used to develop our retention coefficients. Peptide standard 4 makes an ideal internal standard since it is also a component of the five synthetic peptide standards* that were carefully designed to provide a sensitive monitoring of column performance.

Rules for prediction of retention times

These rules apply to linear gradients: starting composition of 100% A, followed by increasing concentrations of B at 1%/min (A = 0.1% aq. TFA; B = 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 (Table I) plus the time for elution of unretained compounds (t_0) and the time correction for the peptide standard (t_s),

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

Determination of t_0. The value t_0 is the time after injection needed for an unretained compound to reach the detector. Compounds such as TFA or β-mercaptoethanol, which are detectable at 210 nm, can be used.

Determination of t_s. The value t_s is obtained by subtracting the sum of the retention coefficients for the peptide standard (ΣR_c^{std}) plus t_0 from the observed retention time of the same peptide [$(t_R)_{std}^{obs}$]:

$$t_s = (t_R)_{std}^{obs} - (\Sigma R_c^{std} + t_0)$$

* Obtained from the Alberta Peptide Institute, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.

Using peptide standard 4 in the peptide standard mixture supplied by the Alberta Peptide Institute:

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

These corrections (t_s and t_0) allow the researcher to use: (a) any HPLC apparatus; (b) RP columns of any length or diameter; (c) reversed-phase packings of any *n*-alkyl chain length and ligand density.

The value of any predictive method in RP-HPLC is assessed by its accuracy in predicting the retention times of peptides not used for determining the retention coefficients. Although excellent separation of our synthetic model peptides was generally obtained under the conditions employed to determine our coefficients, alterations of parameters, such as gradient steepness, flow-rate, temperature, etc., may be required for optimum resolution of peptide mixtures. In a subsequent report[25], we examine the accuracy of our coefficients by applying them to RP-HPLC retention time prediction of a wide range of peptides. In addition, we demonstrate how appropriate modifications of our prediction rules can be made to compensate for factors influencing peptide retention and resolution (gradient steepness, flow-rate, and temperature).

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