Journal of Chromatography, 316 (1984) 359–372
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 463

EXAMINATION OF THEORETICAL PRINCIPLES OF GRADIENT ELUTION AS APPLIED TO REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACIDS

K. A. COHEN*, J. W. DOLAN and S. A. GRILLO
IBM Instruments, P.O. Box 332, Orchard Park, Danbury, CT 06810 (U.S.A.)

SUMMARY

More than twenty different phenylthiohydantoin (PTH)-derivatized amino acids were used in the examination of the relevance and the applicability of theoretical principles that have been postulated earlier to describe gradient elution in high-performance liquid chromatography (HPLC). Reversed-phase HPLC conditions representative of PTH-amino acid assays, *i.e.* chemically modified silica microspheres in conjunction with an aqueous–organic mobile phase, were utilized. As predicted by theory, inevitable compromises between resolution and retention time were observed empirically. However, an *a priori* assumption of equivalent linear solvent strength conditions was shown to be inappropriate for selected PTH-amino acid solutes. These findings were supported by the experimentally determined variability among the different solutes of solvent strength values, *S, i.e.* the slope of the plots of the logarithm of isocratic capacity factor *versus* the fraction of organic modifier.

INTRODUCTION

High-performance liquid chromatography (HPLC) has gained considerable popularity as an analytical as well as preparative technique, serving the separation needs of the biochemical and biotechnological community. The various modes of HPLC have been exploited, including reversed phase, ion exchange, size exclusion and hydrophobic interaction[1–4]. Reversed-phase HPLC has been particularly useful in that it can provide fractionation of a wide variety of biologically important molecules, such as proteins and peptides[1,2,5,6] and amino acid derivatives[6–9].

Frequently, gradient elution has been employed within reversed-phase HPLC for the separation of solutes falling within the compound classes cited above. Although there have been several theoretical treatments of gradient elution[10–16], the typical approach to the optimization of gradients has been phenomenological, for both the polypeptides[17–21] and the various derivatives of amino acids[7,9]. In these reports, critical gradient parameters were identified and examined, but not within the context of gradient elution models. Exceptions to this empirical approach were the work in refs. 22–27 in which general compliance with the optimization of gradient

0021-9673/84/$03.00

steepness according to the gradient postulates of Snyder[10] were observed for proteins and polypeptides. There has not been any analogous investigation for amino acid derivatives.

Inasmuch as the gradient elution model of Snyder was shown to be applicable to various biochemical compounds of comparatively large molecular weight (3–70 kilodaltons), it was selected for examination in this work. Much smaller probes, *i.e.* the phenylthiohydantoin (PTH) derivatives of more than twenty amino acids were investigated. These probes were subjected to gradients of varying steepness, and the resulting effects on resolution were compiled in accordance with the Snyder model.

THEORY

Resolution, R_s, is a quantitative measure of separation and can be expressed for two adjacent bands under isocratic conditions by the following derived relationship[28]

$$R_s = \frac{1}{4} N^{1/2} (\alpha - 1) \frac{k'}{k' + 1} \tag{1}$$

where N is the column efficiency, k' is the average value capacity factor for the two bands, and α is the separation selectivity factor (defined as the ratio of the capacity factor of the later band to the capacity factor of the earlier band). Eqn. 1 shows that there are three chromatographic parameters (N, α and k') that control resolution. Knowledge of how particular chromatographic conditions influence these parameters can be helpful in developing a rational strategy for the purpose of increasing resolution.

For instance, it is widely known that N can be increased by going to longer columns and/or smaller particles. However, because of the square-root dependence of R_s on N, the gain in efficiency leads to only a moderated gain in resolution. A second strategy that can be implemented in order to improve separation is to vary chromatographic conditions that affect α. Dramatic changes in relative retention are possible when either the composition of the mobile phase[29–31] or the composition of the stationary phase is altered[32]. However, the effect of the change in the phase system on α is frequently unpredictable.

The remaining chromatographic parameter in eqn. 1 that can be varied to achieve better separation is k'. The k' term in eqn. 1 is such that resolution will increase with an increase in k'. The increase in resolution is significant for low k' values. On the other hand, as the k' values become greater (*e.g.*, $k' > 10$) resolution increases very slowly.

In view of the longer retention times accompanying these larger k' values, the profitability of the extra resolution becomes marginal, because as k' is increased, the capacity factor term, $k'/(k' + 1)$, approaches unity. This asymptotic dependence of resolution on capacity factor leads to the recommendation that k' be made to fall within the range 2–10. Control of k' can be readily exercised by varying the relative concentrations of the stronger and weaker solvents in the mobile phase. That k' can be manipulated so simply and that the effect of this manipulation is usually favorable and predictable are two reasons why control of the capacity factor should be considered as a means of improving separation.

As stated above, eqn. 1 applies to the isocratic mode. An analogous expression can be derived for the gradient elution mode[10]

$$R_s = \frac{1}{4} N^{1/2} (\alpha - 1) \frac{\bar{k}}{\bar{k} + 1} \quad (2)$$

R_s, N and α in eqn. 2 have the same meaning as they have in eqn. 1. However, a somewhat different term (the average capacity factor, $\bar{k}$) is introduced in eqn. 2. This average capacity factor, $\bar{k}$, accounts for the fact that in the gradient elution mode (unlike the isocratic mode) the composition of the mobile phase is continuously changing. Clearly, due to this change in mobile phase composition, the instantaneous capacity factor of a solute will also change. Customarily, in gradient elution the mobile phase becomes stronger with time; hence, the instantaneous capacity factor of a solute decreases as the gradient progresses. Because the instantaneous capacity factor of a solute is not constant over the course of the gradient, the average capacity factor ($\bar{k}$) is utilized in the resolution equation. Achievement of the maximum resolution in the minimal amount of time is possible by designing gradient conditions so that $\bar{k}$ does not exceed approximately 10.

This $\bar{k}$ used in eqn. 2 applies to the average capacity factor that is characteristic of a particular solute undergoing gradient elution and should not be confused with k' of eqn. 1. (This latter k' is also an average, but of two adjacent and different solutes eluted under isocratic conditions.) For a further explanation of $\bar{k}$, the reader is referred to the original development of this gradient elution theory[10–12,26,27]. Much of the following theoretical treatment is extracted from and based on the gradient elution theory of Snyder[10].

The design of optimum gradient conditions can be facilitated through the use of a term directly related to $\bar{k}$. This term is the gradient steepness parameter b, and is given in the expression

$$b = \frac{1}{1.15\,\bar{k}} \quad (3)$$

The reciprocal relation of b and $\bar{k}$ should be noted. Large values of $\bar{k}$ in gradient elution correspond to small values of b, which would result in long retention. For instance, for a $\bar{k}$ of 10, simple substitution into eqn. 3 yields $b = 0.087$. To achieve the maximum separation in the minimum amount of time, as a guideline, $\bar{k}$ should not exceed 10; hence, values of b should not be less than 0.087. The precise value of b that is selected will depend on what the user assesses to be the best compromise between gradient time and its asymptotic effect on resolution.

Eqn. 4 permits the user to design the gradient with a specific value of b.

$$b = \varphi' S\, V_0/F \quad (4)$$

V_0 is the retention volume of an unretained solute, φ' is the rate of change of organic modifier during the gradient, F is the volumetric flow-rate and S is called the solvent strength. The units for V_0, F and φ' are ml, ml/min and %/min, respectively. The parameter φ' is obtained by dividing $\Delta\varphi$, the fractional composition range of organic

modifier that is spanned during the gradient, by t_G, the time period that the gradient is operated. For example, if a gradient is run from 5% organic modifier to 95% organic modifier over a period of 30 min, then $\varphi' = \Delta\varphi/t_G = (0.95 - 0.05)/30 = 0.03$.

Solvent strength S, a term which is dependent on the organic modifier, has received considerable attention, either directly or indirectly, in numerous studies[12,33–36]. S can be obtained through a series of isocratic experiments in which the capacity factor of a solute is measured at several different φ-values, φ being the fractional composition of the organic modifier in the mobile phase. A plot of log k' *versus* φ is constructed, and S is defined as the slope of the plot, *i.e.* $d(\log k')/d\varphi$.

Typically, for a given solute and a given phase system, such a plot can be closely approximated by a linear mathematical relation. This approximation is generally agreed to be reasonable, particularly when the capacity factor is made to fall within the range 1–10[10–12,16]. When the capacity factor is outside this range, some workers recommend the use of polynomial functions to describe the retention[33].

By manipulation of eqns. 2 and 4, the asymptotic dependence of R_s on t_G in the gradient elution mode can be illustrated[10,22,23] and is shown in Fig. 1. This mathematical development results from the effect on R_s of $\bar{k}$. Eqn. 3 shows the inverse relation of R_s to b. And, finally, R_s is directly related to t_G through eqn. 4 and the discussion thereof. It should be noted that the validity of the asymptotic influence of $\bar{k}$ is contingent upon the other terms in eqn. 2 remaining essentially invariant.

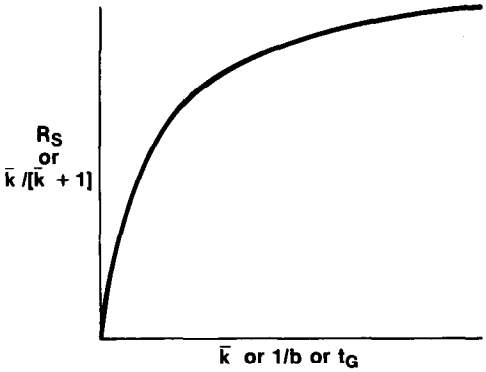

Fig. 1. Predicted (theoretical) dependence of R_s on various gradient parameters.

EXPERIMENTAL

Apparatus

An LC/9533 ternary gradient liquid chromatograph and an LC/9523 variable-wavelength detector were connected to an LC/9540 chromatography data integrator (IBM Instruments, Danbury, CT, U.S.A.).

Column

The pre-column was a normal-phase (silica) column, 50 × 3.2 mm (IBM Instruments 8635354), which was fitted between the pump outlet and the injector. The

analytical column was an IBM 5-μm octadecyl 250 × 4.5 mm (IBM Instruments 8635308).

Mobile phase

The mobile phase consisted of the following solvents: 0.05 *M* sodium acetate buffer, acetonitrile and tetrahydrofuran, the last two being HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.). The sodium acetate buffer was prepared by dissolving HPLC-grade sodium acetate (J. T. Baker, Phillipsburg, NJ, U.S.A.) in water (Organicpure, Sybron/Barnstead, Boston, MA, U.S.A.) and adding 180 μl/l glacial acetic acid (A-38, Fisher Scientific, Springfield, NJ, U.S.A.) resulting in pH 5.81. The buffer was filtered through 47-mm, 0.4-μm polyester filters with a solvent filtration kit (IBM Instruments 8635615). Solvents were degassed by placing them under vacuum for 20 min and then placing them under a slightly pressurized helium atmosphere.

Samples

All of the PTH-amino acids were obtained from Sigma (No. PTH-27, St. Louis, MO, U.S.A.). The amino acids chosen consisted of all of the common, naturally occurring amino acids, as well as several which are often included as internal standards or oxidative by-products in amino acid separation studies. For each amino acid, *ca.* 2 mg was dissolved in 10 ml of 0.05 *M* sodium acetate buffer–acetonitrile (50:50). These samples, stored in the dark at 0–2°C, served as stock solutions and were diluted five-fold with the 0.05 *M* sodium acetate buffer prior to injection.

Chromatography

Approximately 1 nmole of each amino acid was injected. For isocratic elution the injection volume was 1–3 μl, while 20–30 μl were injected for gradient elution. Injections were performed at least in duplicate, and for the measurement of retention times the average was used to calculate capacity factor k'. The void volume of the column was determined at each mobile phase composition by injecting 0.1 *M* potassium nitrate (reagent grade, Fisher Scientific). All mobile phases were mixed by using the solvent mixing capabilities of the LC/9533. Solvents A, B and C in the chromatograph consisted of buffer, tetrahydrofuran and acetonitrile, respectively. Peaks were detected at 268 nm. Sensitivity was typically 0.2 or 0.1 a.u.f.s. In the gradient elution mode, the delay volume of the chromatograph, including the pre-column, was accounted for so that injections were made when the gradient reached the analytical column. All gradients were linear and were run from buffer–acetonitrile–tetrahydrofuran (95:2:3) to buffer–acetonitrile–tetrahydrofuran (37:60:3). All isocratic mobile phases contained 3% tetrahydrofuran and various ratios of acetonitrile and buffer. The flow-rate was always 1.0 ml/min and the temperature was ambient.

RESULTS AND DISCUSSION

Several pairs of adjacent and/or closely emerging PTH-amino acids were subjected to gradient elution analysis, in order to examine the applicability of the asymptotic relationship illustrated in Fig. 1. Gradient steepness was varied through manipulation of b, which was controlled experimentally by the adjustment of t_G. Also,

for each PTH-amino acid, capacity factors were measured as a function of the fractional composition of the organic modifier (acetonitrile) in a series of isocratic separations. From these data, plots of the log k' *versus* φ were constructed.

Such plots for serine and asparagine, two PTH-amino acids that are customarily eluted early in this kind of assay[8], were prepared in Fig. 2. Four data points, each corresponding to an isocratic experiment, were obtained for the description of the retention of each amino acid over a k' range of approximately 1–10. As expected, retention decreased as the fraction of acetonitrile was increased. It was also evident that the plots, which were constructed according to a linear regression analysis, were quite linear. In fact, the correlation coefficients were 0.9992 and 0.9990 for the serine and asparagine plots, respectively. The same regression analysis yielded a slope value (S) of 3.7 for both solutes.

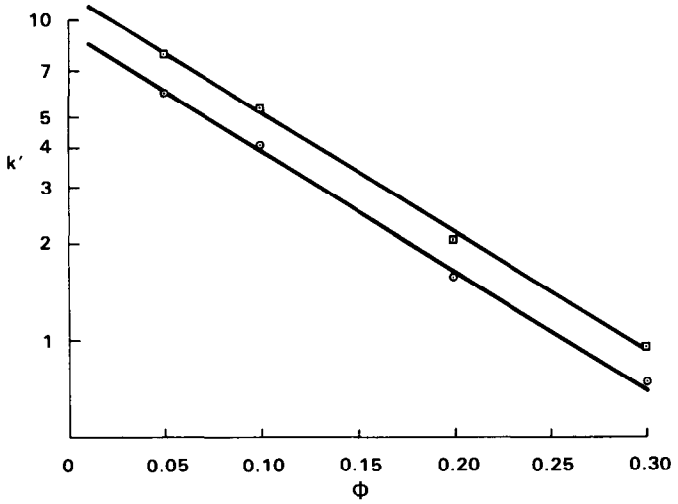

Fig. 2. Plot of isocratic log k' *versus* φ (acetonitrile) for PTH-asparagine (⊙) and PTH-serine (⊡). S = 3.7.

The effect of gradient time on the separation of these two amino acids was studied, as depicted in Fig. 3. Five different gradient times were examined, ranging from about 10 to 75 min. These corresponded to a range of b values of 0.44 to 0.06 (eqn. 4) and to a range of φ' values of 0.056 to 0.008. Better separations were achieved by increasing the gradient time from 10.4 to 41.4 min, as seen in the top three chromatograms. However, as shown in the bottom two chromatograms, when t_G was increased further, the improvement in separation was not significant.

These results were represented more quantitatively in Fig. 4. Resolution as a function of gradient time was plotted for this pair of PTH-amino acids. Resolution, R_s, was calculated according to the well-known equation

$$R_s = \frac{2(t_2 - t_1)}{(w_1 + w_2)} \tag{5}$$

where t_1 and t_2 were the retention times of the earlier- and later-emerging peaks,

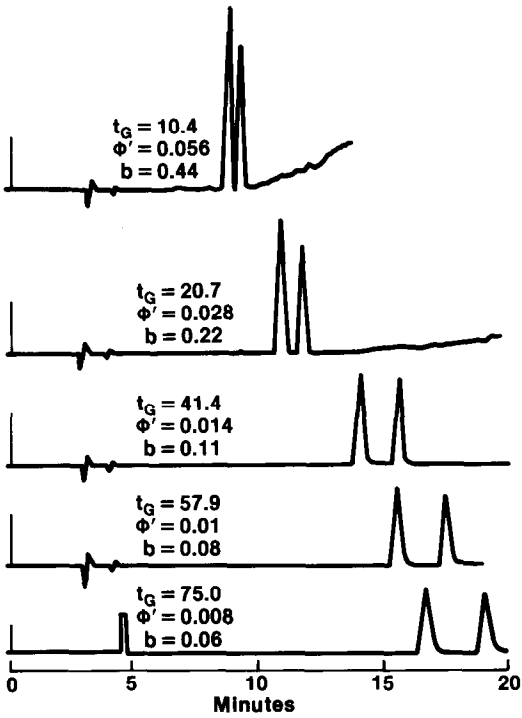

Fig. 3. Effect of gradient time and other gradient parameters on the separation of PTH-asparagine and PTH-serine.

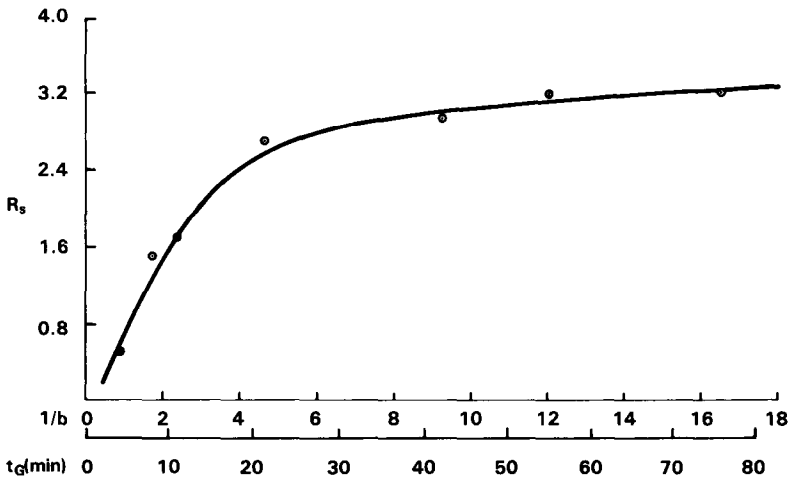

Fig. 4. Resolution of PTH-asparagine and PTH-serine as a function of gradient time and reciprocal gradient steepness.

respectively, and w_1 and w_2 were their peak widths at the baseline, as measured by the tangent method[28]. As predicted above, an asymptotic dependence of resolution on gradient time was obtained. The plot of resolution increased initially but then leveled off. This leveling-off effect occurred at a $1/b$ value of approximately 8, which corresponded to a b value of 0.12. This was consistent with the optimal b value discussed earlier and with the generally recommended range of $0.1 < b < 0.3$ (ref. 10).

The same kind of examination of isocratic and gradient retention was conducted for leucine and phenylalanine, two PTH-amino acids that are typically eluted late in the chromatography of sequenator products[8]. As seen in Fig. 5, the isocratic log k' *versus* φ data were reasonably approximated by linear regression plots over the k' range 1–10. The plots were parallel, having slopes, or S values, of 3.4.

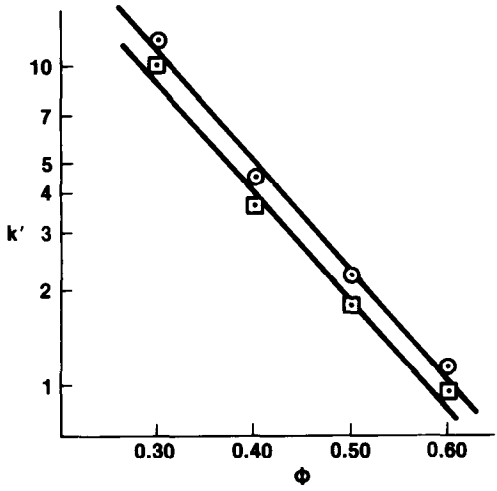

Fig. 5. Plot of isocratic log k' *versus* φ (acetonitrile) for PTH-leucine (⊙) and PTH-phenylalanine (⊡). $S = 3.4$.

In the gradient elution analysis of these two solutes, an increase in t_G initially resulted in an increase in separation (Figs. 6 and 7). However, for gradient times greater than about 30 min, the increases in resolution were marginal, particularly in view of the concomitant increases in elution times. A t_G of 30 min corresponded to a b value of 0.14, again falling within the suggested range 0.1–0.3.

It was evident that the relations in Figs. 4 and 7 were characterized by an asymptotic nature, as predicted. Other closely eluted pairs of PTH-amino acids were tested in a similar fashion. Comparable results were obtained. For example, the effect of gradient time on the resolution between PTH-tryptophan and -lysine showed the same curvature, as illustrated in Fig. 8. Again, the optimal gradient steepness parameter was calculated to be roughly 0.14.

The findings presented thus far have underscored the utility of employing theoretical protocols which have been developed and applied previously for the optimization of resolution in the gradient elution mode[10–12,22–27]. Moreover, it has been reported earlier that several gradient elution schemes that had been highly optimized

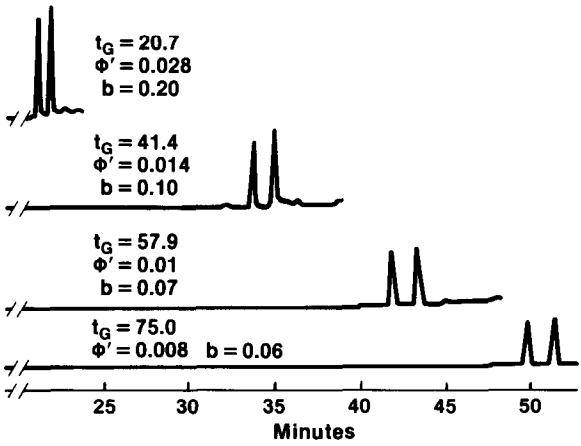

Fig. 6. Effect of gradient time and other gradient parameters on the separation of PTH-leucine and PTH-phenylalanine.

by trial-and-error approaches for the total assay of all PTH-amino acids showed a general compliance with the theoretical optimization protocols discussed here[37]. However, simple extension of the gradient time in order to optimize resolutions, was not directly applicable to every pair of closely emerging PTH-amino acids, as demonstrated below.

The influence of gradient time on the separation of PTH-methionine sulfone from PTH-alanine was studied. As shown in Fig. 9, R_s was not increased with an increase in t_G. Rather, the separation seemed to deteriorate as gradient time was extended. The reason for this anomalous result was apparent in Fig. 10, in which it

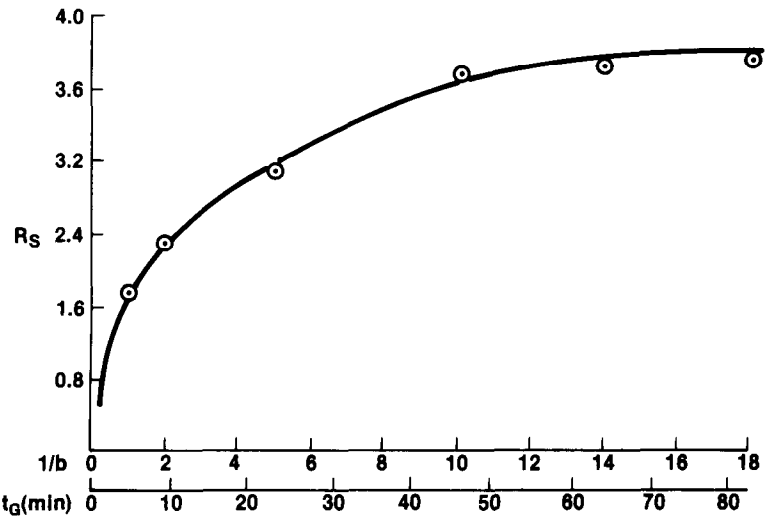

Fig. 7. Resolution of PTH-leucine and PTH-phenylalanine as a function of gradient time and reciprocal gradient steepness.

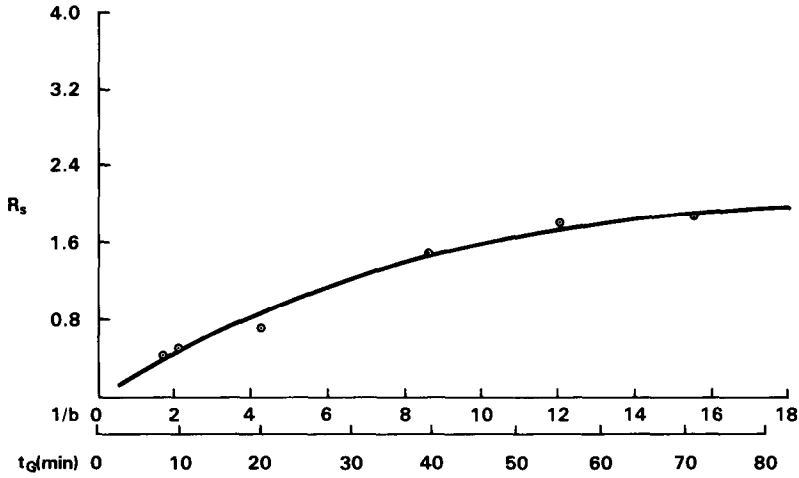

Fig. 8. Resolution of PTH-tryptophan and PTH-lysine as a function of gradient time and reciprocal gradient steepness.

was observed that the slopes of the plots of the log k' *versus* φ were not equal. Thus, by definition, the S values for these two solutes were different: 3.5 and 3.8 for PTH-alanine and -methionine sulfone, respectively. This led to an α in eqn. 2 that did not remain constant for the various gradient times examined in Fig. 9. Pictorially, the change in the separation selectivity factor with a change in t_G (or b) was indicated by the plots not being parallel in Fig. 10. The linearity of the plots, nonetheless, was empirically no less than that for the parallel plots observed in Figs. 2 and 5 (see Table I).

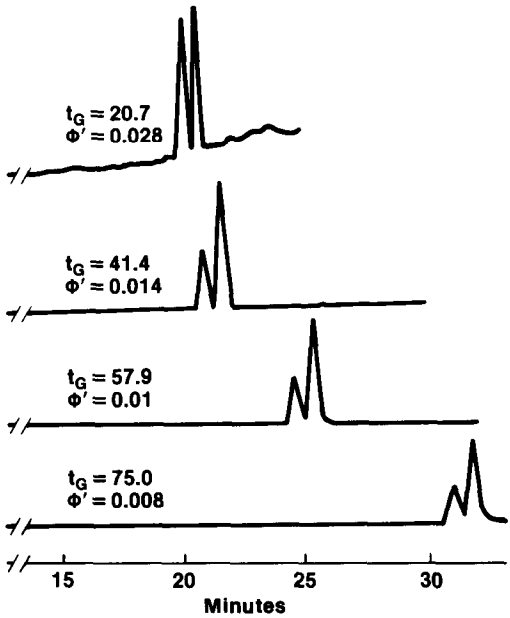

Fig. 9. Effect of gradient time on the separation of PTH-methionine sulfone and PTH-alanine.

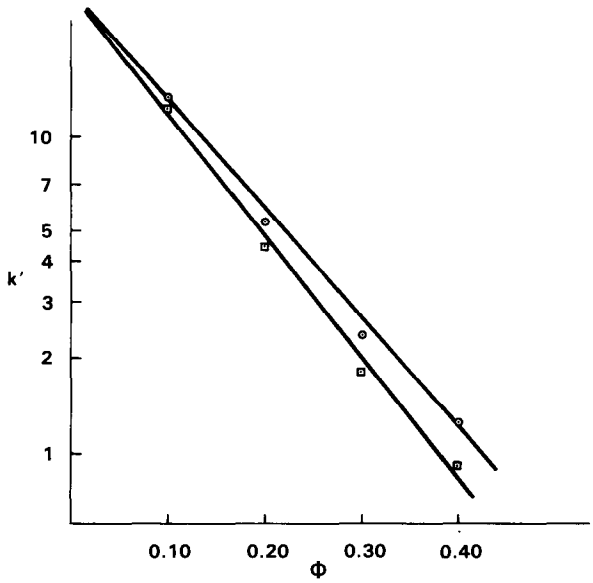

Fig. 10. Plot of isocratic log k' *versus* φ (acetonitrile) for PTH-methionine sulfone (⊡, S = 3.8) and PTH-alanine (⊙, S = 3.4).

Another instance of irregular behavior was seen in the examination of the PTH-derivatives of tyrosine and α-aminobutyric acid. When the gradient time was increased, the resolution decreased at first, to the extent that the two peaks coincided at a t_G of 57.9 min (Fig. 11). Yet, further increases in gradient time beyond 57.9 min provided improved separation. Such behavior was explainable on the basis of the retention data represented in Fig. 12, in which the isocratic plots were not only not parallel but actually intersected in the k' range 1–10. Therefore, the α value did not

TABLE I

EMPIRICALLY DETERMINED S VALUES FOR TWENTY FOUR PTH-AMINO ACIDS

	S, slope	*Correlation coefficient*		*S, slope*	*Correlation coefficient*
CYS-OH	3.9	0.9999	PRO	3.2	0.9949
ASP	3.2	0.9999	MET	3.4	0.9954
GLU	4.9	0.9999	VAL	3.2	0.9916
ASN	3.7	0.9990	NVAL	3.5	0.9947
GLN	4.2	0.9985	TRP	4.0	0.9955
HIS	4.0	0.9997	PHE	3.4	0.9927
SER	3.7	0.9992	ILE	3.2	0.9939
THR	3.5	0.9991	LEU	3.4	0.9950
GLY	3.3	0.9992	NLE	3.4	0.9940
ARG	3.3	0.9895	LYS	3.9	0.9911
METS	3.8	0.9962			
ALA	3.4	0.9969	Range	3.1–4.9	0.9895–0.9999
TYR	4.1	0.9964	$\bar{X}$, S_x	3.6, 0.4	0.9961, 0.0031
ABA	3.1	0.9924	R.S.D.	11%	0.3%

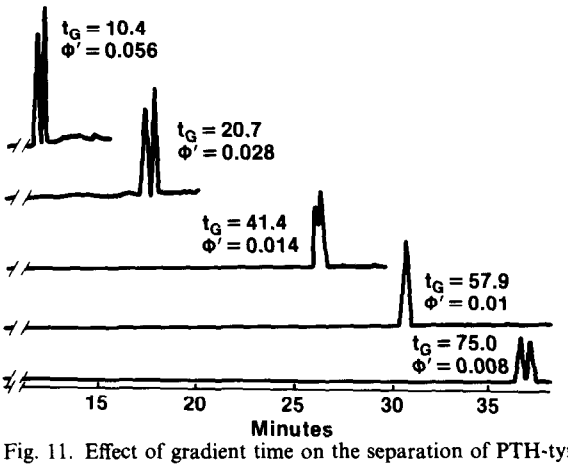

Fig. 11. Effect of gradient time on the separation of PTH-tyrosine and PTH-α-aminobutyric acid.

remain constant as gradient steepness was varied. It followed from the crossover of the plots in the isocratic mode that there might be a t_G that would cause coincidence in the gradient mode. Clearly, the asymptotic relationship of resolution with gradient time was confounded in this as well as the previous case. Mathematical expressions relating to hypothetical retention systems similar to these, *i.e.* compounds of unequivalent S or b values, have been developed previously[10].

Because the nature of the plots of isocratic log k' against φ influenced gradient results so markedly, the isocratic retention behavior of 24 PTH-amino acids was studied. S Values were acquired and computed as described above, and were listed in Table I in roughly the order in which the solutes were eluted during gradient mode. The range of S values was 3.1–4.9, while all plots were essentially linear. No correlation of the value of S to elution order was apparent. This non-uniformity of S

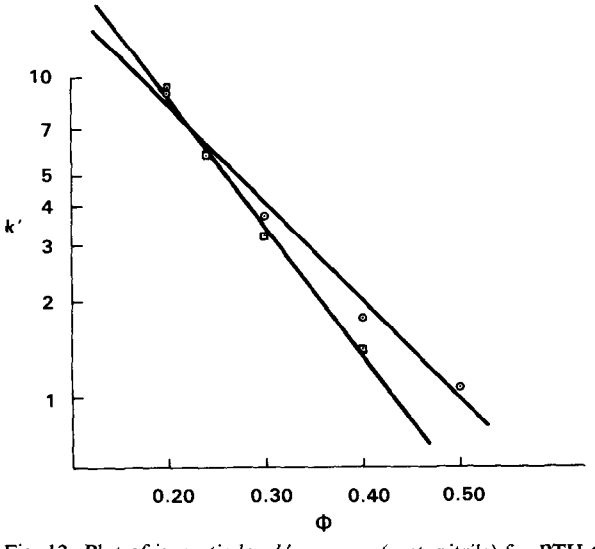

Fig. 12. Plot of isocratic log k' *versus* φ (acetonitrile) for PTH-tyrosine and PTH-α-aminobutyric acid.

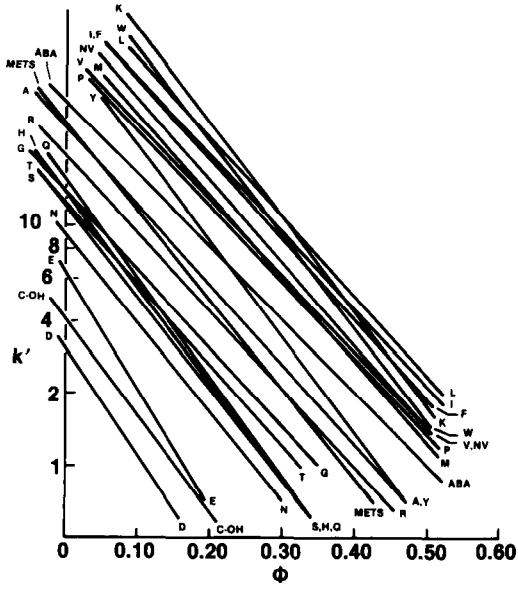

Fig. 13. Plot of isocratic log k' *versus* φ (acetonitrile) for 24 PTH-amino acids. D, aspartic acid; C–OH, cysteic acid; E, glutamic acid; N, asparagine; S, serine; T, threonine; G, glycine; H, histidine; Q, glutamine; R, arginine; A, alanine; METS, methionine sulfone; ABA, α-aminobutyric acid; Y, tyrosine; P, proline; V, valine; M, methionine; NV, norvaline; I, isoleucine; F, phenylalanine; L, leucine; W, tryptophan; K, lysine.

values for solutes of similar k' values in acetonitrile systems has been noted earlier[33]. Correlation to other retention and chemical parameters are currently under scrutiny for this and several other phase systems.

The span of S values is illustrated more graphically in Fig. 13. It was observed that there is an envelope of similar S values, associated with the plots being generally parallel. There are also a number of intersections and divergences, however, which confounded the simple approach of optimizing resolution by increasing gradient time, as discussed. On the other hand, these irregular retention behaviors have been exploited to fine-tune the separation of localized pairs selectively without disturbing the separation of the remaining solutes[37,38].

CONCLUSIONS

In compliance with the gradient elution model of Snyder[10], resolution of similarly retained PTH-amino acids generally showed an asymptotic dependence on gradient steepness, as controlled through the manipulation of gradient time. These solutes were characterized by isocratic log k' *versus* φ plots that were essentially parallel, resulting in constant α values during the course of the gradient. There were, however, selected pairs of these solutes for which the asymptotic relationship was not applicable. These solutes were characterized by plots that were distinctly not parallel. This resulted in a separation selectivity factor that varied during the course of the gradient and thereby confounded the asymptotic relationship. Insight into the variability of the gradient α values was gained through comparison of the S values of adjacent

solutes, derived from isocratic experiments. Overall, the plots illustrating the S values were reasonably linear ($r > 0.9895$) in the k' range 1–10. S values varied from 3.1 to 4.9. There was no observable correlation between S and elution order.

ACKNOWLEDGEMENT

The authors are grateful to Ms. Sandra Britton of IBM Instruments, for preparation of this manuscript.

REFERENCES

1 M. T. W. Hearn, *Adv. Chromatogr.*, 20 (1982) 1.
2 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
3 F. E. Regnier and J. Fausnaugh, *LC*, 1 (1983) 402.
4 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.
5 S. Stein and S. Udenfriend, *Anal. Biochem.*, 136 (1984) 7.
6 M. T. W. Hearn, F. E. Regnier and C. T. Wehr, *Amer. Lab.*, 10 (1982) 18.
7 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
8 M. W. Hunkapiller and L. E. Hood, in C. H. W. Hirs and S. N. Timasheff (Editors), *Methods in Enzymology*, Vol. 91, Academic Press, New York, 1983, pp. 486–493.
9 B. N. Jones and J. P. Gilligan, *J. Chromatogr.*, 266 (1983) 471.
10 L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography: Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, pp. 208–316.
11 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
12 J. W. Dolan, J. R. Gant and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31, and references cited therein.
13 P. Jandera and J. Churacek, *Adv. Chromatogr.*, 19 (1981) 125.
14 H. Engelhardt, *Z. Anal. Chem.*, 277 (1976) 267.
15 C. Liteanu and S. Gocan, *Gradient Elution Chromatography*, Wiley-Interscience, New York, 1974.
16 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 205 (1981) 13.
17 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
18 B. N. Jones, R. V. Lewis, S. Paabo, K. Kojima, S. Kimura and S. Stein, *J. Liquid Chromatogr.*, 3 (1980) 1373.
19 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
20 W. S. Hancock and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 71.
21 J. A. Bietz, *J. Chromatogr.*, 255 (1983) 219.
22 K. A. Cohen, *Ph.D. Thesis*, Northeastern University, Boston, 1982.
23 M. A. Phelan and K. A. Cohen, *J. Chromatogr.*, 266 (1983) 55.
24 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.
25 N. H. C. Cooke, B. G. Archer, M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
26 M. A. Quarry, R. L. Grob and L. R. Snyder, *J. Chromatogr.*, 285 (1984) 1.
27 M. A. Quarry, R. L. Grob and L. R. Snyder, *J. Chromatogr.*, 285 (1984) 19.
28 L. R. Snyder and J. J. Kirkland (Editors), *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 1979.
29 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
30 S. R. Bakalyar, R. McIlwrick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
31 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
32 A. P. Goldberg, *Anal. Chem.*, 54 (1982) 342.
33 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179.
34 C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 53 (1981) 2072.
35 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
36 M. C. Hennion, C. Picard, C. Combellas, M. Caude and R. Rosset, *J. Chromatogr.*, 210 (1981) 211.
37 J. W. Dolan, K. A. Cohen, G. K. Chao and S. A. Grillo, *Gradient Elution HPLC: A Comparison of Theory and Practice for Compounds of Biochemical Interest*, presented at the 187th ACS National Meeting, St. Louis, April, 1984.
38 *Improved PTH Amino Acid HPLC Separation System*, Applied Biosystems, Foster City, CA, 1984.