Separation of Peptide Mixtures by Reversed-Phase Gradient Elution. Use of Flow Rate Changes for Controlling Band Spacing and Improving Resolution

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A general model has recently been proposed for the separation of peptides and proteins using reversed-phase gradient elution liquid chromatography. One application of this model suggests that flow rate, gradient time, or column configuration can be varied for band spacing control in the separation of enzymatic digests of proteins. Here a systematic procedure is described that uses repeated separations with different flow rates to maximize the separation of individual peaks within the chromatogram. From these initial separations it is possible to choose an optimum flow rate for the separation of a given sample. It is important in this approach to identify which bands in the various separations correspond to the same peptide. Various peak-tracking procedures are discussed and illustrated.

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Peptide mixtures such as tryptic digests of proteins are often complex and difficult to separate completely. It is common to observe 20 or more peaks in an initial chromatogram of such samples, with peak size varying widely and several band pairs showing only partial resolution. Under these circumstances it is unlikely that all compounds in the sample will be separated (*1*, *2*). However the latter conclusion assumes that the various compounds have retention times that are randomly distributed across the chromatogram. This may be true of an initial chromatogram, but it is possible to systematically rearrange the peaks within the chromatogram, using various approaches. Thus for the reversed-phase separation of small molecules, it is now common to optimize band spacing via change in the organic solvent or pH used for the mobile phase or by adding ion-pairing reagents (e.g., ref 3). Similar approaches have been reported for rearranging peptide peaks (e.g., ref 4–6) but are not used extensively, probably because volatile mobile phases of low pH are generally preferred, e.g., trifluoroacetic acid/water/acetonitrile.

It is also possible to adjust band spacing in the reversed-phase separation of peptides by changing from one column type to another, e.g., C18, cyano, or phenyl (e.g., ref 7 and 8). However this approach also suffers from certain limitations and is not commonly used; changes in band position for different columns tend to be minor and unpredictable, and it is not possible to continuously vary band position so as to arrive at an optimum overall band spacing.

An ideal approach to controlling peak spacing in the reversed-phase separation of peptide mixtures would have several features. It would be convenient and generally applicable to all samples. It would allow for fine gradations in the degree of peak rearrangements, so as to achieve an optimum overall resolution of the total sample. Finally it is desirable that these changes in peak position be predictable as a function of experimental conditions, because this allows the use of external logic to deduce the presence of unresolved doublets in any chromatogram obtained during separation–optimization. In this paper we describe a preliminary version of such an approach, which is applicable for the usual gradient elution separations of peptide mixtures.

In gradient elution each compound moves through the column with an average capacity factor value k' (equal to $\bar{k}$) that can be adjusted by changes in mobile phase flow rate F, gradient time t_G, or column volume V_m. A change in $\bar{k}$ will generally lead to changes in relative retention time and band position. While variation in F, t_G, or V_m can each lead to rearrangement of peaks within the chromatogram, it appears that a change in flow rate is the most practical. Here we describe the use of this approach for optimizing the separation of complex peptide mixtures.

THEORY

The reversed-phase gradient elution separation of peptides and/or proteins is well described in terms of linear solvent strength (LSS) theory as developed in ref 9 and applied to peptide/protein separations (*10*). The isocratic retention (capacity factor k') of these compounds as a function of the volume-fraction organic, ϕ, in the mobile phase is given as

$$\log k' = \log k_w - S\phi \quad (1)$$

where k_w refers to the value of k' for water as mobile phase ($\phi = 0$) and S is a constant that is characteristic of a given solute and organic solvent in the mobile phase. For acetonitrile as organic solvent (*10*), values of S are approximately related to solute molecular weight M as

$$S = 0.48M^{0.44} \quad (2)$$

although factors other than molecular weight are believed to affect solute S values. Nonlinear plots of $\log k'$ vs. ϕ have been reported by some workers for reversed-phase separation of peptides (e.g., ref 11), but this has little effect on the following discussion. Elsewhere we will show (*12*) that nonlinearity of $\log k'$ vs. ϕ plots can be treated by LSS theory as well as the case of linear plots (where eq 1 applies).

The theory of LSS gradient separation yields a value of the retention time t_g in gradient elution as a function of the isocratic parameters k_0 (initial value of k') and S of eq 1

$$t_g = (t_0/b) \log (2.3k_0b + 1) + t_0 + t_D \quad (3)$$

where the gradient parameter

$$b = t_0 \Delta\phi S/t_G \quad (4)$$

is a function of the solute parameter S and the experimental conditions t_0 (column dead time), t_D (gradient delay time), $\Delta\phi$ (change in ϕ during the gradient), and gradient time t_G (*9*). Further development of this theory yields predictions for change in band spacing as a function of mobile phase flow rate (*10*). For the case of compounds A and B separated in gradient runs where flow rates F_1 and F_2 are employed, we can first define the retention-time differences Δt_g

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(run 1 with F_1)

$$\Delta t_{g1} = (t_g)_a - (t_g)_b \quad (5)$$

and

(run 2 with F_2)

$$\Delta t_{g2} = (t_g)_a - (t_g)_b \quad (6)$$

where subscripts a and b refer to values for compounds A and B, respectively. We are interested in the change in band spacing with change in flow rate F, and this is given (*10*) as

$$\Delta t_{g2} - \Delta t_{g1} = \Delta\Delta t_g = (t_G/\Delta\phi)[\log (F_1/F_2)](1/S_a - 1/S_b) + V_m(C_a - C_b)(1/F_2 - 1/F_1) \quad (7)$$

Here S_a and S_b are S values for compounds A and B and V_m is the column dead volume. The first right-hand-side term of eq 7 is seen to depend on values of S_a and S_b, while the second right-hand-side term is a function of the size-exclusion parameters C_a and C_b for solutes A and B

$$C_i = t_{sec}/t_0 \quad (8)$$

The value of t_{sec} for solute i is equal to the retention time of solute i in an isocratic system (same column and flow rate) where solute i is unretained (for small molecules and/or large-pore packings, $t_{sec} = t_0$).

It has been shown (*10*) that even for the case of large protein molecules the second right-hand-side of eq 7 is small compared to the first term, so that we can write (to a first approximation)

$$\Delta\Delta t_g = (t_G/\Delta\phi) \log (F_1/F_2)(1/S_a - 1/S_b) \quad (9)$$

or

$$\Delta t_g = m \log F + n \quad (10)$$

where m and n are constants for a particular HPLC system and pair of compounds A and B, when only F is varied. Equations 9 and 10 will be most accurate for the case of relatively small peptides separated on larger-pore packings.

The origin of the flow rate dependence of band spacing Δt_g arises as follows. Equations 3 and 4 predict that an increase in F will result in a decrease in t_0 and in b, which in turn will yield elution of any solute in an average mobile phase composition where ϕ is smaller (see ref 13). For solutes of differing S value this will in turn result in a change in the average k' values ($\bar{k}$) during gradient elution. This effect has previously been described in detail (ref 10, Figure 4). As a result, a change in ϕ as a result of change in F also leads to a change in $\bar{k}$ for each solute and a resulting change in their relative separation Δt_g. See also comments in the Appendix.

Equation 4 can be rearranged into the equivalent form

$$b = V_m\Delta\phi S/t_G F \quad (11)$$

where V_m is the volume of mobile phase within the column, varying with the column dimensions. The average k' values during gradient elution are related to b as

$$\bar{k} = 1/1.15b = t_G F/1.15 V_m \Delta\phi S \quad (12)$$

According to eq 12 changes in band spacing can be achieved (as in Figure 1) by varying any one of the separation conditions included in this relationship: gradient time t_G, flow rate F, column volume V_m, or gradient range $\Delta\phi$. Cohen et al. (*16*) have shown such changes in band spacing when only gradient time t_G is changed. Van der Zee and Welling (*17*) have reported similar changes in relative retention for proteins when column volume V_m is varied. Likewise it is possible that other workers (e.g., ref 18), who have isolated a peptide fraction under one set of gradient conditions and then further resolved that fraction with a shallower gradient (larger value of $t_G/\Delta\phi$), have inadvertently made use of changes in band spacing to enhance resolution. The use of flow rate variation is preferable to change in the other variables of eq 12, because a change in F is (a) generally more convenient and (b) less related to concomitant changes in peak capacity (see ref 19, 20), which also affect resolution.

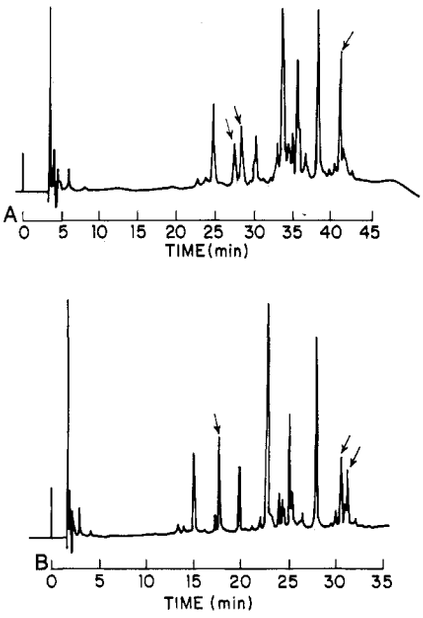


Figure 1. Gradient elution chromatograms for lysozyme digest: Zorbax PEP-RP1 column, 8 × 0.62 cm; gradient of 8 vol % mobile phase B to 40 vol % B in mobile phase A; mobile phase A is 0.1 vol % TFA plus 0.13 vol % morpholine in water; mobile phase B is 0.1 vol % TFA plus 0.13 vol % morpholine in acetonitrile; gradient time 30 min; temperature 35 °C; (A) 0.5 mL/min flow rate, (B) 1.0 mL/min flow rate.

EXPERIMENTAL SECTION

All measurements were made with a Model 8800 liquid chromatograph (Du Pont Clinical and Instruments Division, Biomedical Products Department, Wilmington, DE), which included a Model 870 three-headed pump with a four-solvent gradient mixer and system controller. Samples were injected with either a Model 710B WISP autoinjector (Waters Associates, Milford, MA) or a six-port air-actuated sampling valve (Valco Instruments, Houston, TX) using a 10-μL sampling loop. Either a Model 860 variable wavength detector (Du Pont) or a Model 1040A diode array detector (Hewlett-Packard) was used to detect the samples after separation. The column temperature was 35 °C in all runs.

The columns used were Zorbax PEP-RP1 (Du Pont). These have dimensions of 8 × 0.62 cm and are packed with 5-μm C8 particles having 15-nm-diameter pores. HPLC-grade acetonitrile and water used in this work were obtained from J. T. Baker (Phillipsburg, NJ); trifluoracetic acid (TFA) (Pierce Chemical Co., Rockford, IL) was added (0.1 vol %) to both the water and acetonitrile for gradient elution runs. The lysozyme separations used 0.125 vol % TFA and 0.1 vol % morpholine for improved peak shape. The lysozyme digest was a gift of A. Banes, University of North Carolina, and sperm whale myoglobin (Beckman Instruments) was digested with trypsin according to published procedures (*21*). Data were collected and analyzed by an HP-85 computer (Hewlett-Packard) programmed in BASIC; subsequent plots were made with an HP7470A plotter.

RESULTS AND DISCUSSION

The initial separation of the tryptic digest of lysozyme as shown in Figure 1A used a gradient of 8–40% acetonitrile in 30 min with a flow of 1.0 mL/min. The resulting separation was typical of protein tryptic digests; i.e., there are a large number of peaks of various sizes (areas), many of which appear to separate well, but with indications of some overlap of

Table I. Lysozyme Digest Retention Time[a]

	retention time at the following flow rates, min				
peak ID	0.5 mL/min	1.5 mL/min	2.0 mL/min	3.0 mL/min	4.0 mL/min
1	30.47	12.31	10.03	6.30	5.16
2	32.05	12.91	10.47	6.66	5.16
3	42.27	16.72	13.28	8.72	6.68
4	36.54	16.72	14.17	9.51	7.62
5	43.72	20.84	17.70	12.29	9.91
6	43.72	21.58	18.60	13.12	10.65
7	45.56	25.48	22.54	17.25	15.03
8	51.47	27.63	24.16	18.05	15.03
9	54.09	31.42	28.07	22.02	18.78
10	63.80	37.98	34.30	27.68	24.03
11	68.30	41.78	38.05	31.34	
12	73.51	48.54	44.90	38.77	
13	85.21	60.64	57.20	51.29	
14	94.66	68.36	65.25	59.15	
15	95.63	69.78	66.15	59.98	
16	96.55	70.54	66.96	60.78	
17	100.83	78.18	74.93	69.72	

[a] Zorbax PEP-RP1 column, 8 × 0.62 cm; gradient of 8 vol % mobile phase B to 40 vol % B in mobile phase A; mobile phase A is 0.1 vol % TFA plus 0.13 vol % morpholine in water; mobile phase B is 0.1 vol % TFA plus 0.13 vol % morpholine in acetonitrile; gradient time 120 min; temperature 35 °C.

specific peaks. A second separation, using the same conditions except that the flow was reduced to 0.5 mL/min, is shown in Figure 1B. In general, the chromatograms are similar in overall appearance, but the retention of the peaks is greater in the latter separation, as expected for a slower flow rate. There are some specific flow-related changes in separation, however, as highlighted by the arrows in the early and late portions of these chromatograms. In particular, two peaks are fused in chromatogram A at 1.0 mL/min and resolved in chromatogram B at 0.5 mL/min, while an opposite pattern is observed for a pair of bands in the later part of the chromatogram. These data demonstrate that a change in flow rate can result in changes in band spacing using otherwise identical conditions of separation, as predicted by the theoretical analysis above.

In order to study this effect more thoroughly with the lysozyme digest as a test mixture, a series of runs was made at different flow rates (0.5–4.0 mL/min); retention times for the major peaks (peak areas >0.5% of total sample) in the chromatogram are given in Table I. Here a given peak (e.g., no. 3) is the same peptide in each run. Because the absolute identity of each peak could not be confirmed (no standards available), a peak-tracking procedure was needed to correlate bands from one run to another. We devised such a procedure using a combination of relative peak retention plus peak size (area). An empirical figure-of-merit (F_m) was defined for each pair of peaks from runs 1 vs. 2

$$F_m = \left[\frac{(|\%A_1 - \%A_2|)^{1.5} + 0.2}{\%A_1 + \%A_2}\right][(|\Delta t_{g1} - \Delta t_{g2}|)^{1.5} + 0.2] \quad (13)$$

Here peak x in run 1 is compared with peak y in run 2 to see if they represent the same compound. The smaller the value of F_m for two peaks, the more likely it is that they represent the same compound. The parameters in the above equation are as follows: $\%A_1$, $\%A_2$, the area percent values of the peak in run 1 and run 2, respectively; Δt_{g1}, Δt_{g2}, corrected retention times for the peak in each run. The latter retention times are relative to a reference compound in the latter chromatogram, usually the largest peak. See the Appendix for a further discussion of eq 13.

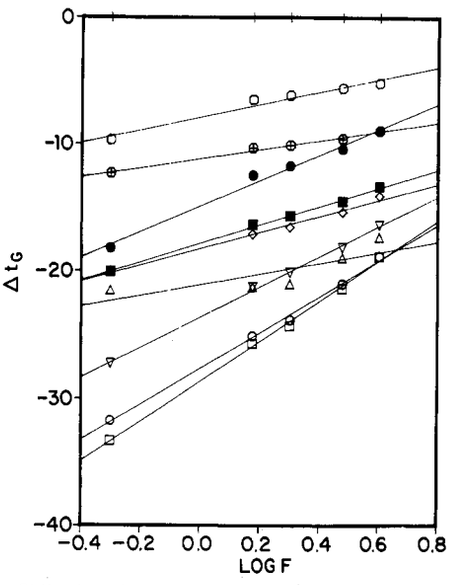


Figure 2. Shifts in relative retention of first 9 peaks in lysozyme chromatogram as a function of mobile phase flow rate. Values of Δt_g refer to retention time t_g for a given compound, minus t_g for the standard peak (no. 10 in Table I). Conditions are the same as in Table I.

Application of the F_m approach of eq 13 to the data of Table I provided preliminary (provisional) assignments of peaks between runs of different flow rate. Equation 10 was then used to confirm these assignments; i.e., properly identified peaks should form straight-line plots when values of Δt_g are plotted against log F as in Figure 2. Using this approach, we would track the major peaks in five separate runs of the lysozyme digest, using a 120-min gradient time and flow rates of 0.5, 1.5, 2.0, 3.0, and 4.0 mL/min. Figure 2 shows plots of the data according to eq 10, where t_g values for each compound are referenced to peak 10 (the first 9 peaks in the lysozyme separation are plotted). Figure 2 demonstrates that as the flow rate is changed, peak retention can change both absolutely and relatively; i.e., there are reversals in band position. Figure 2 also confirms the approximate linearity of these plots, as predicted by eq 10 (scatter in such plots around the best straight lines through data for a given peptide are due in part to overlapping bands, with resulting uncertainity in the exact t_g values of each peptide).

While this peak tracking approach worked well in this case, more complex digests might require further data, such as measurements at additional intermediate flow rates, to properly identify peaks. In addition, other identification techniques, such as the ones described below, may have to be used for additional complementary information.

For the myoglobin tryptic digests, we used a second, complementary technique for peak identification: detection at three wavelengths, 220 nm, 260 nm, and 280 nm. At 220 nm the absorptivity of the peptide amide bond is roughly constant, independent of amino acid type. Therefore band area at 220 nm is proportional to the concentration of a given peptide in the original sample. At 280 nm, only the amino acids tryptophan and tyrosine show significant absorbance, so only peptides or proteins containing these compounds will yield a peak. At 260 nm, phenylalanine is the primary absorbing species (although tryptophan also contributes to the signal). In principle, we could use this absorbance behavior to characterize peptides separated by gradient elution LC. However we have used the 280/220 and 260/220 ratios simply to track peaks as flow rate was changed.

Myoglobin digests were run with a 60-min gradient from 10% to 70% acetonitrile using flow rates of 0.5, 0.61, 0.7, 1.5, and 3.8 mL/min. Plots of these retention data according to

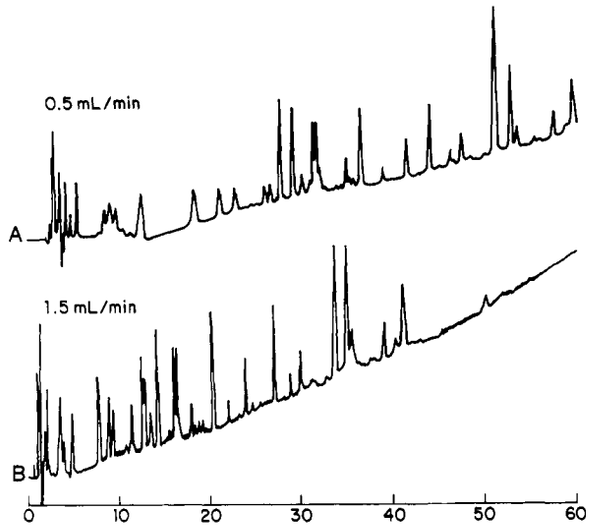


Figure 3. Gradient elution chromatograms for myoglobin digest: Zorbax PEP-RP1 column; linear gradient from 10% B to 70% B in A; mobile phase A is 0.1 vol % TFA in water; mobile phase B is 0.1 vol % TFA in acetonitrile; temperature 35 °C: (A) flow rate is 0.5 mL/min; (B) flow rate is 1.5 mL/min.

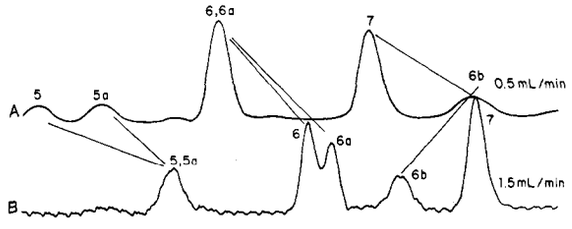


Figure 4. Detail from Figure 3 for myoglobin separation, showing changes in peak position with flow rate: (A) 0.5 mL/min; (B) 1.5 mL/min. Conditions were the same.

eq 10 (as in Figure 2) again gave linear plots for each peptide. The chromatograms for 0.5 mL/min and 1.5 mL/min in Figure 3 show the resulting general change in retention range, as well as some selectivity changes. The early part of the chromatogram is highlighted in Figure 4, where we show the effects of two different flow rates on this set of six peaks. As the flow rate is increased, peaks 1 and 2 (which were separated at 0.5 mL/min) now coelute at 1.5 mL/min. Peaks 3 and 4 (unseparated at 0.5 mL/min) have now begun to separate at the faster flow rate, and peaks 5 and 6 are separated in both runs, but have reversed their elution order. Depending on the particular separation required, one of these conditions might be preferable. In the general case, we would like to separate all six of these compounds, which appears possible at a sufficiently high flow rate (where the sequence of elution should be: peak 2 (first), 1, 6, 3, 4, 5 (last)).

RETENTION OPTIMIZATION

Linear plots as in Figure 2 for all the peptides in a sample can be used to determine the optimum flow rate for achieving maximum sample resolution in each system. Thus the difference in t_g values for any two bands in the chromatogram is also equal to the difference in their Δt_g values; i.e., we want the difference in Δt_g values for the poorest separated band pair in the chromatogram to be a maximum. This can be readily achieved by holding a clear straightedge perpendicular to the x axis of, e.g., Figure 2 and moving the straightedge along the x axis. For some value of F (or log F), the spacing between the poorest separated pair of adjacent bands will be a maximum (best overall resolution), and this optimum value of F will be apparent in this operation. This approach is similar to that described by Laub and Purnell for gas chromatography ("window diagrams", ref 22). An alternative to this graphical approach is to describe the various curves of, e.g., Figure 2 in terms of eq 10 and solve for the optimum flow rate algebraically (via a computer).

Table II. Variability of Band Spacing and Peptide S Values

(a) Example for 120-Min Gradient, Lysozyme Sample (Data of Table I)

F_1	F_2	peak	t_{g1}	t_{g2}	Δt_g	$\Delta\Delta t_g$
0.5	4.0	1	30.47	5.16	25.31	
						1.58
		2	32.05	5.16	26.89	
						8.70
		3	42.27	6.68	35.59	
						−6.67
		4	36.54	7.62	28.92	

(b) Summary and Data Reduction (See Text)

sample	t_G, min	$\Delta\phi$	F_2/F_1	av $\Delta\Delta t_g$	std dev	$\Delta S/\bar{S}$
lysozyme digest	45	0.32	8	0.08	±1.49	0.10
	120	0.32	6	0.43	±3.72	0.10
myoblobin digest	60	0.60	7.6	0.32	±1.31	0.12

Another approach for maximizing separation is as follows. The early and late halves of a chromatogram can be considered separately and optimum flow rates determined for *each* section. Note that initial elution at one flow rate will have little effect on the retention of later peaks eluted at a different flow rate, for gradient separations of peptides. Most of the peptides eluted with the second flow rate will have remained at the column inlet during elution at the initial flow rate (this is typical of peptide separation). That is, programming the flow rate during gradient elution of a peptide mixture offers a means of further varying band spacing within the final chromatogram.

Probability That a Change in Flow Rate Will Result in Complete Resolution of Two Peptide Bands. Retention data as in Table I for lysozyme (at different flow rates) allow us to assess the likelihood that a certain change in flow rate will result in the separation of a peptide pair that is unresolved at some starting flow rate. The general approach is illustrated in Table IIa for a 120-min gradient run (first four bands of lysozyme digest). Values of $\Delta\Delta t_g$ are seen to represent the *change* in spacing between adjacent bands as F is varied, with negative values (as for peaks (no. 3 and 4) indicating peaks that are approaching each other and which will eventually change places in the chromatogram. These data allow us to ascertain how often a change in flow rate can result in peak disengagement. Average values of $\Delta\Delta t_g$ and their standard deviation were determined for each of three cases (Table IIb). The average value of $\Delta\Delta t_g$ (from increase in F) is seen to be small for each example. Of greater interest is the larger *random* variation in $\Delta\Delta t_g$ as expressed by the standard deviation of these various data (Table II). These $\Delta\Delta t_g$ values from Table II (and other data) appear to be normally distributed, meaning that the probability of achieving a certain separation via change in F can be predicted from the Gaussian distribution of $\Delta\Delta t_g$ values. We can relate this variation in $\Delta\Delta t_g$ values to corresponding variations in individual solute S values ($\Delta S/S$). If it is assumed that $S_a \approx S_b$ in eq 9, then we can derive

$$\Delta S/\bar{S} = \Delta\Delta t_g \Delta\phi \bar{S}/t_G \log (F_1/F_2) \qquad (14)$$

and obtain values of $\Delta S/\bar{S}$ from the above data. Here $\bar{S}$ is the average value of S for the sample, estimated from its molecular weight (eq 2). For the lysozyme runs above, it is seen that derived values of $\Delta S/\bar{S}$ are each equal to $(0.01\bar{S})$, confirming eq 9 and 14. Likewise it is seen that $\Delta S/\bar{S}$ for the

myoglobin sample is similar to that for lysozyme ($0.012\bar{S}$), and we can assume that $\Delta S/\bar{S}$ will be close to $0.01\bar{S}$ for most peptide digests.

Starting with the latter conclusion ($\Delta S/\bar{S} \approx 0.01\bar{S}$) for peptide samples and acetonitrile/water gradients, we have carried out a rough analysis of the probability that an 8-fold change in F will result in the measurable separation of two peptide peaks that were initially unresolved ($R_s < 0.8$). From this it appears that the probability of separating an unitially unresolved band pair (final $R_s = 1.0$ or better) via an 8-fold change in F is about 90%, for gradient times as long as 240 min and state-of-the-art columns (e.g., ref 20). Therefore we predict that in nine cases out of ten, flow-rate variation will prove able to resolve initial doublets in the chromatogram. Selection of an optimum overall flow rate, as described above, will then usually allow all peaks that can be separated at any flow rate to be simultaneously resolved with the optimum value of F. When this is observed not to be the case, further use of flow programming should allow a closer approach to optimum overall separation within a single gradient run.

The overall conclusion we can draw is that the use of flow rate changes to improve band spacing is a useful tool. In the general case, however, some band pairs will not be separable by this technique—as is true of any method of varying band spacing within the chromatogram. Since this work was submitted, a similar study by Aguilar et al. (*23*) has appeared.

CONCLUSIONS

The theory of gradient elution (*9*, *10*) suggests that band spacing in peptide mixtures separated by reversed-phase gradient elution can be altered by changes in mobile phase flow rate and that these changes in retention of individual peptide peaks are governed by eq 10. That is, shifts in relative retention vary as the logarithm of flow rate. This suggests a general strategy for maximizing overall sample resolution, i.e., minimizing overlapping peaks in a complex peptide mixture. First, after adjusting gradient time to provide adequate peak capacity (sufficient room in the chromatogram to contain all the peptide peaks likely to be present as described in ref 20), flow rate is varied over wide limits, e.g., 0.5, 1.0, 2.0, and 4.0 mL/min, to generate at least four separate chromatograms. Second, peaks in each chromatogram belonging to the same peptide are identified, based on band size and relative retention, or upon peak height ratios at different wavelengths (e.g., 220, 260, 280 nm). Third, plots of relative retention vs. log (flow rate) are constructed. Finally, an optimum flow rate is determined from these plots to provide for the complete separation of the major peptides in the original sample. In some cases, separation can be further improved by flow-rate programming as described in the text.

In practice we have found this approach to be a useful technique for resolving the major components of an entire peptide mixture or for ensuring the purity of individual peptides of interest. It appears that several peaks in typical protein digests will often correspond to unresolved doublets but that in most cases each such primary-peptide doublet can be resolved by further flow rate variations.

ACKNOWLEDGMENT

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GLOSSARY

b	gradient steepness parameter defined by eq 4
C_a, C_b, C_i	size-exclusion parameters for compounds A, B, and i defined by eq 8; C is equal to one for a totally permeating molecule and is smaller for larger molecules that do not totally permeate the packing pores
F	flow rate of the mobile phase (mL/min)
F_1, F_2	flow rates for two different runs (1, 2) where only flow rate is changed
F_m	a figure-of-merit that compares two peaks from different runs; a small value of F_m implies that the two peaks are the same compound, eq 13
k'	solute capacity factor (isocratic separation)
$\bar{k}$	effective or average k' value in gradient elution (value for peak when it has arrived at the column midpoint), eq 12
k_w	value of k' for water as mobile phase, eq 1
LSS	linear solvent strength; describes gradient elution systems where log k' varies linearly with time during the gradient
M	solute molecular weight (dalton)
m, n	constants for a particular band in gradient runs where only flow rate is varied, eq 10
R_s	resolution function, equal to difference in retention times t_g divided by average bandwidth for a pair of adjacent bands
S	for a given solute in a specified isocratic system, $-\mathrm{d}(\log k')/\mathrm{d}\phi$, eq 1
$\bar{S}$	average S value for two adjacent bands, eq 14
S_a, S_b	S values for two adjacent bands A and B, eq 7
t_D	gradient dwell time; time for solvent introduced into gradient mixer to arrive at column inlet (gradient holdup time) (min)
t_g	retention time for a band in gradient elution (min)
t_{g1}, t_{g2}	value for t_g for two different flow rates (Table II)
t_g	gradient time (min); time during which mobile phase composition is changing
t_o	column dead time (min)
V_m	total volume of mobile phase inside column (mL); column dead volume
Δt_g	difference in t_g values for two peaks in a gradient chromatogram (eq 5 and 6); in Figure 2 and elsewhere, one of the peaks is usually a "standard" peak
$\Delta\Delta t_g$	change in t_g with change in flow rate (eq 7); the change in band spacing of bands A and B as a result of varying flow rate
ϕ	the volume fraction of organic (acetonitrile here) in the mobile phase
$\Delta\phi$	change in ϕ during the gradient
$\%A_1$, $\%A_2$	areas (expressed as percent of total chromatogram) of peaks 1 and 2, eq 13

APPENDIX

Dependence of Values of S on Retention Time t_g. Since S is dependent on solute molecular weight M (eq 2), and retention t_g tends to increase with solute molecular weight, it might be anticipated that adjacent solute bands would have similar M values and therefore similar S values. If this were the case, little change in Δt_g would be anticipated for change in F—since the term $(1/S_a - 1/S_b)$ of eq 9 would then equal zero. Other evidence suggests that this is not the case, and there will be a general tendency for change in t_g as a function of F; i.e., change in flow rate should prove to be a generally useful means of changing band spacing for the purpose of resolving peaks that otherwise coelute. Thus several studies (e.g., ref 14) have shown that t_g values for peptides are not simple functions of peptide molecular weight. Rather peptide retention is also a function of individual hydrophobic and hydrophilic amino acid residues in the peptide. Likewise other studies (*15*) have shown for small molecules and acetonitrile as organic solvent that S values vary markedly with retention (t_R in isocratic systems), in a more or less random pattern as solute structure is varied. Further data presented here confirm

that values of S for typical peptide mixtures are only weakly dependent on peptide t_g values.

Origin of Equation 13 (figure-of-merit procedure). Equation 13 was derived in the following fashion. When chromatographic conditions are changed for a given sample, changes in relative peak position (for different compounds) often occur. For example, a change in mobile phase composition is often used to alter band spacing, and peak reversals are then common. Chromatographers who are familiar with such examples of peak reversal commonly keep track of individual compounds within the sample by comparing between chromatograms on the basis of their relative size and retention. That is, peaks with similar areas and similar retention times are likely to represent the same compound in the two chromatograms. We constructed several representative examples of such peak reversal, using a range of different areas and relative retentions for two peaks. We then studied different functions of relative peak area and peak retention in an effort to provide as accurate a match as possible between peaks representing the same compound. In this way eq 13 was eventually derived. We subsequently tested eq 13 against a number of actual chromatograms where peak identity was known (steroids, herbicides, etc., changing mobile phase composition), and where peak reversal was rather common. For these examples it was found that eq 13 usually gave the correct identifications; i.e., correctly matched the compounds in chromatogram 1 with those in chromatogram 2. Exceptions to this were occasionally noted and found to be related to several effects, one of which is change in peak area with mobile-phase composition. In these cases this difficulty could be overcome by making smaller changes in mobile phase composition between the two chromatograms being compared. The use of eq 13 in the present study is less subject to these errors, because changes in mobile phase composition were less serious than in the latter studies with the steroids and herbicides, and because changes in F can be made small enough to avoid major peak reversals with resulting erroneous peak assignments. Thus we recommend increasing (or decreasing) flow rate slowly for the second run, then by larger amounts in succeeding runs. This practice was used in the myoglobin study, where flow rates selected were 0.5, 0.61, 0.7, 1.5, and 3.8 mL/min. This allows the more accurate assignment of peak identity to the initial runs, following which eq 10 can be used to confirm assignments in later runs.

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Quantitation of Nucleic Acids at the Picogram Level Using High-Performance Liquid Chromatography with Electrochemical Detection

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High-performance liquid chromatography with amperometric detection was used to quantitate nucleic acids at levels down to 100 pg. The method is based on hydrolysis and quantitation of the purine bases. The detection limit for adenine was 0.1 pmol and for guanine was 0.05 pmol. The method compared well with ultraviolet absorption at 258 nm and ethidium bromide fluorescence, for quantiation of DNA from four different sources.

The growing interest in recombinant DNA technology has led to the need for a sensitive and precise method of quantitating nucleic acids. Various methods for quantitating DNA/RNA have been reported; however, each one has some particular drawback with respect to quantition at low levels. The ultraviolet (UV) absorbance measurement at 258 nm is simple to perform, and the detection limit is approximately 100 ng/mL based on an absorptivity of 10^4 cm^{-1} mol^{-1} of phosphate (*1*), but many other compounds absorb UV light in this region. Several colorimetric procedures (*2*) exist that are based on the reaction between ribose and either diphenylamine or orcinol, following hydrolysis of the nucleic acid. These procedures yield detection limits of 1000 ng/mL and are subject to interferences from other compounds (*3*, *4*). Techniques based on the fluorescence of ethidium bromide (*5*, *6*) or 4′,6-diamidino-2-phenylindole (*7*) yield detection limits in the range of 1–100 ng/mL, but these values are based

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