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DESIGN OF OPTIMIZED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC GRADIENTS FOR THE SEPARATION OF EITHER SMALL OR LARGE MOLECULES

I. MINIMIZING ERRORS IN COMPUTER SIMULATIONS

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SUMMARY

Computer simulations can be used to develop high-performance liquid chromatographic gradient elution methods. However, the usefulness of this approach depends on the accuracy of the resulting predictions. Possible sources of error in computer simulation for the prediction of separation based on gradient elution have been investigated. This has in turn led to recommendations for minimizing such errors. With suitable precautions it appears possible to make adequately reliable predictions of separation by gradient elution. Several examples with protein mixtures as samples are reported.

INTRODUCTION

Many samples are relatively complex and difficult to separate by means of high-performance liquid chromatography (HPLC), *e.g.*, mixtures containing a large number (20–30 or more) of major components of similar chemical structure. Such mixtures often require gradient elution. Separations of this type are usually carried out with simple linear gradients, but some samples benefit from the use of more complex gradient shapes. Thus the distribution of bands within the chromatogram may be uneven, suggesting steeper gradients in regions that are relatively empty of bands. Bands within the chromatogram may show increasing bunching for later-eluted compounds, and this favors the use of a curved (convex) gradient[1]. Finally, many samples exhibit pronounced changes in band spacing as the gradient steepness is

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varied[2–5]; such samples may be better separated using segmented (non-linear) gradients.

Gradients of optimal shape are in many instances not obvious, which means that a number of trial-and-error runs are usually necessary before an adequate separation can be achieved. Complex samples of the kind under discussion also often require relatively long run-times for adequate separation (*e.g.*, 1 h or more), which then means a substantial method-development effort. Previous work has shown that this process can be considerably accelerated (and better gradient methods developed) by the use of computer simulation[2–5]. Only two experimental runs are required (linear gradients in which only the gradient time is varied), and then a small computer (*e.g.*, IBM PC) can be used to explore the effects of different gradient conditions on the separation. The initial and final mobile phase compositions can be varied, the gradient time can be changed and gradients of any shape can be studied. Each simulated run requires only *ca.* 1 min or so to carry out and evaluate.

There are two limitations to this approach, however. First, accurate simulations (leading to optimal final conditions) depend on the HPLC equipment used and the choice of conditions for the two initial experimental runs. Significant errors in simulated chromatograms can result when inappropriate equipment or conditions are employed. Second, trial-and-error changes in the gradient can be an inefficient way to approach optimal final conditions. The success of this procedure (whether carried out experimentally or via computer simulation) depends markedly on the experience and insight of the chromatographer. Clearly, it would be helpful to have some rules or generalizations to more guide effectively the empirical optimization of gradient conditions. Finally, the need for (a) sufficiently accurate computer predictions in combination with (b) an effective strategy for optimizing the gradient appears to be greater for the case of higher-molecular-weight samples such as peptides and proteins, for reasons illustrated in the examples of refs. 5, 6 and 28.

In this paper, we explore the causes and effects of errors in computer-simulated predictions of gradient-elution separation. Experimental data relating to this issue will also be presented. The following two papers[5,6] examine the theory of how to design optimal gradients for different situations. Because the problems and opportunities associated with computer simulation are enhanced with macromolecular samples, we shall emphasize the use of gradient elution with this class of samples, particularly biological macromolecules such as peptides, proteins and oligonucleotides.

THEORY AND BACKGROUND

Errors in computer simulation

Retention in gradient elution can be related in a rigorous fashion to certain characteristics of the sample and to the experimental conditions[7–9]. This in turn allows the use of a small number of experimental runs to (a) measure these sample characteristics (parameters) and (b) predict retention as a function of any gradient conditions. However, these relationships apply for "ideal" systems, where all parameters are precisely measurable, the HPLC equipment functions in an ideal manner and certain complicating processes can be ignored (*e.g.*, changes in column performance with time).

In addition, it is convenient to make certain simplifying assumptions concerning

retention in the HPLC system under study. For reversed-phase HPLC (the subject of this paper), computer simulation as described here (DryLab G software) assumes that isocratic retention is given by

$$\log k' = \log k_w - S\,\varphi \qquad (1)$$

where k' is the capacity factor for a given compound when the volume fraction of organic component in the mobile phase (%B) is φ, k_w is the value of k' for water as mobile phase ($\varphi = 0$) and S is a constant for that compound (for fixed experimental conditions other than φ).

In the real world, we need to be concerned about various "non-ideal" effects and approximations such as eqn. 1, and to limit their impact so that resulting computer simulations are as reliable as possible. Previous papers have discussed various non-ideal conditions[10,11] and the failure of eqn. 1[12,13] as these relate to accuracy in computer predictions of retention. However, most of this earlier work has been concerned with isocratic–gradient relationships; *i.e.*, the simulation of gradient retention from starting isocratic runs, or *vice versa*. Here, we desire to simulate gradient retention on the basis of initial (experimental) gradient runs.

Table I summarizes several factors that can limit the accuracy of computer simulations for the prediction of gradient retention (assumes two experimental gradient runs to start). Several of these factors (and other questions that we shall address) can be conveniently studied through the use of computer simulations. As computer simulations (DryLab G) are based on "ideal" conditions, errors caused by variability in different experimental parameters can be assessed by repeating such simulations with different (erroneous) values of each parameter.

TABLE I

ERRORS IN COMPUTER-SIMULATED RETENTION TIMES AS A RESULT OF VARIOUS FACTORS

Factor	*Comment*
(1) Dwell volume, V_D	Error in V_D of $\pm$ 10–20% not important, except for bands eluted at the beginning of the chromatogram
(2) Mixing volume, V_M	Large V_M causes errors in predicted separation for segmented gradients
(3) Flow-rate, F	Error in F seldom has a significant effect on predicted separations
(4) Column dead volume, V_m	Error in V_m has little effect on predicted separations
(5) Change in retention due to change in column, temperature, etc.	The main problem is the change in column retention characteristics due to loss of bonded phase; accurate predictions of separation require that initial experimental runs be carried out within a 48-h period, and the column must be "broken in"
(6) Solvent demixing	Normally has a negligible effect on separation, particularly for large molecules
(7) Failure of eqn. 1	Can lead to errors in extrapolative predictions of separation; DryLab G guards against this possibility
(8) Error in t_g due to band overlap	Can cause appreciable errors in predicted separations
(9) Misassigned bands for initial experimental runs	Can cause major errors in predicted separation; a third run can be used to eliminate these errors
(10) Conformational change in protein molecules	Make sure that sample is fully denatured prior to separation

Dwell volume, V_D. The gradient retention time t_g depends on the volume (V_D) of the HPLC system between the inlet gradient mixer and the column (measured as described in ref. 9). We examined the effect of errors in V_D on computer simulation by using experimental data (two runs with a fifteen component herbicide sample; gradient times, t_G, of 30 and 90 min) plus DryLab G to predict the retention for both intermediate (t_G = 60 min) and extrapolated (t_G = 150 min) gradient times. The numerous band-spacing changes in this sample as a function of t_G (ref. 4) should make this computer simulation sensitive to errors in V_D.

Initial predictions were based on the correct (*i.e.*, "best") dwell volume for our HPLC system, equal to 5.5 ml. Then DryLab simulations were repeated assuming an (erroneous) V_D value of 6.5 ml. These results are compared in Table II. The effect of this +18% error in V_D on the predicted values of the retention time t_g (min) is an average error of −0.2% for the 60-min run and 0.5% for the 150-min run. Fig. 1a and b compare the resulting (simulated) chromatograms for correct *vs.* incorrect values of V_D for the 60-min run. The simulations in Table II also show a constant error of +8% in R_s for all band pairs (both 60- and 150-min runs) as a result of this +18% error in

TABLE II

ERRORS IN SIMULATED RETENTION AS A RESULT OF ERRORS IN DWELL VOLUME

Data for the fifteen-component herbicide sample described in ref. 4; 25 × 0.46 cm I.D. C_8 column; flow-rate, 2 ml/min; correct V_D value is 5.5 ml.

Band No.	*Retention time (min)*						
	5–80%B gradient				*40–80%B gradient*		
	t_G = *60 min*		t_G = *150 min*		t_G = *32 min*		
	V_D = *5.5*	V_D = *6.5**	V_D = *5.5*	V_D = *6.5**	V_D = *5.5*	V_D = *6.5**	*Error*
1	11.99	11.96	14.76	14.85	1.61	1.43	−0.2
2	13.91	13.88	17.87	17.98	1.85	1.60	−0.3
3	14.26	14.23	18.30	18.40	1.91	1.65	−0.3
4	15.50	15.45	21.01	21.16	1.97	1.69	−0.3
5	22.38	22.32	35.78	36.07	2.93	2.44	−0.5
6	24.59	24.53	39.42	39.70	3.35	2.74	−0.6
7	26.02	25.95	46.11	46.48	3.90	3.19	−0.7
8	27.13	27.06	47.49	47.85	4.21	3.55	−0.7
9	27.17	27.10	48.65	49.04	4.32	3.58	−0.7
10	27.94	27.87	49.66	50.03	4.61	3.83	−0.8
11	28.64	38.57	51.81	52.20	4.78	3.98	−0.8
12	30.49	30.42	53.42	53.77	6.88	6.22	−0.7
13	32.96	32.88	59.70	60.08	8.25	7.64	−0.6
14	36.29	36.21	67.65	68.04	10.61	10.1	−0.5
15	49.37	49.29	99.92	100.35	21.86	21.64	−0.2
Average error		−0.2%		−0.5%		−9.5%	

* Erroneous value of V_D.

V_D. As values of V_D will not normally be in error by this much, actual errors in the predicted values of t_g and R_s for every band pair should be adequately small. Similar results were obtained for an assumed (erroneous) value of V_D = 4.5 ml, rather than 6.5 ml.

For isocratic predictions from gradient data, it was shown previously that errors in values of V_D lead to generally larger errors in predicted values of retention time t_R[13] and resolution R_s[14]. Early bands in a gradient run can exhibit "pre-elution"[10], *i.e.*, isocratic migration under the influence of the volume V_D of the starting mobile phase when the starting %B in the gradient is sufficiently large. The predicted separation of such bands should therefore be more sensitive to errors in V_D. This is indeed so, as illustrated for the same herbicide sample in Fig. 1c and d. Here, a higher %B is used initially (40% *vs.* 5% in Fig. 1a and 5), and simulated chromatograms are compared for correct (5.5 ml) and incorrect (6.5 ml) values of V_D.

As seen in Table II, use of the incorrect value of V_D (6.5 ml) causes an average error in predicted retention times of 9.5%. The largest errors occur for bands that are eluted near $t_0 + t_D$, equal to about 4.0 min for the examples in Table II. The effect on the relative separation, however, is less significant, as can be seen by comparing Fig. 1c and d; the overall appearances of these two chromatograms are similar.

Mixing volume, V_M. The mixing volume, V_M, of a gradient HPLC system measures the tendency of the system to round the ends of the gradient as a result of mobile-phase dispersion during its passage between the mixer and column inlet. This is illustrated in Fig. 2a. As discussed in ref. 10, this rounding of the gradient is greater for

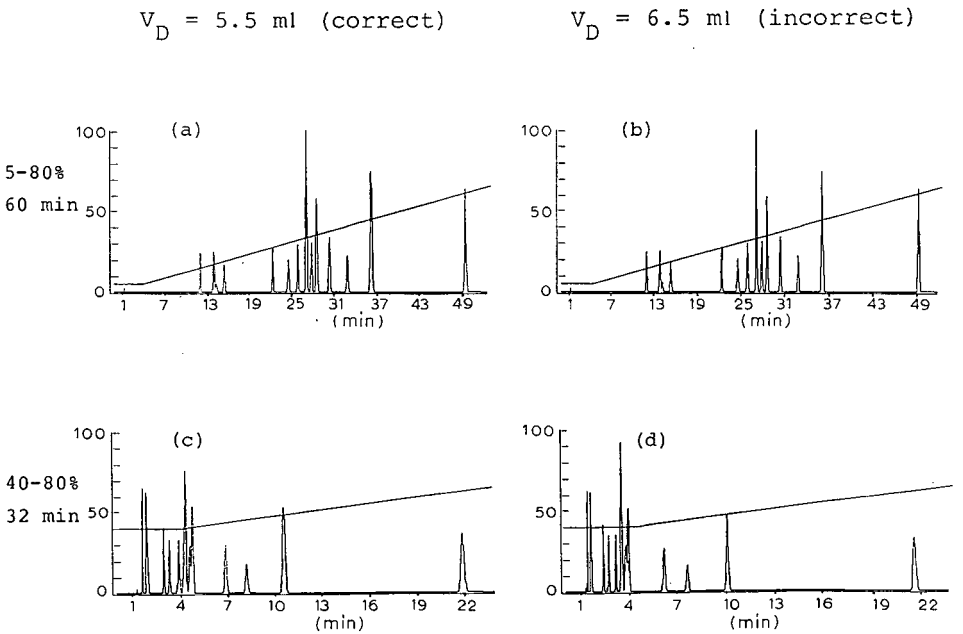


Fig. 1. Effect of error in the value of V_D on prediction of gradient elution separation. Sample, fifteen-component mixture of herbicides[4]; column, 25 × 0.46 cm I.D. Zorbax C_8; 5–80% acetonitrile–water gradients; flow-rate, 2 ml/min; gradient times t_G are indicated.

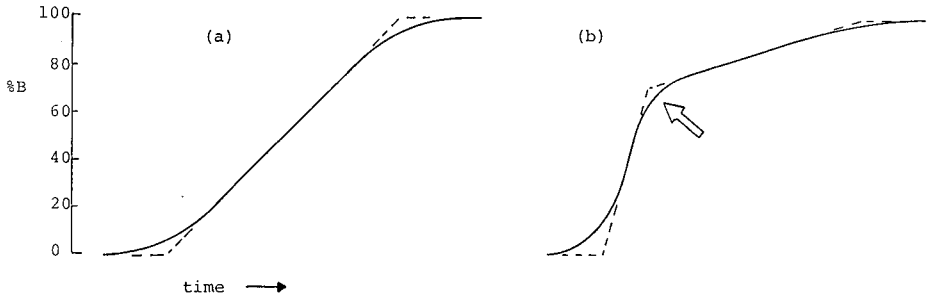


Fig. 2. Rounding of gradient due to a large mixing volume, V_M. (a) Linear (unsegmented) gradient; (b) segmented gradient.

larger values of V_M, and is smaller for larger values of $t_G F$, where F is the flow-rate. As rounding of the gradient primarily affects the retention of bands at the beginning and end of the gradient, mixing-volume effects can be minimized by starting the gradient earlier and ending it later (smaller values of φ at the beginning of the gradient and larger values of φ at the end). For linear gradients and modern HPLC equipment (with values of $V_M < 2$ ml), in most instances gradient rounding is not a serious contribution to errors in predicted values of t_g. This is discussed more fully elsewhere[15]. One conclusion that emerges from the above discussion, however, is that the computer simulations of gradient runs will generally be less accurate for early bands in the chromatogram.

Rounding of the gradient as in Fig. 2a can also affect the retention of bands that are eluted in the middle of the chromatogram when segmented gradients are used (Fig. 2b). This can in turn lead to errors in computer-simulated separations. As discussed in the following paper[5], however, the resulting errors in the prediction of retention time should be smaller than for initial rounding of the gradient.

Mobile phase flow-rate, F. The effect of errors in flow-rate, F, on gradient retention was discussed in ref. 10. Errors in F can arise from faulty pumping or from inadequately compensated mobile phase compression effects, and also from errors in selecting the correct flow-rate. The separation of biological samples such as peptides and proteins often involves lower flow-rates and lower operating pressures, and for these conditions errors in flow-rate will usually be much less than 1%.

We can derive an equation relating errors in t_g (δt_g) to errors in flow-rate. The gradient retention time t_g is given approximately by[7–9]

$$t_g = (t_0/b)\,[\log(2.3k_0 b)] + t_0 + t_D \tag{2}$$

where

$$b = V_m \Delta\varphi\, S/t_G\, F \tag{3}$$

(see below and the Glossary of Symbols for the definitions of these commonly used terms). Combining eqns. 2 and 3 yields

$$t_g = (t_G/\Delta\varphi S)\,[\log(2.3k_0) + \log(V_m\, \Delta\varphi S/t_G F)] + (V_m + V_D)/F \tag{4}$$

Differentiating eqn. 4 with respect to F gives the error in t_g (δt_g) as a function of error in F (δF):

$$\delta t_g = -\{[t_G/(2.3\ \Delta\varphi\ S\ F)] + [(V_m + V_D)/F^2]\}\ \delta F \quad (5)$$

For a typical example involving a protein separation, we might have the following conditions (corresponding to the 60-min 30S ribosomal-protein runs in this study): gradient time t_G = 60 min, gradient range $\Delta\varphi$ = 0.2, S = 30, F = 0.7 ml/min, column dead-volume V_m = 2.5 and V_D = 5.5 ml. A 1% error in F (δF = 0.007) would then result in an error in t_g of 0.2 min, or about 0.4% for the average band. However, this error will be about the same for all bands in the sample (assuming the S values are roughly constant), meaning that little change in relative retention will result. Typical errors in flow-rate (see ref. 10) should therefore have little impact on predicted t_g values from computer simulation, and a negligible effect on predicted values of R_s.

Column dead volume, V_m. Our computer-simulation software (DryLab G) assumes that V_m equals a constant fraction of the total column volume (62%). This fraction can actually vary by ±10–20% for different columns, leading to potential errors in simulated t_g values. Simulations as in Table II were therefore repeated for a change in V_m of +20%. The effect on predicted values of retention and resolution (for t_G = 60 and 150 min, as in Table II) was negligible: a 0.1–0.3% decrease in retention time and a 1–3% increase in resolution. It can be concluded that errors in our assumed value of V_m (based on a 62% void volume) can be ignored. Similar conclusions have been drawn for the effect of error in V_m on the prediction of isocratic retention from gradient data[13]. This suggests that errors in V_m will have little effect on t_g values for early bands (unlike the errors in V_D; see above).

Change in sample retention. For a given separation (fixed conditions), sample retention can vary as a result of unintended changes between replicate runs: uncontrolled temperature, changes in the column with continued use, errors in assigning various parameters (flow-rate, gradient conditions, etc.). In a previous study[10,11] most of these effects have been analysed in detail; it was shown that such errors are not usually significant insofar as separation is concerned. However, that study was based on neutral sample molecules (dialkylphthalate) and unmodified organic–water mixtures as the mobile phase.

In reversed-phase separations of peptide and protein samples, low-pH mobile phases containing buffers or ion-pairing agents are often used. These mobile phases typically degrade the column by removing the alkyl-bonded phase[16,17], so that sample retention tends to change during use; usually the retention of all compounds decreases with time. Changes in sample retention can lead to errors in computer simulations. If the retention changes between the two initial experimental runs used in computer simulation, the derived sample parameters (S, k_w) will be in error, as will subsequent predictions of retention. If the retention changes after the two initial runs, predictions from computer simulation will not match the corresponding experimental runs.

Solvent demixing. This refers to uptake by the column of organic solvent during the gradient[11]. Errors due to this effect are greater for high-surface-area columns and for steep gradients. Neither of these conditions is likely in typical protein separations*

* Separations of proteins generally employ wide-pore packings that have low surface areas; similarly, the large S values found for proteins require relatively flat gradients for acceptable values of b (eqn. 3).

and this effect can therefore be considered unimportant. Even for small-molecule samples and steep gradients, errors due to solvent demixing are unlikely to have much effect on predictions of separation via computer simulation, because these errors cancel for adjacent bands.

Failure of eqn. 1. In several studies it has been observed that larger peptides and proteins obey eqn. 1 within experimental error[18–20], whereas other studies have

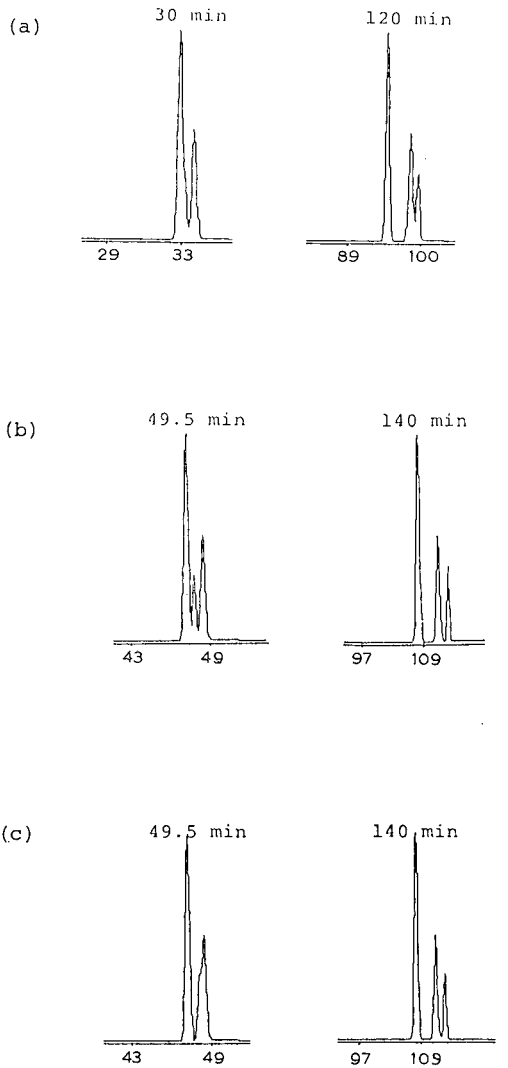


Fig. 3. Effect of error in input t_g values on accuracy of predicted chromatograms; hypothetical example of Table III (see text). (a) Input data; (b) simulated chromatograms for optimal gradient times (49.5 and 140 min) predicted from incorrect t_g value for band 2 (32.91 min in 30-min run); (c) simulated chromatograms as in (b), except correct value of t_g (33.19 min) used as input.

reported modes curvature in plots of log k' *vs.* φ for peptide and protein samples[21,22]. The general effect of log k'–φ curvature on computer simulation by DryLab G software has been examined for small-molecule samples[4,12,13], with the conclusion that significant errors are not introduced in interpolative predictions of t_g. Extrapolative predictions must be treated with greater caution, however.

Error in retention times due to band overlap. This problem is illustrated in Fig. 3 and Table III. Two experimental runs are shown (simulations) for t_G equal to 30 and 120 min. Two bands overlap in the 30-min run (t_g = 32.91 and 33.19 min), whereas all three bands are resolved in the 120-min run. The usual procedure in this instance is to use the observed ("average") retention time (32.91 min) for each of the two overlapping bands, as the correct retention times for each of the two bands are not known. The use of this procedure, followed by trial-and-error computer simulations, led to the prediction that acceptable separations could be obtained either with t_G = 49.5 or 140 min ("optimized simulations" in Fig. 3). However, repeating these simulations with correct values of t_g for all bands (Table III) gave the "experimental" runs in Fig. 3 (corresponding to what would have been observed if actual experiments had been carried out).

It is clear from Fig. 3 and Table III that significant errors in the computer simulations have resulted because of the initial errors in t_g (bands 1 and 2, 30-min run). This particular example represents an extreme case, corresponding to significant resolution in the overlapped band pair, but just short of the resolution required to measure t_g for the second band. The actual resolution is R_s = 0.6 for bands 1 and 2 in the 30-min run, with a 4:1 ratio for the band areas of the two compounds. The relative error in the predicted resolution of band 2 is greatest for small changes in retention as a result of changing t_G, *e.g.*, 49.5-min run (simulated) *vs.* 30-min run (experimental). Means for dealing with this kind of error in computer simulation are discussed under Results and Discussion.

Misassigned bands. Computer simulation requires that the bands in the second

TABLE III

EFFECT OF RETENTION ERRORS DUE TO BAND OVERLAP ON COMPUTER SIMULATIONS

Examples are simulated by DryLab G. Conditions: dwell volume, 5.5 ml; 25 × 0.46 cm I.D. column; flow-rate, 1 ml/min; 5–100%B gradient; three bands.

t_G (min)	Retention time, t_g (min)			Resolution, R_s	
	Band 1	Band 2	Band 3	Bands 1 and 2	Bands 2 and 3
30	32.91	33.19	33.71		
120	95.13	99.70	98.60		
49.5*	47.23	47.91	48.59	1.2	1.2
49.5**	47.23	48.20	48.59	1.7	0.7
140*	108.0	114.0	112.1	3.1	1.6
140**	108.0	113.8	112.1	3.1	1.4

* Simulation based on incorrect value of t_g (32.91 min) for band 2 in 30-min run.

** Simulation based on correct value of t_g (33.19 min) for band 2 in 30-min run.

experimental run be matched with those in the first run, *i.e.*, if compound A is responsible for band 1 in run 1, it is necessary to identify the band in run 2 that contains compound A (and so on for all other bands). In some instances this presents no difficulty, because band size and relative retention make these assignments obvious. When this is not so, it may be necessary to run a third experiment (of intermediate gradient time) and compare this chromatogram with that predicted by computer simulation from the first two runs. Errors in band assignment will usually be obvious from such a comparison[3–5].

Changes in protein conformation during separation. Several studies have shown that the tertiary structure of a protein molecule can change during reversed-phase HPLC[23,24]. In principle, such changes in conformation could lead to errors in predicted separations based on computer simulation. Changes in conformation during HPLC can be minimized by experimental conditions that favor denaturation of the sample, and such conditions also favor the improved separation of most protein samples[23,24].

EXPERIMENTAL

Equipment and software

The HPLC system was a DuPont 8800 liquid chromatograph, consisting of an 8800 gradient controller, an 870 pump, and two 862 variable-wavelength detectors connected in series for detection at both 214 and 280 nm (DuPont, Wilmington, DE, U.S.A.). The column compartment was equipped with a manual Rheodyne injection valve fitted with a 50-μl loop. A 5.0 × 0.46 cm I.D. precolumn was positioned between the pump and injector, and an in-line 2-μm filter was placed between the injector and analytical column. The dwell volume of the system was equal to 6.8 ml for detection by the second detector (at 214 nm; all chromatograms shown here were monitored at 214 nm). Analog data were digitized and archived by a Nelson Analytical Series interface (Nelson Analytical, Cupertino, CA, U.S.A.). Computer simulations were carried out using DryLab G software (LC Resources, Lafayette, CA, U.S.A.).

Materials

Reagents and solvents. Acetonitrile was of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Water was deionized and further purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Trifluoroacetic acid (TFA) was of Sequanal grade (Pierce, Rockford, IL, U.S.A.). Triethylamine (TEA) was Gold Label grade (Aldrich Chemical, Milwaukee, WI, U.S.A.).

Columns. Most of the separations for the ribosomal proteins were carried out on 25 × 0.46 cm I.D. Zorbax BioSeries Protein PLUS columns (DuPont). In earlier studies (Table V), columns from other suppliers (Synchrome, West Lafayette, IN, U.S.A.; Separations Group, Hesperia, CA, U.S.A.) were also used (see ref. 6 for details).

Samples. Ribosomal protein samples were prepared from *E. coli* bacteria as described by Kerlavage *et al.*[25]; see ref. 28 for further details.

Procedures. All gradients were formed from water plus acetonitrile [containing 0.085% (v/v) TFA and 0.1% (v/v) TEA, pH 2.8] plus acetonitrile. Columns were re-equilibrated by running a 10-min reversed gradient, followed by at least 20 min of the starting mobile phase. The flow-rate was 0.7 ml/min in all instances.

RESULTS AND DISCUSSION

Comparison of experimental and predicted retention for several protein samples

30S ribosomal protein sample. This sample contains a total of 21 proteins having molecular weights between 8000 and 28 000 Da. In the following two papers[5,6] and elsewhere[28] we have presented examples of computer simulation for the 30S ribosomal proteins; the predicted separations closely match experimental runs based on the same (reversed-phase) gradient conditions. These examples[5,6,28] illustrate our current ability to use computer simulation for protein samples in place of trial-and-error experimental runs.

It is instructive to review a number of other (earlier and unreported) comparisons that were made in our laboratory, before we fully appreciated what steps are necessary to achieve accurate computer simulations (see the following section for recommendations). Over a period of several months, we carried out separations of the 30S ribosomal proteins on several different columns, but with generally similar gradient conditions (26–46% acetonitrile–water gradients, 0.7 ml/min; see Experimental). In each instance gradient runs were repeated for gradient times of 60, 120 and 240 min. It was therefore possible to compare the ability of computer simulation (based on two of these runs) to predict the results of the third run. Previous studies[4,13] suggest that interpolative predictions will generally be more reliable than extrapolative simulations, *i.e.*, prediction of the 120-min run from the 60- and 240-min runs will be more accurate than prediction of the 240-min run from the 60- and 120-min runs. Likewise, the use of two initial runs differing in gradient time by a larger factor (*e.g.*, four-fold for 60- and 240-min runs) will also allow more accurate simulations[12]. The gradient equipment was also checked[10] to ensure satisfactory operation.

Table IV illustrates the kind of accuracy that we have observed for simulated runs when appropriate precautions are taken. In this series of runs, we were able to recognize 17 distinct bands. The 60- and 240-min experimental runs were used to predict retention times for the 120-min run. Table IV lists experimental (expt.) and predicted (calc.) retention times (t_g); retention-time differences for adjacent bands ($t_j - t_i$ for bands i and j) are also listed. Values of $t_j - t_i$ are proportional to resolution; predicted values of this quantity must therefore be reliable within $\pm$10–20% if computer simulation is to be useful for method development.

We see from the example in Table IV that retention times are predicted with acceptable accuracy ($\pm$0.2 min or $\pm$0.5%). This is better (by a factor of about 5) than the average accuracy of similar predictions in the case of small-molecule sample[4]. One reason is that gradient retention times for large molecules (having large S values) vary much less with changes in the experimental conditions. Other factors being equal (similar gradient-slope values b), a change in k' (or k_0) during gradient elution (due to a change in experimental conditions) causes a change in t_g (δt_g) that is proportional to $1/S$.

Retention time differences ($t_j - t_i$) are also accurately predicted in Table IV ($\pm$0.2 min or $\pm$5%). When two bands are close together (barely resolved), as with bands 15 and 16 or 16 and 17, the relative error in $t_j - t_i$ is generally larger.

Table V summarizes similar comparisons as in Table IV for eleven different sets of runs. These experiments were carried out over a period of about 1 year and involved six different columns. During most of this time we ignored the potential problems

caused by change in column retention characteristics with time. In some instances, these data for computer simulation were carried out on new columns, prior to conditioning the column (by injecting protein samples until constant retention and recoveries were observed for a given sample). In other instances, the three runs for computer simulation were not completed within a period of 48 h. It is instructive to compare the accuracy of computer simulation as a function of these variables; this information is provided in Table V.

The format of Table V requires comment. Consider run series G (which is detailed in Table IV) as an example. The designations "NO"–"NO" indicate that the column was conditioned before use, and all three runs for comparison were carried out within a period of 2 days. The error in predicted values of t_g is 0.2 ± 0.3 min, meaning that the average error was +0.2 min, and the random scatter around this value was 0.3 min (1 S.D.). The relative standard deviation of all t_g values *vs.* experimental values was 0.5%. The relative standard deviation of predicted values of $t_j - t_i$ was 5%. These values may be compared with the results in Table IV.

If we group the results in Table V according to whether the three runs were carried out within a 2-day period and/or a new column was used, we obtain the results

TABLE IV

COMPARISON OF PREDICTED *VS.* ACTUAL RETENTION TIMES FOR 30S RIBOSOMAL PROTEIN SAMPLE (DRYLAB SIMULATION)

Run series G of Table V; 60- and 240-min runs used to predict 120-min run. Column, 25 × 0.46 cm I.D. BioSeries Protein PLUS; flow-rate, 0.7 ml/min; 26–46% acetonitrile–water gradients.

Band No.	t_g *(min)*		*Error (min)*		*Error (%)*	
	Expt.	*Calc.*	t_g	$t_j - t_i$	t_g	$t_j - t_i$
1	22.0	22.9	0.9	−0.6	4.0	5
2	33.7	34.0	0.3	0.0	0.9	0
3	47.0	47.3	0.3	−0.2	0.6	3
4	53.4	53.5	0.1	−0.1	0.2	2
5	58.5	58.5	0.0	0.0	0.0	0
6	60.1	60.1	0.0	0.1	0.0	8
7	61.0	61.1	0.1	−0.1	0.2	5
8	63.3	63.3	0.0	0.0	0.0	0
9	67.1	67.1	0.0	0.1	0.0	1
10	80.7	80.8	0.1	−0.3	0.1	12
11	83.2	83.0	−0.2	0.6	0.2	–
12	83.2	83.6	0.4	0.2	0.5	7
13	86.5	86.5	0.0	−0.2	0.0	2
14	100.2	100.0	−0.2	0.4	0.2	–
15	100.2	100.6	0.4	−0.5	0.4	36
16	101.6	101.5	−0.1	0.3	0.1	18
17	103.3	103.5	0.2	–	0.2	–
Average absolute error			±0.2	±0.2	±0.5	±5

TABLE V

COMPARISON OF PREDICTED *VS.* ACTUAL RETENTION TIMES FOR 30S RIBOSOMAL PROTEIN SAMPLES (DRYLAB SIMULATION)

Summary of several run series as in Table IV (60- and 240-min runs used to predict 120-min run). Conditions similar to those in Table IV, except for the use of different columns. See text for further details.

*Column**	*New column?***	*Runs in >2 days?****	*Errors in retention (min, ±1 S.D.)*	
			t_g	$t_j - t_i$
A	NO	YES	0.6 ± 0.5 (±1.4%)	±7
B	NO	NO	−0.1 ± 0.2 (±0.4%)	±3
C	YES	NO	0.8 ± 0.3 (±1.6%)	±10
D	YES	YES	0.8 ± 0.4 (±1.7%)	±8
E	NO	YES	0.8 ± 0.4 (±1.6%)	±8
F	NO	YES	0.7 ± 0.6 (±1.9%)	±6
G	NO	NO	0.2 ± 0.3 (±0.5%)	±5
H	NO	YES	0.9 ± 0.6 (±2.1%)	±6
I	NO	YES	0.4 ± 0.3 (±0.8%)	±8
J	YES	YES	4.0 ± 1.6 (±8.4%)	±12
K	NO	NO	−0.1 ± 0.2 (±0.4%)	±5

Summary:

Run conditions	*Error in predicted data (%)*	
	t_g	$t_j - t_i$
Conditioned column; all runs completed within 2 days	±0.4	±4
Unconditioned column, or completion of all runs required >2 days	±1.3	±7
Unconditioned column and completion of all runs required >2 days	±5	±11

* See Experimental section for columns.
** "NO" indicates that the column was conditioned before use for these runs.
*** "NO" indicates that the three runs for this comparison were carried out within a 48-h period.

summarized at the bottom of Table V. These summary results clearly demonstrate the need to condition the column prior to computer-simulation experiments, and to complete these runs within a 2-day period. However, the data in Table V also depend on the stability of the HPLC column under the conditions of separation. In later studies, using a newly developed, more stable column for reversed-phase protein HPLC (BioSeries Protein PLUS; DuPont), we experienced fewer problems with time-dependent changes in column retention.

Table V also shows that good comparisons between predicted and experimental gradient separations are possible when the above precautions are taken. However, it is expected that once a gradient method has been developed, it will be applied (with the same or equivalent column) over a period of weeks or months. The data in Table V suggest that during this time sample retention will change. The obvious question is

then the effect that this will have on separation. If the separation changes enough over a period of about 1 week to require redevelopment of the method, this could be a serious problem.

The data in Table V show a tendency toward shifts in retention that are larger than random variations in retention. This suggests that whereas shifts in retention do occur with continued use of the column, changes in relative retention (and resolution) will be smaller. This is borne out by the variation in values of $t_j - t_i$, which change more slowly with continued use of the column than do values of t_g, *e.g.*, for "NO–NO" *vs.* "YES–YES" cases in Table V (summary at the bottom of the table), variation in t_g increases 12-fold (5% *vs.* 0.4%), but the $t_j - t_i$ values show only a 3-fold increase in variability (11% *vs.* 4%).

If loss in resolution is experienced with continued use of a given column, it is possible to use computer simulation to modify the method. In the simplest case, the original input runs for computer simulation can be used to predict how the gradient should be modified to pull two overlapping bands apart. If a significant loss of resolution is experienced for several band pairs, two new runs (*e.g.*, with $t_G = 60$ and 240 min) can be carried out and the method redeveloped from the beginning*.

Runs B and G in Table V were carried out under conditions that should insure reliable predictions by computer simulation. It was of interest to examine the accuracy obtained when predicting retention for the 240-min run by using the 60- and 120-min runs as inputs for DryLab G (extrapolative prediction). When this was done, the same accuracy in the predicted values of t_g ($\pm 0.45\%$) and $t_j - t_i$ ($\pm 4\%$) was observed. In this instance, it is seen that comparable accuracy in computer predictions is obtained in either the 120-min or 240-min run (suggesting that curvature in the plots of log k' *vs.* φ in this system is minimal).

The accuracy of computer simulation for early eluted bands was also studied by starting the gradient at a higher acetonitrile concentration (as in Fig. 1c). Two runs with 26–46% actonitrile and gradient times of 60 and 240 min were used as inputs for computer simulation. Under these conditions, no bands showed significant pre-elution. Gradient steepness was next maintained constant (0.33%B/min), while the starting %B was varied from 21 to 41%. These experiments are summarized in Fig. 4, together with corresponding predicted separations from computer simulation. Experimental and predicted retention times are also summarized in Table VI. It is seen in Table VI that the overall precision of computer simulation for these examples (all bands) is about ± 0.4 min in retention time. However, bands pre-eluting near $t_0 + t_D = 13.4$ min ($8 < t_g < 20$ min; indicated by triple asterisks in Table VI) show about a 3-fold poorer agreement (± 1.4 min). This is not surprising, for the reasons discussed above. However, the effects of these errors on the predicted separations in Fig. 4 are seen to be of minor significance.

50S ribosomal protein sample. This sample contains a total of 32 proteins with molecular weights between 5000 and 30 000 Da. Several runs were carried out initially, varying only the gradient time. These data (summarized in Table VII) allow further comparisons of the accuracy of computer simulation for protein samples. Two runs with gradient times of 192 and 768 min (21–69% acetonitrile–water gradients) were

* This assumes that excess band broadening is not observed. If it does occur, the column may have to be replaced.

TABLE VI

EXPERIMENTAL *VS.* COMPUTER-SIMULATED RETENTION TIMES FOR GRADIENT RUNS WITH VARYING INITIAL %B (DRYLAB SIMULATION)

Data for 30S ribosomal protein sample. Conditions as in Table IV, except for gradient time and range.

*Band No.**	*Retention time (min)***							
	21–51%		*31–51%*		*36–56%*		*41–61%*	
	Expt.	*Calc.*	*Expt.*	*Calc.*	*Expt.*	*Calc.*	*Expt.*	*Calc.*
1	40.5	40.0	5.6	5.5		3.7		3.7
2	46.9	46.4	10.8	12.7***		4.1		3.7
3	51.9	51.5	20.2	20.7***		4.3		3.7
4	54.6	54.3	23.9	23.9		4.9		3.7
5	56.5	56.2	26.1	26.0		5.5		3.7
6	57.0	56.7	26.6	26.5		5.9		3.7
7	57.0	57.7	26.6	26.6	6.3	6.4		3.7
8	58.7	58.4	28.3	28.3	7.0	7.4		3.7
9	61.0	60.8	30.7	30.7	9.4	11.0***		3.8
10	68.2	67.8	37.9	37.8	21.1	22.1		4.1
11	68.8	68.4	38.7	38.4	23.2	23.0		4.6
12	68.8	68.4	38.7	38.4	23.2	23.3	5.6	5.2
13	70.1	69.7	39.9	39.7	25.0	24.3	5.6	5.2
14	70.7	70.3	40.6	40.3	25.0	25.1	5.6	5.5
15	71.6	71.1	41.1	41.1	25.8	25.9	6.1	5.8
16	78.7	78.1	48.4	48.1	33.1	33.0	14.4	15.8***
17	78.7	78.1	48.4	48.1	33.1	33.1	14.4	15.8***
18	80.1	79.4	49.7	49.4	34.4	34.4	17.1	18.1***
19	85.4	85.1	55.2	55.1	40.0	40.1	25.0	25.0
Average error:								
All bands	±0.4 min		±0.3 min		±0.3 min		±0.6 min	
Bands marked with	±0.6%		±0.9%		±1.3%		±4.1%	
triple asterisks			±1.2 min		±1.6 min		±1.3 min	
			±8%		±17%		±9%	

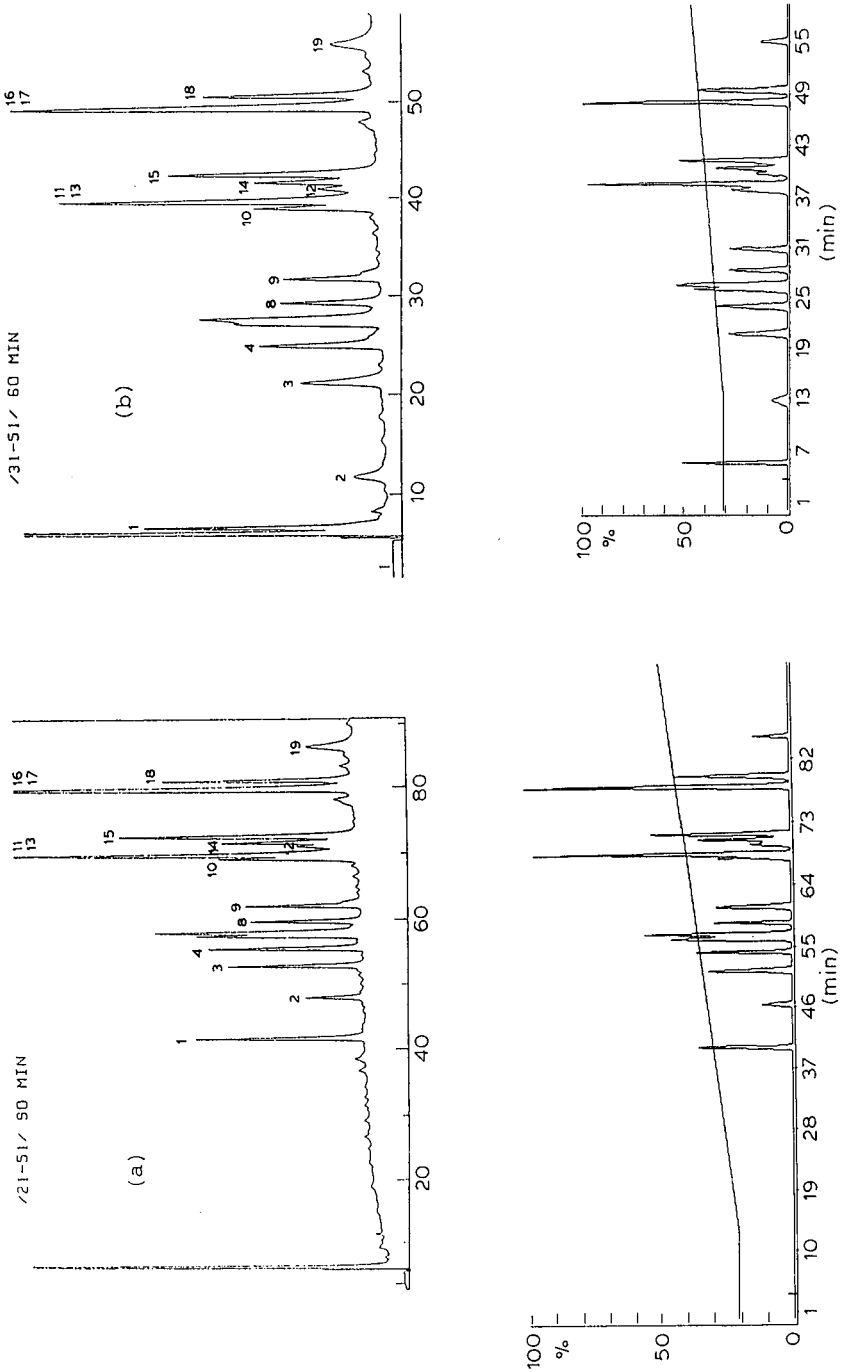
* Bands numbered in order of retention; band 1 is the second band in the chromatogram (the first band is omitted).

** 21–51% run has t_G = 90 min; other runs have t_G = 60 min.

*** Bands eluted within 8–20 min, *i.e.*, close to $t_0 + t_D$ = 13 min.

used as input data for computer simulation. The experimental retention-time data for several runs of intermediate t_G value are compared with computer-simulated values in Table VII, with generally good agreement (±0.8%). Predictions of average resolution are seen to be adequate (±4% overall).

In the following paper[5], a four-segment gradient for the optimized separation of this sample is described. Experimental and predicted retention times for this run agreed within ±0.5% (±0.8 min) and the average resolution agreed within ±8%.


/21-51/ 90 MIN
(a)
/31-51/ 60 MIN
(b)
100
%
50
0
(min)

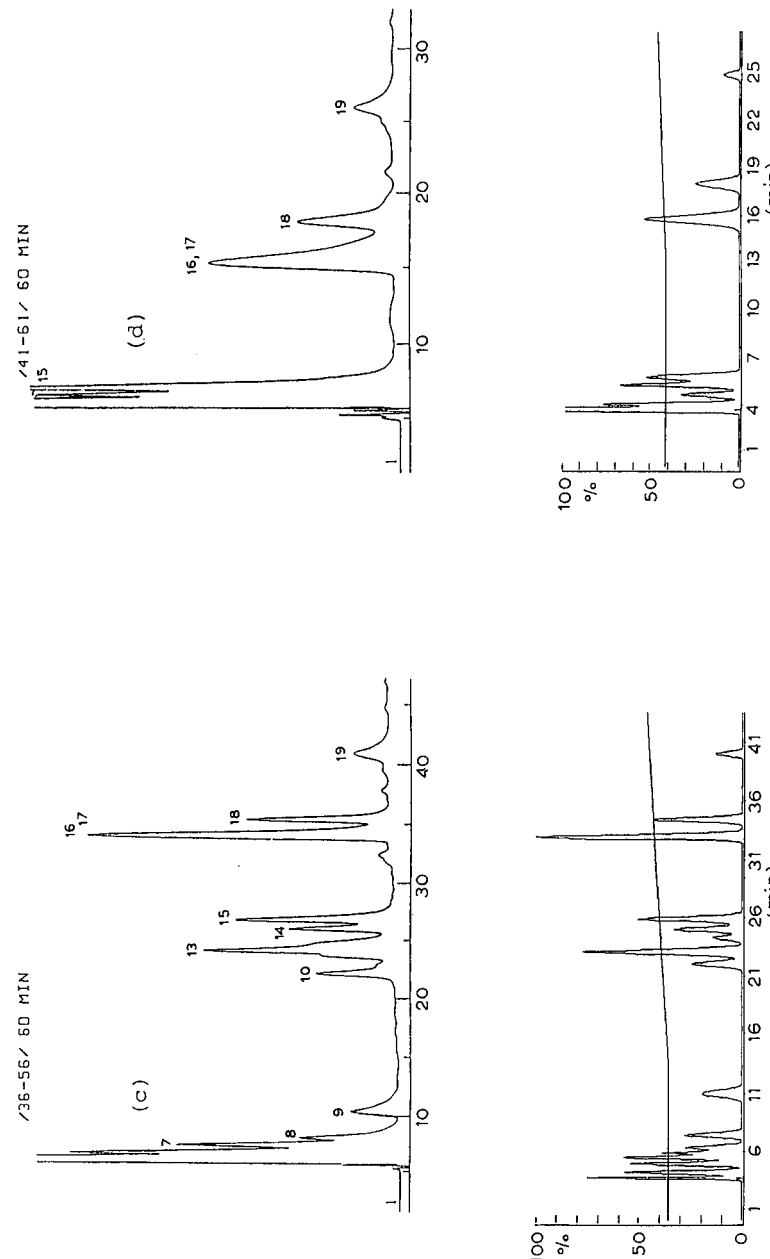


Fig. 4. Comparison of experimental and simulated chromatograms for 30S ribosomal sample (initial %B varying). Gradient steepness held constant (3 min/%); flow-rate, 0.7 ml/min. Input data for 26–46% B gradients; t_G = 60 and 240 min.

TABLE VII

COMPARISON OF PREDICTED *VS.* ACTUAL RETENTION TIMES FOR THE 50S RIBOSOMAL PROTEIN SAMPLE (DRYLAB SIMULATION)

Conditions as in Table IV, except for gradient range and time; 21–69% acetonitrile–water gradients. Experimental data for runs with t_G = 192 and 768 min (0.7 ml/min) were input into DryLab G.

*Band No.**	*Retention time (min)***							
	240 min		*288 min*		*480 min*		*576 min*	
	Expt.	*Calc.*	*Expt.*	*Calc.*	*Expt.*	*Calc.*	*Expt.*	*Calc.*
1	30.4	30.1	31.8	32.2	37.8	38.9	41.3	41.6
2	44.5	44.5	48.6	49.5	65.7	68.2	73.8	76.8
3	66.0	66.0	74.8	75.5	109.0	111.9	126.1	129.5
4	70.6	70.4	79.9	80.6	117.3	120.2	135.8	139.3
5	81.1	81.1	92.8	93.4	138.5	141.2	161.6	164.5
6	82.1	82.3	94.1	94.8	140.4	143.3	164.0	166.8
7	87.4	87.4	99.7	100.6	148.5	151.5	173.2	176.0
8	90.3	90.4	104.1	104.6	156.4	159.4	184.4	186.0
9	90.3	90.7	104.1	105.0	157.9	161.1	186.3	188.5
10	93.1	93.4	107.6	108.5	164.2	167.5	194.5	196.4
11	94.7	95.3	109.6	110.4	166.3	169.5	196.4	198.3
12	95.6	95.5	110.3	111.0	168.9	171.8	200.0	201.6
13	98.7	98.7	114.5	114.3	172.3	174.9	203.5	204.4
14	97.7	97.9	113.1	113.9	173.9	176.8	206.4	207.7
15	98.7	99.2	114.5	115.4	175.9	178.8	208.8	209.9
16	100.6	101.1	116.3	117.3	178.1	181.0	210.4	212.1
17	105.6	106.0	122.6	123.3	188.4	190.9	223.6	224.0
18	107.8	108.2	125.1	126.0	192.9	195.4	228.8	229.4
19	113.4	113.3	132.3	132.1	204.0	205.3	242.4	241.1
20	113.4	113.5	132.3	132.4	204.0	206.0	243.6	243.0
21	114.5	114.9	132.3	134.2	207.1	210.1	246.8	247.4
22	121.7	121.8	141.0	142.2	219.4	221.8	261.0	260.8
23	121.6	122.2	141.0	142.9	221.7	224.6	265.0	264.8
24	127.8	128.2	149.2	150.5	235.9	238.8	283.3	282.5
25	135.8	136.7	158.3	159.9	247.5	250.5	295.4	294.9
26	141.3	142.3	165.1	167.0	260.6	264.5	313.1	312.7
27	145.2	146.3	169.8	171.6	267.7	271.4	321.0	320.5
28	173.3	174.7	203.3	204.7	319.5	321.6	380.3	378.6
29	178.7	180.2	209.7	211.1	329.7	331.4	391.9	390.1
Average error, all bands:								
t_g	±0.4 min		±1.0 min		±2.6 min		±1.5 min	
	±0.4%		±0.8%		±1.4%		±0.7%	
R_s***	±5%		±4%		±3%		±5%	

* Bands numbered in order for 768-min run; one band pair overlaps in all gradients; two other bands omitted.

** Gradient times indicated for each run.

*** Average absolute error in $t_j - t_i$ divided by average value of $t_j - t_i$.

TABLE VIII

COMPARISON OF PREDICTED *VS.* ACTUAL RETENTION TIMES FOR IL-2 MUTEINS AND OXIDIZED DERIVATIVES (DRYLAB G SIMULATIONS)

Column, 25 × 0.46 cm I.D. C_8; 35–60% acetonitrile–water gradients (0.1% TFA); flow-rate, 2 ml/min. Data from ref. 26.

*Compound**	*Retention times, t_g (min), for indicated gradient times*				
	Experimental values			*Calculated values*	
	20	*40*	*80*	*40***	*80****
Ala^{1}Cys^{125}:					
M/S	20.02	33.18	57.43	33.28	57.02
O/S	21.04	35.03	61.06	35.21	60.34
M/O	22.18	36.30	62.50	36.71	60.87
O/O	23.56	38.99	67.78	39.41	66.11
Ala^{1}Ser^{125}:					
M/S	18.08	29.60	50.87	29.70	50.46
O/S	18.98	31.41	53.90	31.36	54.10
M/O	19.59	31.94	54.75	32.18	53.78
O/O	20.98	34.61	59.74	34.82	58.90
Error (1 S.D.):					
t_g				±0.25 (0.7%)	±1.02 (1.8%)
$t_j - t_i$				±0.17 (9%)	±0.64 (20%)

* M refers to an oxidized methionine, S to an oxidized sulfide bridge; thus M/S is the methione-oxidized disulfide form of the molecule; in ref. 19, M/S is referred to as A-ox, O/S as B-ox, M/O as A-red and O/O as B-red.

** Calculated from 20- and 60-min runs.

*** Calculated from 20- and 40-min runs.

Other examples from the literature

IL-2 muteins. Kunitani *et al.*[26] reported gradient retention data for 30 muteins* and/or oxidized derivatives of interleukin-2 (IL-2, molecular weight 14000 Da), including runs for each compound at three different gradient times (20, 40 and 80 min). These data can further test the accuracy of computer simulation in the same way as the preceding example for the ribosomal proteins. Two gradient runs can be used to predict retention for the third run (only t_G varying). Table VIII illustrates this for two different muteins and their various oxidized derivatives.

The predicted (calculated) t_g values for the 40-min run are in good agreement with experimental values (±0.7%), as are the retention time differences ($t_j - t_i$, ±9%). For the entire set of 30 compounds from ref. 25, retention times for the 40-min run agree within ±0.9% (1 S.D.).

* "Muteins" refer to related proteins which differ only in the substitution of one or a few amino acids (by other amino acids) in the polypeptide chain.

The comparisons for the 80-min run in Table VIII are much poorer, and the retention order of two bands is predicted incorrectly ($Ala^{1}Ser^{125}$ O/S and M/O). For the entire 30 compounds, the error in t_g was $\pm 2.3\%$. The poor results found for the 80-min runs may reflect (a) the use of initial t_G values (20 and 40 min) that are too similar, and/or (b) the use of extrapolative prediction for the 80-min runs *vs.* interpolative prediction for the 40-min runs. However, these factors did not limit the accuracy of extrapolative predictions for the 30S ribosomal proteins (above), suggesting that column variability may have been less well controlled in the IL-2 study in ref. 26 (*cf.*, discussion of Table V). DryLab G software tests for both (a) initial t_G values (experimental inputs) that are too similar and (b) computer predictions that involve excessive extrapolation. The user is alerted to possible errors in computer-simulated results when either of the latter tests fails. In either instance, more accurate computer simulations can be obtained by carrying out an additional experimental run (new value of t_G) and inputting the new data into the computer.

Nuclease muteins. Ford and Smith[27] reported gradient retention times for thirteen muteins of nuclease. Each compound was run with three different gradient times, 10, 20 and 60 min. Values of t_g for the 20-min runs agreed with computer simulated values (using 10- and 60-min runs) within ± 0.15 min (1 S.D.), corresponding to $\pm 1.2\%$. Retention time differences ($t_j - t_i$) were in much closer agreement (± 0.04 min).

These various studies (30S and 50S ribosomal proteins, IL-2 and nuclease muteins) suggest that computer simulation can be useful for purposes of method development when care is taken in the choice of conditions for the initial experimental runs.

Recommendations for the use of computer simulation with protein samples

The preceding discussion provides guidelines for maximizing the accuracy of computer-simulated separations by gradient elution, as summarized in Table I. Problems 5 and 7–9 are seen to be potentially the most serious. The impact of these sources of error in computer simulation can be minimized by the following procedures.

(1) Use HPLC systems with low mixing volumes, V_M, especially when using segmented gradients.

(2) When carrying out the initial two experimental runs, collect data in sequential runs within a 48-h period and use columns that have been previously conditioned by running several protein samples.

(3) Select initial gradient times that differ by a factor of at least 3–4 (*e.g.*, t_G values of 30 and 120 min); samples having higher molecular weights require longer gradient times.

(4) When entering data for two bands that overlap excessively in one of the initial (experimental) runs, be aware that the predicted resolution for these two bands can be in error. See further discussion below.

(5) Make sure that bands for the two initial runs are properly assigned for entry into DryLab G; this can be checked by comparing a simulated chromatogram for one run, based on band areas for the other. Alternatively, carry out a third experimental run with an intermediate gradient time and compare this chromatogram with one predicted by computer simulation. Errors in initial band assignment will result in major errors in the predicted retention of only a few bands, with most bands showing

much better agreement between experimental and computer-simulated runs. For further discussion, see ref. 3.

So far, we have said little about the problem of inaccurate t_g values from the initial experimental runs (illustrated in Fig. 3). Errors of this type are less common, inasmuch as special circumstances are required to create discrepancies as large as those of Fig. 3. One approach to dealing with this problem is as follows. First, be aware that errors of this type are possible when two bands overlap in one of the initial runs so as to yield only one value of t_g for the two bands. Second, when a simulated (optimized) chromatogram exhibits discrepancies as in Fig. 3 which seems attributable to band overlap, try to adjust the t_g values for the initially overlapped bands in a reasonable manner, so as to achieve better prediction of the final optimized run. With this new set of computer-simulation input values, it should be possible to largely correct for errors of the type illustrated in Fig. 3 (see also the further discussion of Fig. 5 in the following paper[6]). Note finally that the use of columns with large plate numbers minimizes the problem of inaccurate t_g values in the input data.

CONCLUSIONS

Computer simulation can be used to greatly reduce trial-and-error experiments in the laboratory during the development of HPLC methods based on gradient elution. However, the effective use of computer simulation requires sufficient accuracy in the predicted results. A number of experimental factors can lead to errors in predictions of separation via computer simulation. We have examined these various factors from both a theoretical and an empirical standpoint; practical recommendations are presented so as to ensure accurate predictions by computer simulation.

Experimental parameters or conditions that can play a significant role in affecting predictions of separation by computer simulation include: (a) the mixing volume, V_M, of the HPLC system, (b) changes in retention due to alteration of the column during use, (c) non-linearity of plots of log k' *vs.* percentage of organic compound for sample components, (d) errors in the measurement of experimental retention times t_g and (e) failure to recognize band reversals in the two experimental runs required for computer simulation. Each of these (and other) factors were examined in detail.

When experimental conditions were chosen to minimize these errors in computer simulation, good agreement was found between experimental and predicted separations for several protein samples. This in turn allows the use of computer simulation to facilitate the design of optimal gradients for the separation of protein samples.

GLOSSARY OF SYMBOLS

These apply to both this and the following two papers[5,6]. Reference to the following paper[5] is noted by use of "II" (*e.g.*, eqn. II-3 is eqn. 3 in ref. 5; Fig. II-2 is Fig. 2 in ref. 5); III refers to ref. 6

A, B, C.. bands or compounds in sample
b gradient steepness parameter, defined by eqn. 3
F mobile phase flow-rate (ml/min)

k' solute capacity factor (isocratic separation)
$\bar{k}$ average or effective value of k' for a band during gradient elution; equal to $1/1.15b$
k_f value of k' for band in gradient elution at the time the band leaves the column (eqn. II-2)
k_0 value of k' for a mobile phase having the same composition as at the start of the gradient (φ_0)
k_w value of k' for water as mobile phase ($\varphi = 0$)
n gradient shape parameter (eqn. III-4)
N column plate number
RRM relative resolution map, *e.g.*, Fig. II-12
R_s resolution of two adjacent bands; equal to the difference in retention times divided by the average baseline bandwidth
S defined by eqn. 1; equal to $d(\log k')/d\varphi$
S.D. standard deviation
t time after injection of sample and start of gradient (min)
t_D dwell time of gradient equipment (min); equal to V_D/F
t_g solute retention time in gradient elution (min)
$(t_g)_{10\%}$ time at which a band has migrated 10% of the distance through the column (min); eqn. II-5
t_i, t_j retention times t_g for adjacent solute bands i and j
t_G gradient time (min)
t_0 column dead-time (min)
V_D dwell volume of gradient elution equipment (ml); volume between (and including) mixer and column inlet
V_m dead volume of column (ml)
V_M mixing volume of gradient elution equipment (ml); usually equal to volume of gradient mixer
δF error in assumed value of F (eqn. 8)
δt_g error in a measured value of t_g (eqn. 8)
$\Delta\varphi$ change in mobile-phase composition (φ) during the gradient
φ mobile-phase composition (volume fraction of organic solvent in a water–organic solvent mixture)
φ_e mobile-phase composition (value of φ) in which a band is eluted from the column
φ_0 value of φ at the beginning of the gradient
φ^* value of φ which two adjacent gradient segments share; corresponds to the φ value at the end of the preceding segment and the φ value at the beginning of the following segment; φ^* equals φ_0 when the first segment is due to V_D and the second segment is the first gradient segment.

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