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FACTORS AFFECTING RETENTION AND RESOLUTION OF PEPTIDES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Retention of small peptides (20 residues or less) on reversed-phase columns can be predicted by summing the contribution to retention of each amino acid and end group. The high correlation ($r = 0.98$) for 100 peptides of predicted *versus* actual retention times indicates that conformation and sequence have minor effects on retention.

Resolution depends not only on the column, but also on the mobile phase composition and flow-rate, the gradient rate and the size and composition of the peptides themselves. These factors were studied using 9 small peptides. Column efficiency (height equivalent to a theoretical plate) in isocratic separation improved linearly with decreasing flow-rate and decreasing log molecular weight of peptide. Resolution in gradient separations was primarily a function of gradient rate; flow-rate had little effect on resolution, but did affect peak height. A compromise must be made between analysis time and resolution: separation with a given mobile phase can be improved by using slower gradients, but only at the expense of exponentially increasing analysis time.

INTRODUCTION

The separation of peptides and proteins by reversed-phase high-performance liquid chromatography (HPLC) is a useful technique in many areas of research. Optimum utilization of this separation method requires a knowledge of the factors that affect retention and resolution. For reviews, see refs. 1–3. Separations are usually made with reversed-phase columns eluted at a low flow-rate with mixtures of water, organic solvent and salts, and with gradients of increasing concentrations of organic solvent. Although the gradient rate and flow-rate affect resolution, the exact nature of the relationship has not been clear. This paper will describe quantitative empirical

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studies of these variables as well as the influence on resolution of the kind of salts and organic solvents used in the mobile phase.

It was previously shown[4] that the retention of small peptides primarily depends on amino acid composition, and that retention can be predicted by summing the hydrophobic contribution to retention of each amino acid and end group ("retention coefficients"). Subsequently, the published retention coefficients for glutamate and several other amino acids were shown to require revision[5]. These amino acids had occurred in only a few of the 25 peptides used in the previous study[4]. We have now increased the number of peptides examined to 100 in order to obtain more accurate values for the retention coefficients. The experiments confirm the hypothesis that it is the amino acid composition that primarily determines retention of small peptides.

METHODS

Data for calculation of retention coefficients were obtained by chromatographing 100 peptides on a Bio-Rad (Richmond, CA, U.S.A.) ODS column (10-μm particle size, 25 cm × 4.0 mm). Most of the peptides were purchased from Sigma (St. Louis, MO, U.S.A.), Peninsula Labs. (San Carlos, CA, U.S.A.) or Calbiochem (Los Angeles, CA, U.S.A.). Samples of pure peptides were also kindly provided by Drs. R. de Castiglione, Carlo Erba, Milan, Italy (caereulein), J. Shields, Eli Lilly, Indianapolis, IN, U.S.A. (4-D-phe–met–enkephalin amide) and R. Schwartz, NIH, Bethesda, MD, U.S.A. (cytochrome *c* 81-104). Trypsinized human hemoglobin A was a gift of Dr. W. Schroeder, CalTech, Pasadena, CA, U.S.A.

The HPLC system used consisted of two Altex Model 110 pumps controlled by a Rockwell AIM65 microcomputer, a 1-ml magnetically stirred mixing chamber, a Rheodyne sample injector with 20-μl loop, a Bio-Rad column, an Altex variable-wavelength detector and a strip-chart recorder. In some experiments, an LDC automatic sample injector with 20-μl sample loops was employed. A voltage/frequency (V/F) converter was connected to the ultraviolet (UV) detector in order to provide analog/digital conversion. The AIM65 microcomputer provided a low-cost versatile way both to control the two pumps for gradient generation and to perform on-line data analysis. The AIM65 was programmed in both BASIC and assembly languages. First a program in BASIC accepted inputs of flow-rate and gradient rate. Then, an assembly language subroutine configured the AIM65's 16 input/output lines and 2 timers: the free running timer was set to give interrupts each 1.2 sec (0.02 min); the timer/counter was used as a counter to count the pulses from the V/F converter; 2 output lines controlled the Altex pumps; 2 other output lines were connected to OPTO-22 solid state relays to control the sample injector or to shut off the system power at the end of a series of runs; another output line was connected via a 10-MΩ resistor to the chart recorder input to provide pen deflection at the beginning and end of peak integration. Each 1.2 sec, the BASIC program updated the calculations for controlling the pumps and entered a subroutine to calculate whether the current digitized value of UV absorbance was on the baseline or at the beginning or end of peaks. Each data point during the peak was stored for calculation of the variance (σ^2) and bandwidth (4σ) of that peak[6]. The number of theoretical plates (N) for that peak was calculated[6] by retention time/σ^2.

The algorithm for computation of retention coefficients was that described previously[4]. Since the number of data points exceeded the capacity of the calculator previously used, the program was rewritten in FORTRAN for use on a PDP 11 computer. The peptides were chromatographed with linear gradients of acetonitrile (0.75%/min) starting with either 0.1 *M* $NaClO_4$–0.1% H_3PO_4 or 0.1 *M* NaH_2PO_4–0.2% H_3PO_4 at 0 min after injection. The acetonitrile (Fisher HPLC grade) contained 0.1% H_3PO_4.

Twenty two peptides were isolated from a tryptic hydrolysate of human hemoglobin A by chromatographing a 10-nmol sample on a Bio-Rad column (5 μm particle size) with phosphate mobile phase and acetonitrile gradient (0.4%/min). Fractions of the column effluent were collected. Aliquots were evaporated to dryness and hydrolysed in 6 *M* hydrochloric acid for identification of the peptides by amino acid analysis. Other aliquots were chromatographed on a 10-μm Bio-Rad column under the standard conditions (0.75%/min acetonitrile, with both phosphate and perchlorate mobile phases) for determination of retention times.

RESULTS

Fig. 1 shows the correlation between predicted and actual retention times for the 100 peptides tested, and lists the peptides in order of their elution by the NaH_2PO_4–acetonitrile mobile phase. The high degree of correlation ($r = 0.98$) confirms the hypothesis that composition is the major factor affecting retention of small peptides. Although the average deviation of predicted from actual retention is small (2.8 min), the deviation for some individual peptides is quite large: 9.3 min for hemoglobin βT5, 9.1 min for arg–gly–tyr–ser–leu–gly and 5 min for dynorphin$^{1-13}$. Table I lists the retention coefficients obtained in $NaClO_4$ and NaH_2PO_4 for 100 peptides as well as the data previously reported based on 25 peptides. As described earlier[4], amino acids with aromatic or aliphatic side chains have a marked positive contribution to retention, while amino acids with neutral or polar side chains have no effect or a small negative contribution to retention. However, the earlier study with $NaClO_4$ considerably overestimated the negative effect of glutamate, arginine and aspartate, and underestimated the negative effect of histidine. The data obtained with the perchlorate and phosphate mobile phases are generally similar. However, basic groups such as lysine, arginine, histidine and amino terminal groups have a more negative effect on retention with the phosphate mobile phase. The phosphate is probably acting as a hydrophilic ion pairing agent.

The size of peptides has little effect on retention as shown in Fig. 2. For example, dipeptides can have very short or very long retention times; the same is true for polypeptides of any given size.

Factors affecting retention

The effect of flow-rate on column efficiency was examined for 10 peptides ranging in molecular weight from TRH (mol.wt. 362) to insulin (mol.wt. 6000). Mobile phases of mixtures of $NaClO_4$ and acetonitrile (10–50%) were chosen for each peptide to give a capacity ratio (k') of 1–3. Fig. 3 shows the results for several of these peptides together with data for a small neutral molecule commonly used as a column test substance (methyl benzoate). The number of theoretical plates obtained at 1 ml/min for methyl benzoate for the 10-μm particle size column ($N = 6670$) and 5-μm column ($N = 16,334$) are similar to the values in the test chromatogram provided by the manufacturer. The efficiency of the columns for peptides becomes worse with

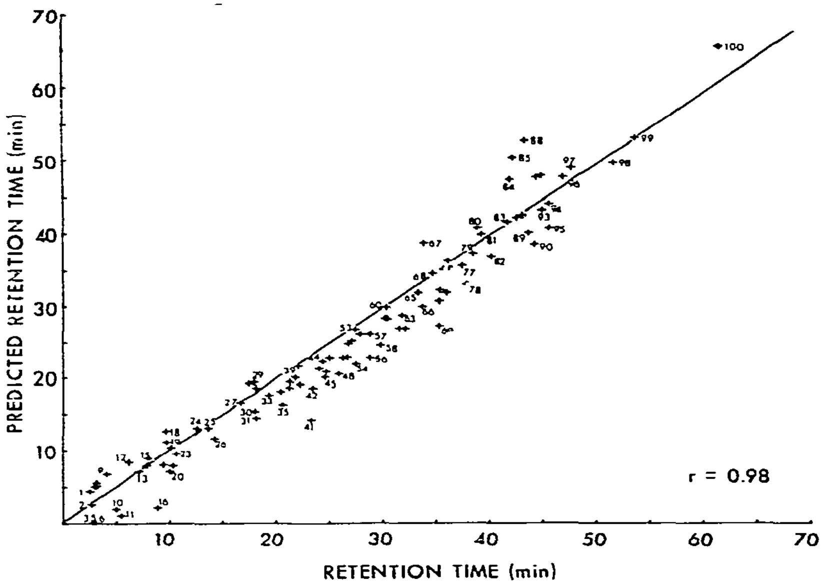

Fig. 1. Correlation of actual retention times *vs.* times predicted by summation of retention coefficients for amino acids and end groups. Numbers adjacent to the data points indicate the peptides listed below in order of elution by the NaH_2PO_4 mobile phase. HB-αT7 = tryptic peptide number 7 (from the *N* terminus) of α-chain of human hemoglobin A. TRH = thyrotropin-releasing hormone. LHRH = luteinizing hormone releasing hormone. Eledoisin related peptide = lys–phe–ile–gly–leu–met–NH_2. 1 = Tri-glycine (3); 2 = ala–his (2); 3 = gly–his–lys (3); 4 = hexa-glycine (6); 5 = HB–βT7 (4); 6 = HB–αT7 (4); 7 = glu–ala (2); 8 = HB–β6 (2); 9 = tri–ala (3); 10 = gln–gly (2); 11 = tuftsin (4); 12 = gly–val (2); 13 = tyr–lys (2); 14 = HB–βT15 (2); 15 = penta–ala (5); 16 = HB–αT2 (4); 17 = HB–αT14 (2); 18 = val–val (2); 19 = tyr–ala (2); 20 = lys–tyr (2); 21 = tyr–gly (2); 22 = NAc–penta–ala (5); 23 = TRH (3); 24 = lys–phe (2); 25 = pro–tyr (2): 26 = met–met (2); 27 = gly–gly–phe (3); 28 = trp–gly (2); 29 = trp–ala (2); 30 = lys–trp (2); 31 = HB–αT1; 32 = trp–gly (2); 33 = HB–α3; 34 = delta sleep subst. (9); 35 = HB–β1 (10); 36 = gly–trp (2); 37 = ala–trp (2); 38 = phe–met (2); 39 = leu–leu (2); 40 = val–tyr–val (3); 41 = arg–gly–tyr–ser–leu–gly (6); 42 = arg–gly–tyr–ala–leu–gly (6); 43 = pro–trp (2); 44 = tyr–phe (2); 45 = met–phe (2); 46 = vasotocin (9); 47 = val–trp (2); 48 = gly–gly–phe–met (4); 49 = tyr–gly–gly–phe (4); 50 = tri–tyr (3); 51 = trp–tyr (2); 52 = lys–vasopressin; 53 = HB–αT4 (17); 54 = HB–αT11 (7); 55 = arg–vasopressin (9); 56 = HB–βT14 (14); 57 = HB–βT11 (9); 58 = HB–βT3 (13); 59 = phe–phe (2); 60 = met–enkephalin–NH_2 (5); 61 = HB–βT13 (12); 62 = lys–bradykinin (10); 63 = phe–gly–gly–phe (4); 64 = met–enkephalin (5); 65 = oxytocin (9); 66 = bradykinin (8); 67 = dynorphin 1–13 (13); 68 = LHRH (10); 69 = HB–βT2; 70 = leu–leu–leu (3); 71 = trp–phe (2); 72 = angiotensin II (8); 73 = α-endorphin (13); 74 = leu–enkephalin (5); 75 = eledoisin-related peptide (6); 76 = HB–αT6 (16); 77 = tyr–8-substance P (11); 78 = trp–trp (2); 79 = gastrin 12–15 (4); 80 = neurotensin (13); 81 = α–MSH (13); 82 = HB–αT5 (9); 83 = substance P (11); 84 = HB–βT9 (16); 85 = HB–βT4 (12); 86 = caerulein–SO_4 (10); 87 = pigeon cytochrome *c* 81–104 (23); 88 = HB–βT5 (21); 89 = sheep cytochrome *c* 81–104 (23); 90 = eledoisin (11); 91 = vasoactive intestinal peptide (29); 92 = somatostatin (14); 93 = caerulein (not sulfated) (10); 94 = HB–αT9 (29); 95 = tri–phe (3); 96 = cholecystokinin 26–33 (not sulfated) (8); 97 = ranatensin (11); 98 = glucagon (29); 99 = tetra–phe (4); 100 = penta–phe (5).

increasing solute molecular weight, or with increasing flow-rate of the mobile phase. The data suggest that for high resolution in isocratic separations, flow-rates should be kept to less than or equal to 1 ml/min except for very small peptides. The main cause of band spreading of these peptides is likely to be due to diffusion, since salts and acid were added to the mobile phase to minimize adsorption. Since diffusion is proportional to the log of molecular size (and thereby related to log molecular weight), the

TABLE I

RETENTION COEFFICIENTS OF AMINO ACID RESIDUES

*Amino acid (N)**	*NaClO₄*** (*N = 25*)	*NaClO₄* (*N = 100*)	*NaH₂PO₄* (*N = 100*)
Tryptophan (24)	18.1	17.1	15.1
Phenylalanine (45)	13.9	13.4	12.6
Leucine (31)	10.0	11.0	9.6
Isoleucine (10)	11.8	8.5	7.0
Tyrosine (37)	8.2	7.4	6.7
Cystine (5)	−2.2	7.1	4.6
Valine (26)	3.3	5.9	4.6
Methionine (22)	7.1	5.4	4.0
Tyrosine-sulfate (1)	6.5	2.4	3.7
Amino- (91)	−0.4	4.6	0.9
Proline (30)	8.0	4.4	3.1
-Amide (18)	5.0	4.4	4.9
Pyroglutamyl- (7)	−2.8	2.8	2.9
Carboxyl- (81)	6.9	2.2	1.6
N-Acetyl- (2)	3.9	6.6	3.8
Alanine (29)	−0.1	1.1	1.0
Glutamate (14)	−7.5	0.7	1.1
Glycine (50)	−0.5	−0.2	0.2
Arginine (23)	−4.5	−0.4	−2.0
Histidine (19)	0.8	−0.7	−2.2
Aspartate (19)	−2.8	−1.6	−0.5
Threonine (18)	1.5	−1.7	−0.6
Lysine (37)	−3.2	−1.9	−3.0
Glutamine (16)	−2.5	−2.9	−2.0
Serine (17)	−3.7	−3.2	−2.9
Asparagine (18)	−1.6	−4.2	−3.0

* Numbers in parentheses are the number of peptides used for calculation of retention coefficients that contain that amino acid. The predicted retention times for peptide = the sum of the retention coefficients for the amino acids and end groups plus t_0 (the time for elution of unretained compounds, 2 min).

** Data for $NaClO_4$ ($N = 25$) taken from ref. 4.

slope of the plots in Fig. 3 should be proportional to log molecular weight of solute. That this is approximately true is shown in Fig. 4. The moderate correlation (r = 0.87) indicates that while size is in fact the major cause of band spreading, considerable adsorptive or other non-ideal factors contribute to bandspreading. The main point to be gained from Figs. 3 and 4 is that the molecular weight of the compounds being chromatographed has an influence via diffusion on the efficiency achievable with any given column.

We next examined the influence of various mobile phase compositions on column efficiency. Retention times and peak widths were determined with the same mobile phase as used for the derivation of retention coefficients: a gradient of 100% A ($NaClO_4$–H_3PO_4) to 40% A–60% B (acetonitrile–H_3PO_4); but a faster gradient rate was used (2.5%/min). As shown in Table II, if methanol was substituted for acetonitrile and the gradient rate changed to 3.5%/min B, similar retention times to those obtained with acetonitrile were obtained, but the peak widths were slightly but consistently worse than with acetonitrile. If a propanol gradient (1.8%/min B) was

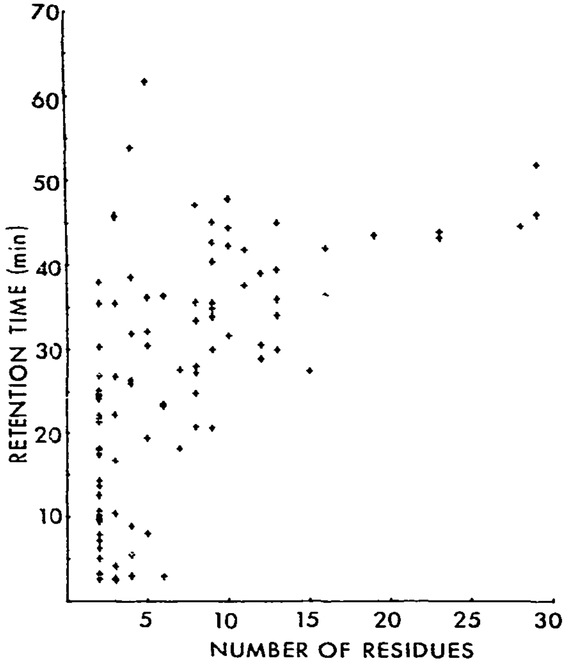

Fig. 2. Lack of correlation of retention of peptides with number of amino acid residues.

substituted for acetonitrile, the retention times were again similar, but peak broadening was much worse than with the acetonitrile. If the $NaClO_4$ is removed from the mobile phase (Table II, column 4), the retention times are only slightly affected (except for insulin which was not eluted), but the peak widths became worse, espe-

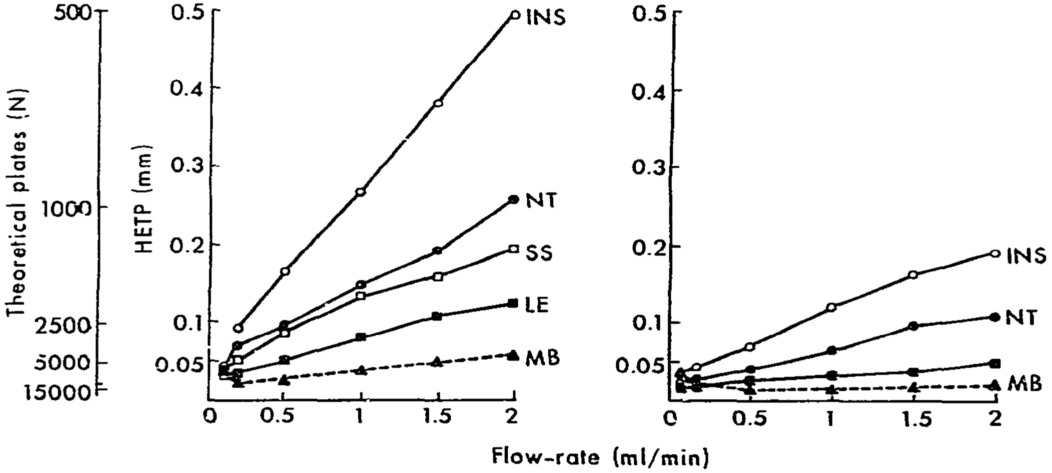

Fig. 3. Decrease in column efficiency with increasing flow-rate and solute molecular weight. MB = methyl benzoate, LE = leu-enkephalin, SS = somatostatin, NT = neurotensin, INS = insulin. Peptides were chromatographed isocratically at room temperature with $NaClO_4$–H_3PO_4–acetonitrile mobile phases. Left: 10-μm column; right: 5-μm column.

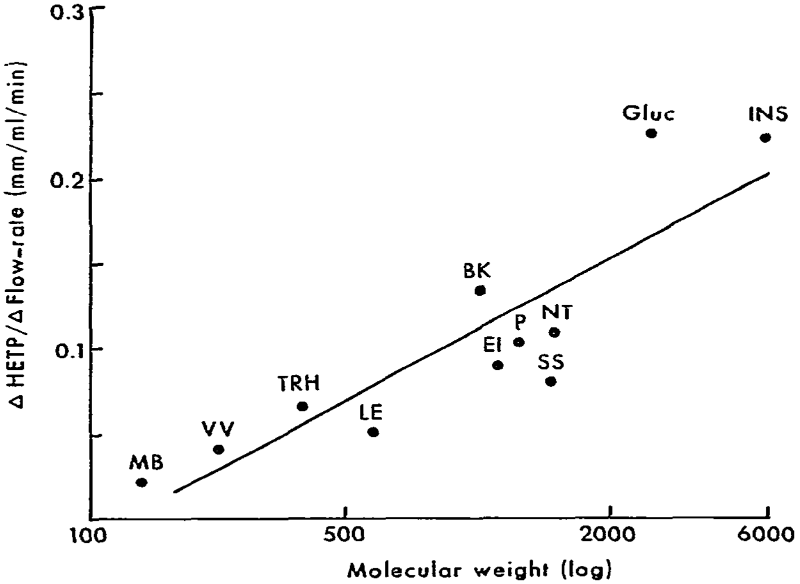

Fig. 4. Flow-rate dependency of column efficiency on solute molecular weight. VV = val–val, TRH = thyrotropin-releasing hormone, EL = eledoisin, BK = bradykinin, Gluc = glucagon.

cially for those peptides with multiple amino groups (probably due to adsorption to free silanol groups). Increasing the ionic strength above 0.1 *M* (column 5) had little effect on retention or column efficiency, but increasing the acidity of the mobile phase with 0.6 *M* $HClO_4$ (column 6) did improve peak width although at the cost of potential damage to equipment and safety hazard to the operators. Replacement of $NaClO_4$ with NaH_2PO_4 decreased the retention of most peptides (in agreement with data from Table II). The peak widths were also decreased. However, there was little if any net improvement in overall resolving capacity since the sharper peaks were merely compressed into a narrower elution range. Replacement of the $NaClO_4$ with trifluoroacetic acid (column 8) had little effect either on the retention or the peak width, except for insulin which was not eluted.

We also studied the effects of gradient rate and flow-rate on the ability of the columns to resolve highly complex mixtures. As a measure of this ability, we chose "average peak capacity", which can be defined as $(t_{\text{last}} - t_0)$/(average bandwidth) where t_0 = elution time of unretained compounds, t_{last} = retention time of the last compound to be eluted (in our study insulin). The average bandwidth is the average of the (4σ) values for a mixture of peptides with a wide range of size, charge and lipophilicity: TRH, gly–trp, tri–tyr, met-enkephalin, bradykinin, neurotensin, substance P, somatostatin and insulin. The average peak capacity is a measure of the number of compounds which can be separated with unit resolution in a chromatographic run. Fig. 5 shows the average peak capacity on a Bio-Rad column (10-μm particle size) calculated with these 9 peptides as a function of flow-rate and gradient rate.

The peak capacity (resolution) improved as the gradient rate decreased from very fast gradients (5%/min or 0–100% in 20 min) to very slow gradients (0.5%/min

TABLE II

EFFECT OF MOBILE PHASE COMPOSITION ON RETENTION (Tr) AND PEAK WIDTH (4σ)

ACN = acetonitrile, MeOH = methanol, PrOH = propanol, TFA = trifluoroacetic acid. Values are expressed as % relative to the conditions in column 1. Samples were chromatographed on a 10-μm particle size column at 1 ml/min with an acetonitrile gradient of 2.5%/min, a propanol gradient of 1.8%/min or a methanol gradient of 3.5%/min.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Solvent		ACN	MeOH	PrOH	ACN	ACN	ACN	ACN	ACN
Salt		NaClO$_4$ (0.1 *M*)	NaClO$_4$ (0.1 *M*)	NaClO$_4$ (0.1 *M*)	–	NaClO$_4$ (0.6 *M*)	–	NaH$_2$PO$_4$ (0.1 *M*)	
Acid		H$_3$PO$_4$ (0.1%)	H$_3$PO$_4$ (0.1%)	H$_3$PO$_4$ (0.1%)	H$_3$PO$_4$ (0.1%)	H$_3$PO$_4$ (0.1%)	HClO$_4$ (0.06 *M*)	H$_3$PO$_4$ (0.1%)	TFA (0.1%)
Benzaldehyde	Tr	100	98	100	101	97	94	102	98
	4σ	100	114	130	97	100	97	95	102
Tri-tyrosine	Tr	100	104	92	98	98	94	87	97
	4σ	100	116	130	112	100	91	88	94
Met-enkephalin	Tr	100	102	94	99	99	95	88	97
	4σ	100	115	140	122	103	94	90	94
Neurotensin	Tr	100	98	102	118	99	96	80	95
	4σ	100	117	172	257	100	97	91	116
Somatostatin	Tr	100	100	103	119	102	100	84	95
	4σ	100	130	167	260	91	88	84	128
Bradykinin	Tr	100	97	95	122	101	97	77	93
	4σ	100	110	166	535	115	88	88	104
Substance P	Tr	100	102	107	129	100	99	83	95
	4σ	100	156	163	690	87	96	96	90
Insulin	Tr	100	94	105	Not	99	98	93	Not
	4σ	100	160	177	eluted	104	95	118	eluted

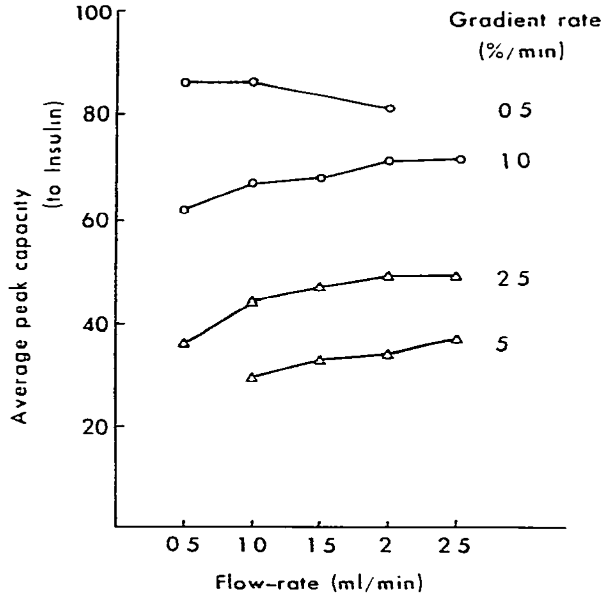

Fig. 5. Increase in resolution with decreasing gradient rate. Peak capacity was calculated as described in Results for separation of nine small peptides by acetonitrile gradients.

or 0–50 % in 100 min). There was relatively little effect of the flow-rate on resolution under the gradient conditions studied. However, as shown in Fig. 6, flow-rate does have a marked effect on sensitivity due to the dilution occurring with increasing flow-rate. Decreasing the gradient rate also decreased the average peak height (Fig. 6)

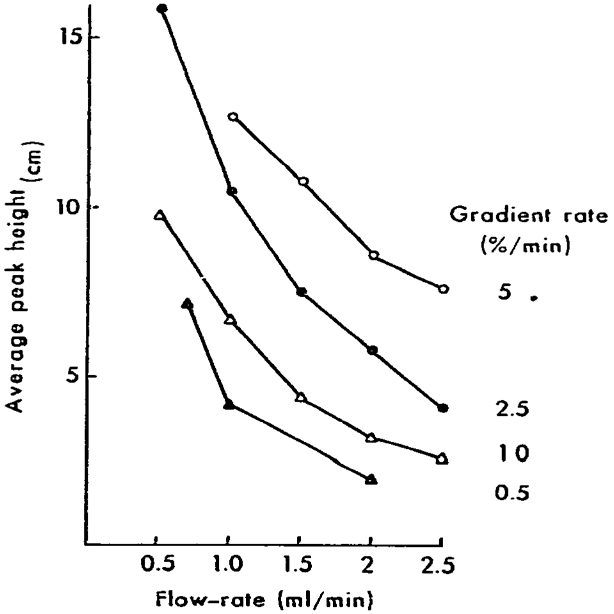

Fig. 6. Increase in peak height with decreasing flow-rate.

although this effect can be minimized by decreasing the flow-rate. It is apparent that a compromise must be made between the desire for a short analysis time and the desire for maximum resolution (Fig. 7). The tradeoff between analysis time and resolution for a column with 5-μm particle size is also shown in Fig. 7. The 5-μm column was superior to the 10-μm column in terms of resolution under each gradient condition; however, the 10-μm column can provide high resolution if slow flow-rates are employed. These effects are shown qualitatively in Fig. 8. Separation of tryptic peptides is far superior in a 90-min gradient to that seen with a 15-min gradient. Increasing the analysis time still further to 10 h however, caused only a small improvement in separation, but caused a substantial decrease in peak-height. It can be noted that the elution order of several compounds is reversed with changes in gradient rate (compare the peaks eluting at 27 min in Fig. 8b, and at 150 min in Fig. 8c). The cause of this effect is not clear. However, where substances differ markedly in molecular weight, we found that given changes in % organic solvent produce much larger changes in *k′* for large-molecular-weight substances than for small molecules. For example, we found that increasing the percentage of acetonitrile from 40 to 50% decreased the *k′* for methyl benzoate from 4.0 to 2.0, while the same change decreased the *k′* for insulin from 4.6 to 0.6. A similar effect was seen by Lewis *et al.*[7] who noted that serum albumin (mol.wt. 68,000) was totally retained at 32% propanol, but rapidly eluted with 34% propanol. A nomogram previously published[8] as an aid for choosing mobile phase composition can therefore not be applied to polypeptides and proteins. That nomogram showed the relationship between *k′* and organic solvent content for ten compounds (all small molecules) which varied widely in polarity. Similar data, plus values obtained with six peptides are shown in Fig. 9. It is clear that the relationship between solvent strength and % organic solvent depends both upon the solute polarity and the solute molecular weight.

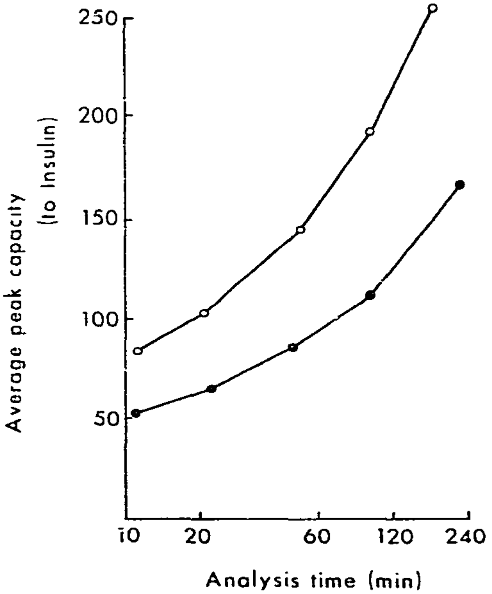

Fig. 7. Tradeoff between analysis time and resolution. Analysis time is the time to elute the most hydrophobic compound of the test mixture (insulin). ○, 5-μm column; ●, 10-μm column.

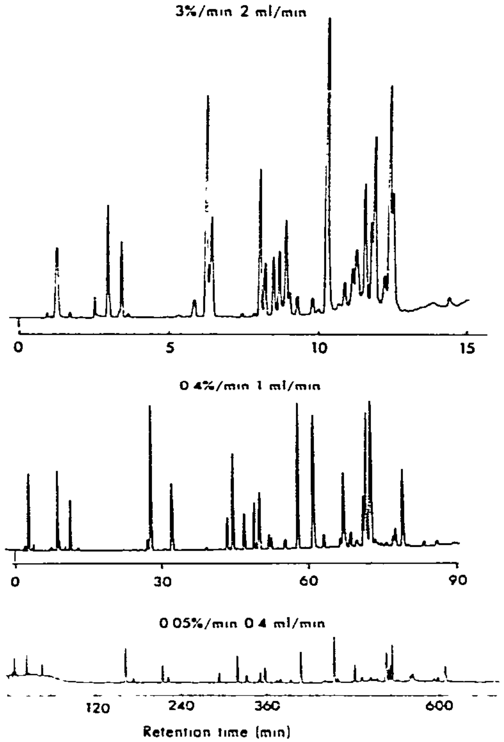

Fig. 8. Separation of trypsinized hemoglobin A. Aliquots (10 nmol) were chromatographed with phosphate–acetonitrile gradients on a 5-μm particle size column at different gradient and flow-rates. Detection was by absorbance at 210 nm (0.5 a.u.f.s.).

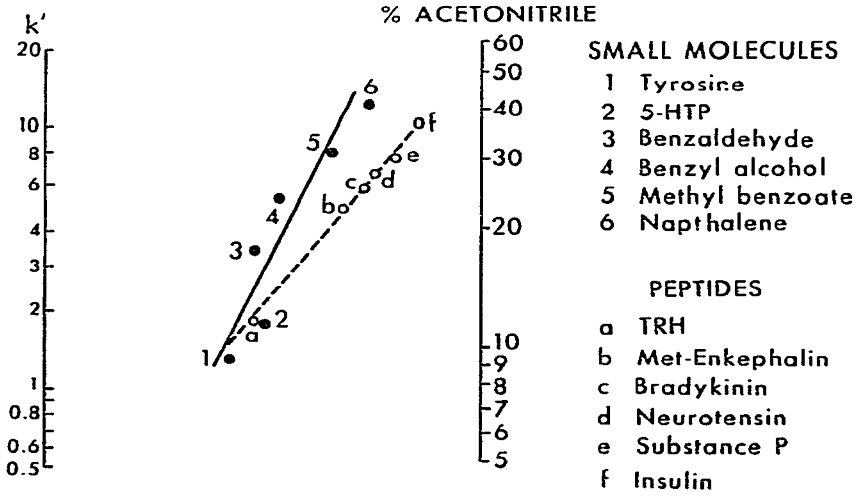

Fig. 9. Nomogram for choosing mobile phase conditions. If the retention at one % acetonitrile is known, the concentration of solvent to achieve any given k' can be predicted for peptides and low molecular weight substances.

DISCUSSION

The concept that the chromatographic behavior of peptides could be predicted on the basis of amino acid composition had been tested already in 1951. Using small peptides synthesized by Emil Fisher, Knight[9] and Pardee[10] showed that R_F values on "normal-phase" paper chromatography could be predicted with some accuracy. Sanger[11] showed that the relationship between R_F and composition was not absolutely accurate since peptides containing the same amino acids in different order could frequently be separated.

The need for revision of the previously published retention coefficients became clear when it was discovered that the retention coefficient values for glutamate, aspartate and tyrosine–SO_4 were in error. In the case of tyrosine–SO_4, the only peptide tested which contained this group was caerulein. It was shown[12] that most of the available commercial "caeruleins" are not the sulfated decapeptide claimed by the suppliers. As a result, the retention coefficients previously obtained for tyrosine and tyrosine–SO_4 were similar, while one would expect the contribution to retention of the tyrosine–SO_4 to be considerably less than that of tyrosine. When authentic sulfated and unsulfated caerulein (a gift of Dr. R. de Castiglione) were chromatographed, the sulfated compound did in fact elute about 4 min earlier than the unsulfated compound using both phosphate and perchlorate mobile phases.

It was shown[4] by using data obtained from the literature[13], that the retention of a variety of peptides could be predicted with an average error of about 4 min. However, using data from tryptic digests of hemoglobin[14] Schroeder[5] observed that predictions of retention of several peptides were grossly in error. For example, fragments αT4 and αT11 eluted at essentially the same times under his chromatographic conditions, but the calculated retention times were −0.1 min and 27.4 min, respectively. It seemed likely that the discrepancy was primarily due to the three glutamate residues which had been thought to have a large negative effect (−7.5 min) on retention. Glutamate and aspartate residues were absent from most of the hormonal peptides in the original data base, and in the data which had been used as a test. The revised data (Table I) with an N value of 14 for glutamate- and an N value of 19 for aspartate-containing peptides show that the effects of glutamate and aspartate on retention at acid pH are in fact quite small. The high correlation between actual and predicted retention times indicates that composition must be the major factor determining retention. However, the difference between actual and predicted times for any given compound (average 2.9 min) indicates that smaller roles are played by other factors including conformation, size, sequence, charge distribution and polarity. The possibility of predicting retention times under gradient conditions[4] was in part based on the hypothesis that linear gradients should provide linear increases in solvent strength. Since this latter hypothesis does not appear to be true[8], it is perhaps not surprising that addition of a given residue to a small peptide may have a slightly different effect than adding it to a large peptide. It is instead somewhat surprising that the contribution of each residue appears to be nearly additive, *i.e.* that each residue of small peptides interacts with the chromatographic matrix without positive or negative effects on the interaction of the other residues with the matrix. Otherwise, predicted retention would deviate sharply from actual retention with increasing molecular weight, whereas the deviation does not begin to be large except with compounds with greater than 20 or 30 residues where folding to internalize hydrophobic residues is likely to be important.

The above results were obtained with peptides containing only L-amino acids. Replacement of an L- with a D-amino acid can markedly change retention. For example, L-leu–L-phe can be readily separated from L-leu–D-phe, and L-4-phe–met–enkephalin amide can be readily separated from D-4–phe–met–enkephalin amide. The latter two compounds were chromatographed under a variety of pH, temperatures, salts and organic solvents in an attempt to find conditions which minimized this steric effect. Under all the conditions tested, these diastereoisomers were separable.

Factors affecting resolution

Peak sharpness in peptide separations depends not only on the column, but also on the mobile phase composition, and the chromatographic conditions of flow-rate and gradient rate, and the size and charge of the peptides themselves. It is now generally known that excellent separations with "well capped" reversed-phase columns can be achieved if a mobile phase is used which minimizes adsorption. Many workers use acid pH values and high concentrations of salts for this purpose, but volatile acids such as 0.1 % trifluoroacetic acid are useful when it is necessary to recover the peptide from the eluate[15]. This low concentration of trifluoroacetic acid is apparently not sufficient to cause ion pairing with basic residues. The data in Table II show that addition of trifluoroacetic acid did not increase the retention of any of the small peptides, even those containing several basic groups (*e.g.* bradykinin), over that seen with mobile phases containing only H_3PO_4.

Gradients of propanol gave poorer resolution with the peptides tested than gradients of acetonitrile. However, propanol has been advocated for large polypeptides and small proteins which may be insoluble in even high concentrations of acetonitrile[3].

The experiments on the effects of flow-rate on column efficiency were carried out with two columns, but only one mobile phase. It has been reported[16] that there can be differences in the flow-rate dependency of efficiency with different mobile phases. For example, with a salt-containing mobile phase, there was a greater flow-rate dependency, and a larger apparent plate count for a small protein (cytochrome *c*) than with a mobile phase without salts[16]. This finding may reflect the absence and presence, respectively of significant adsorptive effects.

The above experiments dealt with the general problem of resolution: maximizing the separation of a complex mixture of peptides. The resolution of any two specific peptides can be improved not only by increasing the overall resolving power of the system, but also by changing the selectivity. For example, we found similar column efficiencies with phosphate and perchlorate mobile phases. However, shifting of peaks relative to each other by the two mobile phases was such that overall resolution was superior with the phosphate mobile phase. For other peptides, the reverse might be true. Larger changes in selectivity can be achieved in some cases with lipophilic ion-pairing agents for compounds that differ in number of basic groups, or by changes in pH for compounds that differ in their content of acid residues.

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