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Computer simulation of gradient elution separation

Accuracy of predictions for non-linear gradients

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ABSTRACT

An experimental study was carried out for the separation of a series of homologous 2-ketoalkanes by reversed-phase gradient elution. Both linear and non-linear gradients were used. Computer simulation was applied to these same separations, and comparisons were made between experimental and predicted results (values of retention time, retention time difference and bandwidth). Errors in the predicted separation were generally small and similar for both linear and non-linear gradients. Somewhat larger errors (retention time differences) were found in non-linear gradient separations for bands eluting after a change in gradient steepness. This can be attributed to the dispersion (rounding) of the gradient as it passes through the high-performance liquid chromatographic equipment. Computer simulation was demonstrated to provide predictions that are adequate for the purposes of developing an optimized gradient separation.

INTRODUCTION

Computer simulation based on DryLab software has been shown to be a useful tool for high-performance liquid chromatography (HPLC) method development [1–3]. The ability to predict separation as a function of experimental conditions allows the chromatographer to explore a much wider range of conditions than would normally be possible in the laboratory. The accuracy of such simulations in practical applications has been documented by a number of different workers [4–8]. Several studies [9–12] have also discussed potential errors in computer simulation from a theoretical standpoint.

In the present investigation, we have examined errors in computer simulation that can arise from the use of non-linear gradients, *e.g.*, segmented or step gradients. A few samples of computer simulation for the prediction of separation based on non-linear gradients have been reported [2,5–8], but no systematic study of the errors associated with these kinds of gradients has yet been reported. Our findings are also

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relevant to the use of microbore columns, where gradient elution imposes special requirements for the equipment used for these separations [13,14].

THEORY

Gradient equipment

The theory of computer simulation for gradient elution has been described [9,15–17]. The use of non-linear gradients introduces additional complexity, but these

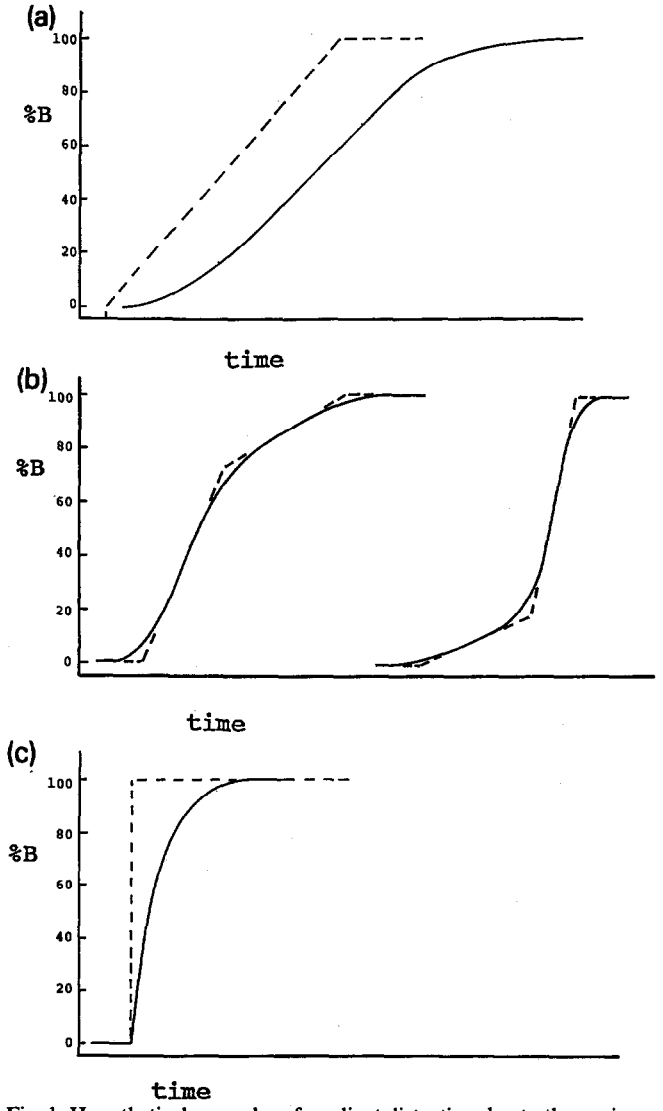

Fig. 1. Hypothetical examples of gradient distortion due to the equipment. (a) Gradient delay and rounding for a linear gradient; (b) gradient rounding for two-segment gradients; (c) gradient rounding for a step-gradient. ——— = Experimental gradient; – – – = input (intended) gradient. See text for further details.

effects can be accounted for by the basic model on which DryLab G software is based; see the discussion of refs. 17 and 18. Further complications are created by the use of "non-ideal" equipment, which produces gradients that do not conform exactly to the desired gradient [19,20]. Every gradient system can be considered "non-ideal" to some degree, but continued advances in equipment design have reduced the importance of these effects.

Fig. 1a illustrates the most common consequences of "non-ideal" gradient equipment. Here the dashed curve is the gradient selected by the user, while the solid curve is the actual gradient delivered by the system. The actual gradient is delayed by a time t_D (the equipment "dwell time"), and the ends of this gradient are rounded by dispersion within the equipment. The effects of gradient dwell time on the separation are easily described and are accounted for in the DryLab G software [20]. Gradient rounding has been discussed in detail for linear gradients [19]; this equipment non-ideality usually has only a minor effect on separations that make use of linear gradients [20], because only bands that elute at the beginning or the end of the gradient are affected. DryLab G/plus does not correct for the effects of gradient rounding by the equipment.

Fig. 1b illustrates the rounding of two representative non-linear (segmented) gradients. In this case, the dashed curves correspond to the input gradients (with correction for the dwell time t_D), and the solid curves are the actual gradients. In this figure it is seen that a decrease in steepness of the second gradient segment leads to low %B values near the point that joins the two segments, and *vice versa* for the case where the second segment is steeper. This should result in simulated retention times that are too small for the first case and too large for the second case —in the region where gradient steepness changes. Sample bands are more likely to be present near the middle of the chromatogram than at its ends, so that errors in predicted retention times due to gradient rounding should be more important in the examples of Fig. 1b than in 1a.

Step gradients as in Fig. 1c can lead to larger deviations of actual %B values (solid curve) from the input gradient (dashed curve). Gradients of this kind are used less often; one example is a step gradient at the end of a separation, in order to remove strongly retained sample components (late eluters) from the column. This application is not usually intended to separate the late eluters from each other, but rather to remove them from the column as quickly as possible. In this situation, errors in predicted retention times (computer simulation) are generally unimportant.

Quantitative relationships (linear or segmented gradients). The rounding of the ends of a linear gradient (Fig. 1a) can be described exactly, as discussed in ref. 19. The maximum deviation in %B for the actual gradient (δ%, see Fig. 2b) occurs at the beginning (or end) of the intended gradient. This deviation is related[a] to the so-called mixing volume V_M of the gradient equipment and the gradient volume ($V_G = t_G F$, where t_G is gradient time and F is flow-rate)

$$\delta\% = 37 V_M / V_G \tag{1}$$

[a] Eqn. 1 can be obtained from Table I of ref. 19 (see values of δ% as a function of V_M/V_G).

The deviation of the actual from intended gradients at other times during the separation is always less than $\delta\%$ and can be predicted if values of V_M and V_D are known for the equipment [19].

The effect of gradient rounding on predicted retention times for linear gradient elution should be minor for values of $V_M/V_G < 0.1$. This means that such effects are avoidable either by selecting equipment with a small value of V_M or by increasing V_G (by using higher flow-rates or longer gradient times).

Gradient rounding near the juncture of two gradient segments (Fig. 1b) is more difficult to describe in mathematical form, and an explicit solution for this case has not been reported. Intuitively it seems obvious that the maximum deviation in the gradient ($\delta\%$) will be less than for the corresponding linear-gradient case, *i.e.*, $\delta\%$ for the steeper segment, since $\delta\%$ approaches zero as the steepness of the two segments becomes more similar, and $\delta\%$ approaches the value for the steeper segment as the difference in steepness for the two segments becomes large.

Quantitative relationships (step gradients). The shape of an actual step gradient as in Fig. 1c can be related to the mixing volume V_M [21]

$$\%B = 100\,(e^{V/V_M} - 1)/e^{V/V_M} \tag{2}$$

Here V is the delivered volume of mobile phase following the start of the gradient (equal to tF, where t is time), and the dwell time is ignored. Correcting for the dwell time we have

$$\%B = 100\,[e^{(V-V_D)/V_M} - 1]/e^{(V-V_D)/V_M} \tag{3}$$

where the dwell volume $V_D = t_D F$. When the actual gradient in Fig. 1c equals 63% B, $V/V_M = 1$ (eqn. 2). This allows a convenient measurement of V_M for the equipment, based on a blank step-gradient run.

Other distortions of the gradient. Aside from the effect of equipment characteristics on gradient distortion, other processes within the column can lead to gradient rounding [22]. Since the column pressure typically varies during a gradient run, solvent compressibility can lead to corresponding changes in flow. The strong solvent component (B) is also selectively adsorbed by the column packing (solvent demixing), leading to a further lag in the experimental *vs.* input gradients. Solvent demixing works in an opposite direction to gradient rounding (causing a decrease in %B; *cf.* Fig. 2b), so as to partially cancel its consequences.

EXPERIMENTAL

Equipment

An LC/9533 ternary-gradient liquid chromatograph (IBM Instruments, Danbury, CT, USA) was used with a Model 7125 sample injector (Rheodyne, Cotati, CA, USA) having a 20-μl loop. A circulating water bath controlled the temperature of the column compartment at 23 ± 0.2°C. A variable-wavelength UV detector (Model 9523, IBM Instruments) was used at 274 nm. Chromatograms were processed with a 3390A reporting integrator (Hewlett-Packard, Palo Alto, CA, USA), which supplied values of retention times, band areas and area/height ratios. The area/height ratio was used to determine bandwidths and plate number (N) values [23].

The equipment dwell volume V_D was determined from (a) blank gradients as in Fig. 1a and (b) comparisons of simulated *vs.* experimental retention times; see refs. 12 and 20 for details. Measurement of blank gradients with flow-rates of 1 or 2 ml/min gave values of V_D = 4.1–4.5 ml. Retention time measurements for an isocratic mobile phase of 73% B were also compared with values predicted by DryLab G/plus (10–100% B in 20 and 60 min used as input). The latter procedure gave V_D = 3.9 ml. An average of these three measurements was assumed: V_D = 4.2 ml. The extra-column band broadening of the HPLC system was reported in ref. 12; σ_{ec} = 0.04 ml.

Materials

Columns were Zorbax C_8 (25 × 0.46 cm I.D., 5-μm particles, MacMod Analytical, Chadds Ford, PA, USA). Column plate number was evaluated at frequent intervals using a test mixture of uracil, acetophenone, nitrobenzene, methyl benzoate and toluene (acetonitrile–water, 70:30, as mobile phase, 0.8 ml/min). The plate number for new columns (toluene solute) was N $\approx$ 14000. The column dead time t_0 was measured from the retention time of sodium nitrate (methanol–water, 70:30, as mobile phase): t_0 = 1.43 min at F = 1.5 ml/min.

The sample used in the present study was a homologous series of nine 2-ketoalkanes: C_5–C_{13} (Aldrich, Milwaukee, WI, USA, or Fluka, Ronkonkoma, NY, USA). The large differences in retention for adjacent bands permitted accurate measurements of retention time and width (or N) for each band, *i.e.*, all bands were well resolved.

The following gradient separations used 0.1% H_3PO_4 (J. T. Baker, Phillipsburg, PA, USA) in water (distilled and deionized [24]) for solvent A, and 0.1% H_3PO_4 in acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) for solvent B. Methanol was obtained from Burdick & Jackson (Muskegon, MI, USA).

Computer simulations

DryLab G/plus software (LC Resources, Lafayette, CA, USA) was used with a liquid chromatography simulator (IBM-PC/XT personal computer plus a 8087 math coprocessor, also from LC Resources). Simulations of bandwidth required values of the column parameters x = 0.75 and y = 0.40 (see ref. 12 for details). Values of the Knox-parameter A for these columns were determined by means of DryLab G/plus and data for the 2-ketoalkane sample: A = 0.90. For additional details on the simulation and measurement of bandwidth and plate number values, see ref. 12.

RESULTS AND DISCUSSION

Mixing volume V_M of the present HPLC system

Fig. 2a and b shows a blank gradient for the equipment used in these studies. The value of δ% from Fig. 2b equals 6.1%, from which V_M = 0.8 ml was determined from Eqn. 1 (F = 0.5 ml/min, t_G = 10 min). The gradient volume V_G = 5 ml for Fig. 2a and b was deliberately small, in order to magnify the effects of gradient rounding and facilitate the measurement of V_M.

The blank gradient of Fig. 2c is composed of two segments of differing steepness; it shows the expected rounding of the gradient at a time after starting the gradient of about 16 min (where the two segments join). The magnitude of this

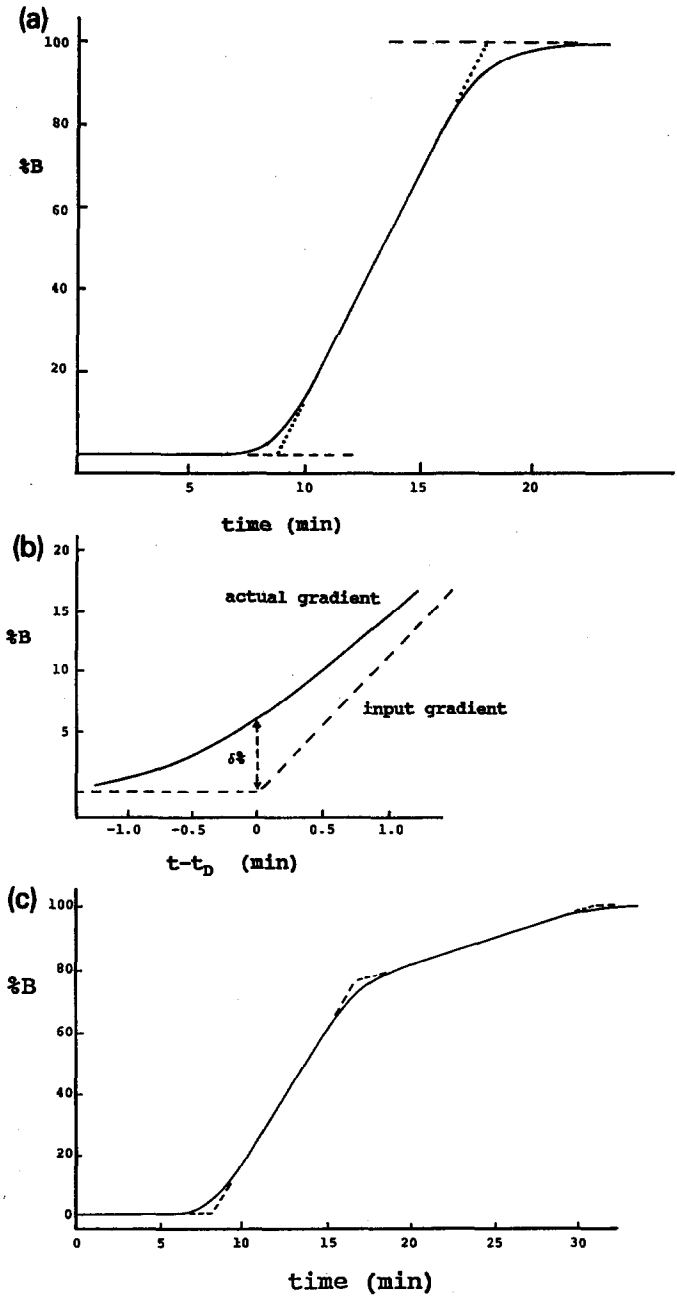

Fig. 2. Experimental examples of gradient distortion by the present HPLC system. (a) Gradient rounding for a linear gradient; 0–100% B in 10 min, 0.5 ml/min; (b) expansion of initial gradient from (a); (c) gradient rounding in a two-step gradient; 0–75–100% B in 0–10–27 min, 0.5 ml/min.

rounding (value of δ%) is comparable to that for the beginning of the initial (steeper) segment, as predicted (see discussion of Theory section). Again, a small gradient volume was used for the first segment of Fig. 2c, in order to magnify the effects of gradient rounding.

Computer simulations for linear gradients

Our primary goal in the present study was to evaluate possible errors in computer simulations for non-linear gradient systems. We wished to compare these errors (for non-linear gradients) with the well-understood [8–11] errors that arise in computer simulations for linear gradient systems. Therefore we first investigated the computer simulation of representative linear gradient separations of the 2-ketoalkane sample. Conditions were selected for these experiments (with one exception) so that sample components were not eluted near the beginning or end of the gradient. In this way, errors due to the rounding of the gradient at its ends were minimized.

Retention time errors. Table I summarizes data for a run with a very steep gradient: 10–100% B in 6 min (15% B/min). There is generally good agreement (Fig. 3) between experimental and calculated (simulated) retention times (t_g), except for the last three bands. The errors in t_g for the latter bands are seen to increase from C_{11} to C_{13}. These errors in t_g have an even larger effect on the retention time differences Δt_g for adjacent bands (values of Δt_g are proportional to resolution R_s). Consequently, the average error in predicted values of Δt_g is rather large (± 23%; see Table I). This is

TABLE I

COMPARISON OF EXPERIMENTAL AND PREDICTED RETENTION TIMES FOR THE SEPARATION OF THE 2-KETOALKANE SAMPLE BY LINEAR GRADIENT ELUTION (DATA OF FIG. 3)

Conditions: 10–100% B, 1.5 ml/min; other conditions as in Experimental section. All simulations based on 20- and 60-min gradient runs as input.

Band	Retention times (min)		Retention time errors (min)[a]	
	Expt.	Calc.	t_g	Δt_g
C_5	7.33	7.48	0.15	−0.06
C_6	8.06	8.15	0.09	−0.07
C_7	8.66	8.68	0.02	−0.03
C_8	9.19	9.16	−0.03	−0.02
C_9	9.67	9.62	−0.05	−0.07
C_{10}	10.14	10.02	−0.12	−0.10
C_{11}	10.61	10.39	−0.22	−0.18
C_{12}	11.11	10.71	−0.40	−0.29
C_{13}	11.67	10.98	−0.69	
Average error[b]			±0.20 (2.2%)	±0.10 (23%)

[a] Difference between experimental and predicted (DryLab) values; t_g is the retention time; Δt_g is the retention time difference for adjacent bands (proportional to resolution R_s).

[b] Average absolute error; % error is average absolute error divided by average retention time or retention time difference.

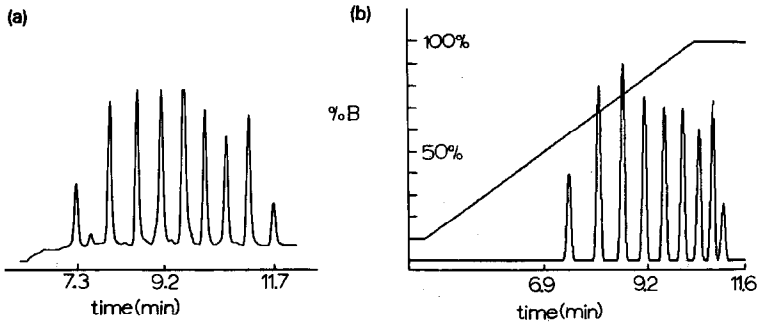

Fig. 3. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with a linear gradient in 6 min. Conditions: 10–100% B, 1.5 ml/min, other conditions in Experimental section. Simulations based on 20- and 60-min runs as input.

also apparent in Fig. 3, where the simulated chromatogram shows much less resolution of the last two bands than is found experimentally.

The larger errors in t_g and Δt_g for the last three bands of Fig. 3 are due to a combination of effects. First, for this rather steep gradient (15% B/min), there is a considerable rounding of the end of the gradient. The maximum error δ% can be estimated equal to 3.4% B for this separation. Second, as can be seen in Fig. 3b, the last three bands are eluted after the completion of the linear gradient (during the subsequent gradient hold at 100% B). Bands that elute near or after the end of the gradient will be particularly affected by gradient rounding.

When gradient steepness is reduced for the separation of the 2-ketoalkane sample, gradient rounding becomes less severe. Less steep gradients also result in the earlier elution of all bands, so that all bands elute before the end of the gradient. This

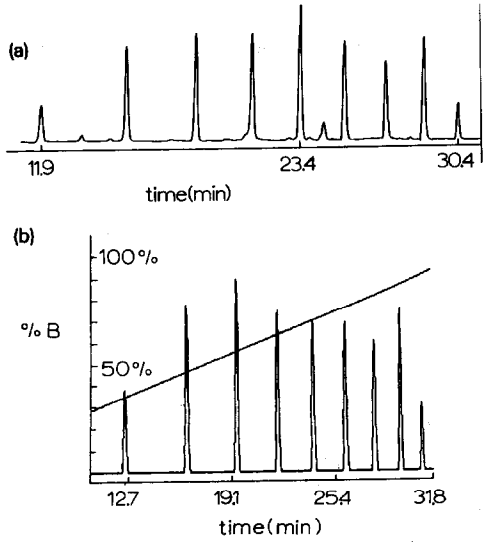

Fig. 4. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with a linear gradient in 30 min. Conditions: 10–100% B, 1.5 ml/min, other conditions in Experimental section. Simulations based on 20- and 60-min runs as input.

TABLE II

SUMMARY OF COMPARISONS OF EXPERIMENTAL AND PREDICTED RETENTION TIMES FOR THE SEPARATION OF THE 2-KETOALKANE SAMPLE BY LINEAR GRADIENT ELUTION (AS IN FIG. 3)

Conditions as in Fig. 3. All simulations based on 20- and 60-min runs as input.

Gradient conditions		Average t_g (min)	Errors	
t_G (min)	%B/min		t_g (%)	Δt_g (%)
6	15	9.5	±2.2	±23
12	7.5	13	1.7	3
30	3	22	2.8	1
90	1	46	1.3	2
150	0.6	65	1.1	3
Average errors (%)			±1.8 (±1.7)[a]	± 6 (±2)[a]

[a] "Best" values, excluding 6-min run.

is illustrated in Fig. 4 for separation of this sample in a time t_G = 30 min (3% B/min). Now the last band (t_g = 30 min) elutes well before the end of the gradient ($30 + t_0 + t_D$ = 34.5 min). As a result of earlier sample elution plus reduced gradient rounding, the experimental retention times for the last three bands agree much more closely than in Fig. 3, and the average error in predicted values of Δt_g is now only ±1% (*vs.* ±23% in Fig. 3).

Table II summarizes data for several gradient runs of varying steepness. With the exception of the (very steep) 6-min gradient, the average error in retention times is ±1.7%, and the error in values of Δt_g is only ±2%. This represents reasonable agreement between experimental and predicted retention times, when compared with previous examples of computer simulation for linear gradients [5–8,11,12].

Isocratic separations. DryLab G/plus can also be used to predict isocratic separation as a function of %B, based on the same gradient input data used in Figs. 3 and 4 and Tables I and II. Comparison of experimental and predicted retention times for isocratic runs is relevant to the present study of non-linear gradient elution, since some (non-linear) gradients consist of an initial gradient followed by a hold (or *vice versa*). In the latter cases, some sample bands may be eluted under isocratic conditions. Fig. 5 shows experimental and simulated chromatograms for isocratic separation of the 2-ketoalkane sample with 65% B as the mobile phase. Retention times agree within ±8.2%, and Δt_g values agree within ±12% (average values). Similar data for other isocratic separations are summarized in Table III.

The accuracy of these isocratic predictions (Table III) is poorer than for corresponding predictions of gradient separation (Table II). Additional errors in computer simulation are introduced by the conversion of gradient (input) to isocratic (predicted) data (see discussion of refs. 19 and 22). The even larger errors in Table III for B > 70% are due to excessive extrapolation of data derived from the input gradient runs. If the latter data are excluded, the average error is ±8% for retention times and ±11% for Δt_g and resolution. This is adequate for the purpose of method development based on computer simulation.

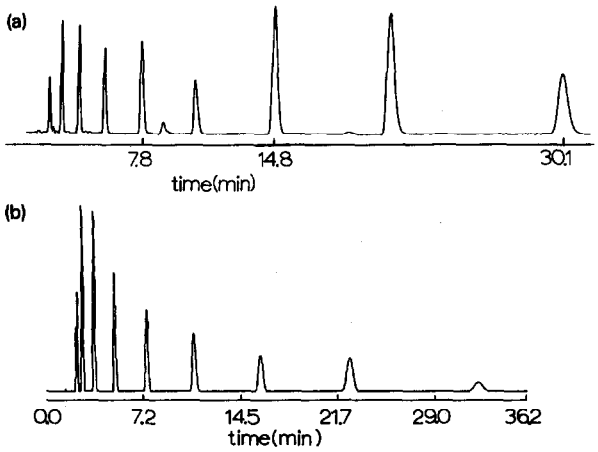

Fig. 5. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample isocratically. Conditions: 65% B, other conditions as in Fig. 3. Simulation based on 20- and 60-min runs as input.

Bandwidth errors. Errors in bandwidths for the various linear gradient and isocratic runs of Tables II and III are summarized in Table IV. The average error in predicted values of bandwidth is about ±10%. These errors are similar to those observed in a preceding study of the separation of *o*-phthalaldehyde-derivatized amino acids by gradient elution [12] and are also adequate for method development purposes.

Computer simulations for non-linear gradients

Additional experiments as in Tables II–IV were carried out for the separation of the 2-ketoalkane sample using non-linear gradients of various types: two-segment gradients, partial gradients with isocratic holds, step gradients and more complex gradients. In each case, comparisons were made of experimental and simulated data

TABLE III

SUMMARY OF COMPARISONS OF EXPERIMENTAL AND PREDICTED RETENTION TIMES (t_R) FOR THE SEPARATION OF THE 2-KETOALKANE SAMPLE BY ISOCRATIC ELUTION (AS IN FIG. 5)

All simulations based on 20- and 60-min runs as input.

B (%)	Average t_g (min)	Errors	
		t_R (%)	Δt_R (%)
60	16	7	9
70	8.4	9	13
80	5.2	14	18
90	3.8	21	23
Average errors (%)		±13	±16

TABLE IV

SUMMARY OF COMPARISONS OF EXPERIMENTAL AND PREDICTED BANDWIDTHS FOR THE SEPARATION OF THE 2-KETOALKANE SAMPLE BY LINEAR GRADIENT OR ISOCRATIC ELUTION (FOR RUNS OF TABLES II AND III)

Conditions: as given in table; other conditions as in Fig. 3. All simulations based on 20- and 60-min runs as input.

Conditions	Bandwidth data[a]	
	Average value (min)	Error[b] (%)
Linear gradients (min)		
6	0.40	±11
12	0.43	5
30	0.60	6
90	1.20	7
150	1.61	18
Average error (%)		± 9
Isocratic runs (%B)		
60	1.42	±12
70	0.81	11
80	0.54	12
90	0.46	15
Average error (%)		±12

[a] Baseline bandwidth.
[b] Average of absolute errors; % error is average absolute error divided by average bandwidth.

for retention time, retention time difference and bandwidth. Attempts were also made to relate unusually large errors to gradient rounding effects, wherever the latter were known to be significant.

Non-linear gradients: retention data. Several separations that involved non-linear gradients were carried out and verified by computer simulation. Typical two-segment gradients are illustrated in Figs. 6 and 7, while Figs. 8–10 show a linear gradient with an initial isocratic hold, a step gradient, and a complex gradient, respectively. Comparisons of experimental and simulated runs are summarized in Tables V (retention data) and VI (bandwidth data), respectively.

Table V shows that the overall accuracy of computer simulation for these ten non-linear gradient separations was ±3.4% for retention times t_g and ±10% for retention time differences Δt_g. These values can be compared with the average errors for linear gradients (Table II): ±1.7% for t_g and ±2% for Δt_g (data for 6-min run excluded). Thus, computer simulation for non-linear gradients is less accurate *vs.* linear gradients, but still adequate for method development purposes.

A distinction should be made between segmented gradients and the other non-linear gradients of Table V. The latter gradients include isocratic holds and are therefore expected to yield somewhat less accurate predictions; thus the data of Table III for isocratic separation suggest ±13% in t_R and ±16% in Δt_R. For the non-linear

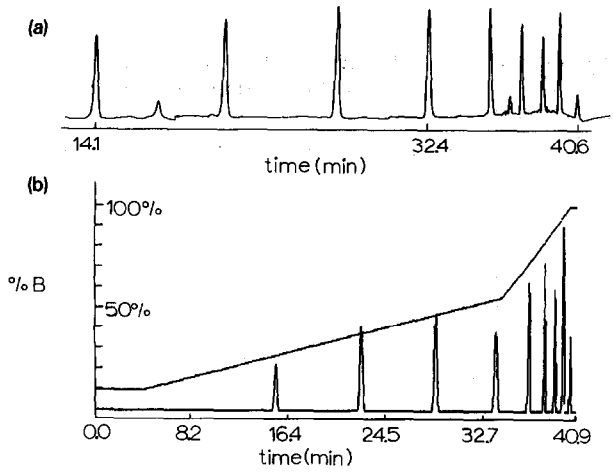

Fig. 6. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with a two-segment gradient. Conditions: 10–55–100% B in 0–30–36 min; other conditions as in Fig. 3. Simulation based on 20- and 60-min runs as input.

gradient separations of Table V which do not include an isocratic hold (two-segment gradients), the accuracy of predicted retention times ($\pm 2.4\%$ in t_g, $\pm 4\%$ in Δt_g) is not very different from the accuracy of linear gradient simulations ($\pm 1.7\%$ in t_g, $\pm 2\%$ in Δt_g; Table II).

Non-linear gradients: bandwidth data. A comparison of experimental and predicted bandwidths for these non-linear gradient separations is given in Table VI. The

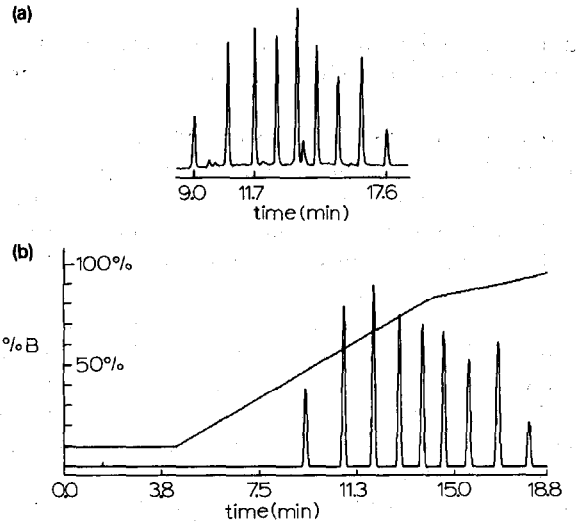

Fig. 7. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with a two-segment gradient. Conditions: 10–85–100% B in 0–10–20 min; other conditions as in Fig. 3. Simulation based on 20- and 60-min runs as input.

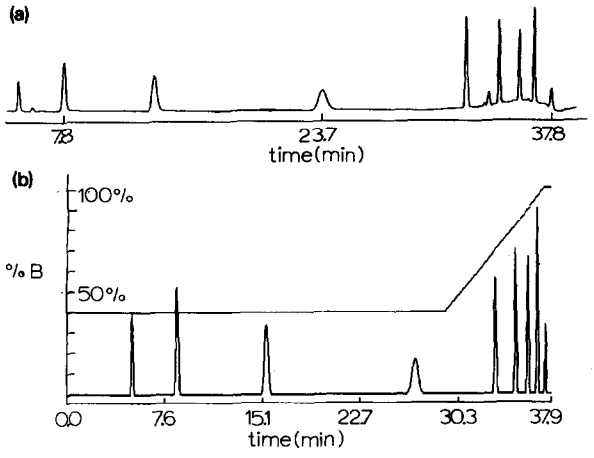

Fig. 8. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with an initial isocratic hold followed by a linear gradient. Conditions: 40–40–100% B in 0–25–33 min; other conditions as in Fig. 3. Simulation based on 20- and 60-min runs as input.

average error in predicted bandwidths for all ten runs is ± 11%, which is similar to the 9–12% error found for linear gradient or isocratic separation (Table IV). It appears that bandwidths are predicted as accurately for non-linear gradient elution as for separations based on linear gradients.

Further analysis of data of Tables V and VI. If gradient rounding or other "non-ideal" effects contribute to error in non-linear gradient elution, larger relative errors would be expected for bands that elute in that part of the chromatogram where

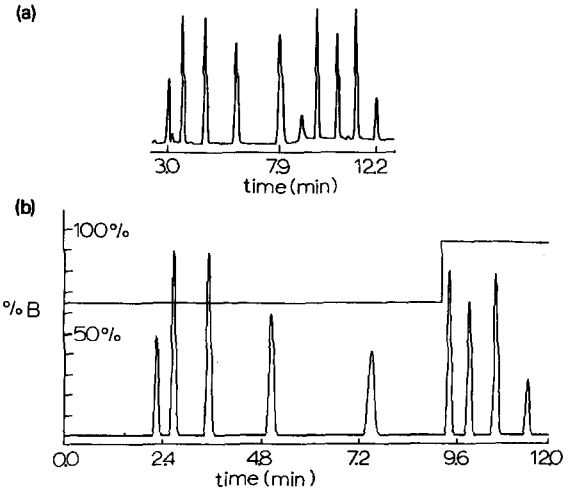

Fig. 9. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with a step gradient. Conditions: 65–65–95–95% B in 0–5–5–60 min; other conditions as in Fig. 3. Simulation based on 20- and 60-min runs as input.

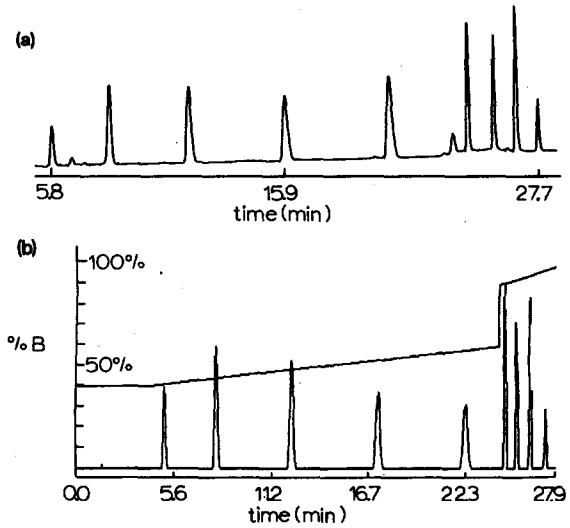

Fig. 10. (a) Experimental and (b) simulated chromatograms for separation of a 2-ketoalkane sample with a complex gradient. Conditions: 40–60–90–100% B in 0–20–20–30 min; other conditions as in Fig. 3. Simulation based on 20- and 60-min runs as input.

TABLE V

SUMMARY OF COMPARISONS OF EXPERIMENTAL AND PREDICTED RETENTION TIMES FOR THE SEPARATION OF THE 2-KETOALKANE SAMPLE BY NON-LINEAR GRADIENT ELUTION (AS IN FIGS. 6–10)

Conditions as listed in table; other conditions as in Fig. 3. All simulations based on 20- and 60-min runs as input.

Gradient conditions	Average t_g (min)	Errors	
		t_g (%)	Δt_g (%)
Two-segment gradient			
10–85–100% B in 0–10–20 min	31	±1.5	± 5
10–55–100% B in 0–30–36 min	31	2.5	6
10–75–100% B in 0–14–25 min	17	2.6	2
10–70–100% B in 0–26–33 min	26	3.1	3
Gradient hold			
40–40–100% B in 0–25–33 min	25	3.3	21
10–85–85% B in 0–10–20 min	14	0.9	6
Step gradient			
65–65–75–75% B in 0–5–5–60 min	9	5.2	10
65–65–95–95% B in 0–5–5–60 min	8	8.8	22
Complex gradient			
40–75–90–100% B in 0–5–5–15 min	9	2.2	6
40–60–90–100% B in 0–20–20–30 min	18	3.5	14
Average errors (all data)		±3.4	±10
(Two-segment gradients)		±2.4	± 4

TABLE VI

SUMMARY OF COMPARISONS OF EXPERIMENTAL AND PREDICTED BANDWIDTHS FOR THE SEPARATION OF THE 2-KETOALKANE SAMPLE BY NON-LINEAR GRADIENT ELUTION (FOR RUNS OF TABLE V)

Conditions as given in table; other conditions as in Fig. 3. All simulations based on 20- and 60-min runs as input.

Gradient conditions	Bandwidth data[a]	
	Average value (min)	Error (%)
Two-segment gradient		
10–85–100% B in 0–10–20 min	0.47	± 8
10–55–100% B in 0–30–36 min	0.71	9
10–75–100% B in 0–14–25 min	0.53	7
10–70–100% B in 0–26–33 min	0.67	8
Gradient hold		
40–40–100% B in 0–25–33 min	0.90	14
10–85–85% B in 0–10–20 min	0.50	8
Step gradient		
65–65–75–75% B in 0–5–5–60 min	0.71	11
65–65–95–95% B in 0–5–5–60 min	0.50	16
Complex gradient		
40–75–90–100% B in 0–5–5–15 min	0.46	9
40–60–90–100% B in 0–20–20–30 min	0.71	11
Average errors (all data)		±11
(Two-segment gradients)		± 8
(Other gradients)		±10

[a] Baseline bandwidths.

the gradient changes steepness. We will refer to this time during the gradient as the "critical" time t_c. For various reasons, it can be argued that errors in Δt_g (expressed in %) provide a good measure of the inaccuracy of simulated retention times. Errors in Δt_g are also much more significant in method development than are errors in t_g. Fig. 11 summarizes the dependence of retention errors measured in this way as a function of retention time (position of the bands in the chromatogram) relative to the critical time t_c. The x-axis of Fig. 11 is in units of $(t - t_c)/t_G$, which is a measure of normalized retention relative to t_c. The data of Fig. 11 are from the four two-segment gradient separations of Table V.

It is obvious from Fig. 11 that errors in Δt_g are consistently larger for bands eluting after the critical time t_c. Based on gradient rounding effects, it might be assumed that these errors should reach a maximum when the average band retention $t = t_c$, but this is clearly not the case. The larger errors for bands eluting after t_c can be rationalized in terms of the elution history of each band as it migrates through the column. That is, a band eluting at a time $t_g = t_c$ has been exposed only to the gradient (mobile phase) preceding t_c, so that (small) errors in this part of the gradient will

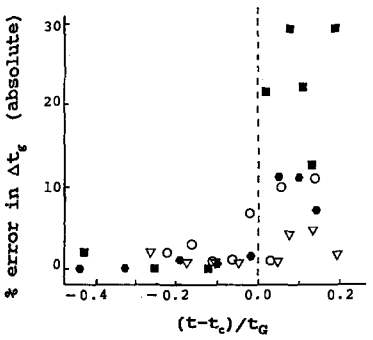

Fig. 11. Error in predicted values of Δt_g as a function of when the two bands elute from the column. The vertical dashed line corresponds to an elution time equal to the "critical" time t_c. Data for two-segment runs of Table V. (○) 10–85–100% B in 0–10–20 min; (■) 10–55–100% B in 0–30–36 min; (▽) 10–75–100% B in 0–14–25 min; (●) 10–70–100% B in 0–26–33 min. Other conditions as in Fig. 3.

result in less error in predicted values of t_g. However, bands eluting after a time t_c are affected to a greater extent by errors in the gradient before and after t_c. That is, the error in t_g (and Δt_g) is a summation of errors in that part of the gradient (preceding t_g) that causes the migration of the band through the column (see Discussion of ref. 15). The rather sharp rise in error that occurs for bands that have eluted just after a time t_c (Fig. 11) is nevertheless rather remarkable.

It is interesting to examine the average error for each of the runs of Fig. 11, for the case where bands elute after the critical time t_c. These average errors for bands eluting after t_c in each of the four runs of Fig. 11 are summarized in Table VII. Retention errors caused by gradient rounding (Fig. 1b, first example) should lead to increasingly negative errors in t_g as $t_g - t_c$ increases (beyond $t_g \approx t_c$). This should result in negative errors in Δt_g when the second gradient segment is steeper, and *vice versa* when the first segment is steeper. This is observed for all four separations of Table VII. Similarly, these errors should be larger for a bigger change in steepness (%B/min) between the two gradient segments; again this is confirmed in Table VII. Thus the correlations of Fig. 11 can be explained in terms of gradient rounding effects.

TABLE VII

AVERAGE ERROR IN VALUES OF Δt_g AND RESOLUTION R_s FOR TWO-SEGMENT GRADIENTS OF TABLE V FOR BANDS ELUTING AFTER THE CRITICAL TIME t_c

Gradient conditions (%/min)		*Average error*[a] *(%)*	
1st segment	2nd segment	Δt_g	R_s
7.5	1.5	+ 7	+10
1.5	7.5	−23	−14
4.6	2.3	+ 4	− 1
2.3	4.3	−11	− 4

[a] Actual (not absolute) values.

If this explanation of the post-t_c errors of Fig. 11 is correct, we should also expect to see corresponding errors in predicted bandwidths, *e.g.*, a decrease in an experimental t_g value due to rounding (elution of the band in a higher %B) should lead to a decrease in bandwidth, so that the error in resolution is less than predicted from the error in Δt_g. This is observed, as seen in Table VII, for errors in R_s; the latter are generally smaller than are errors in Δt_g. Finally, it is seen in Table VII that errors in predicted values of R_s are seldom greater than $\pm 10\%$.

Mobile phase demixing (in the absence of gradient rounding) would lead to a qualitatively similar correlation of errors and band retention (Fig. 11). However, errors due to demixing should increase gradually for increasing values of $t_g - t_c$, beginning at $t_g = t_c$. This is not observed in Fig. 11, suggesting that gradient rounding is the major factor in these larger errors for later-eluting band pairs.

Gradient elution using microbore columns and equipment

The preceding discussion suggests that gradient rounding will become significant (in terms of its effect on separation), when the mixing volume $V_M > 1$ ml (for a column whose diameter is 0.46 cm). Reliable predictions of gradient-elution separation based on computer simulation therefore require $V_M < 1$ ml. As the column diameter d_c is decreased, flow-rates must be decreased by a factor of d_c^2, if a similar separation is to be maintained. This in turn means a reduction of gradient volume V_G by the same factor. If gradient rounding is to be held at the same level, the equipment mixing volume V_M must be reduced by a similar factor. This means that mixing volumes must be less than 50 μl for a microbore system ($d_c = 0.1$ cm). This is a rather severe requirement, requiring careful design of the gradient system [13,14].

CONCLUSIONS

Computer simulation based on DryLab G/plus software was evaluated for non-linear gradients of various kinds. Errors in retention time, retention time differences (proportional to resolution R_s) and bandwidth were generally small and similar in magnitude to those found for linear gradients. These results suggest that computer simulation is sufficiently accurate as a tool for developing methods based on non-linear gradients.

An analysis of observed errors in predicted retention time differences showed that larger errors are found for bands eluting after the "critical" time t_c, where t_c is the time during the gradient when gradient steepness changes. These larger errors appear to be due to gradient rounding caused by dispersion of the gradient within the HPLC equipment. This suggests that gradient equipment which exhibits less dispersion of the gradient (a smaller "mixing volume" V_M) should provide experimental data which are in better agreement with computer simulation, since DryLab G/plus ignores gradient rounding. Computer simulation should therefore prove less reliable for the prediction of segmented-gradient separations (when gradient steepness changes by more than $\pm 4\%$/min between segments), if gradient equipment with $V_M > 1$ ml is used.

For the special case of microbore gradient elution, rounding of the gradient is a potentially more serious problem, because of very small values of the gradient volume V_G (due to low flow-rates). Unless the mixing volume V_M (gradient dispersion) for

microbore systems is reduced to a very small value (< 50 μl), observed separations will be less predictable and computer simulation can be seriously in error.

The present study provides additional confirmation of the general theory of gradient elution as applied to non-linear gradient systems [18].

SYMBOLS

B	solvent B in mobile phase A–B; acetonitrile or methanol in present study
d_c	column internal diameter (cm)
F	mobile phase flow-rate (ml/min)
N	column plate number
R_s	resolution of two adjacent bands
t	average value of t_g for two adjacent bands (Fig. 11; min)
t_c	"critical" time for a non-linear gradient (min); time at which gradient steepness (measured at outlet of column) changes (min)
t_D	dwell time of gradient HPLC system (min)
t_g	retention time in gradient elution (min)
t_G	gradient time (min)
t_0	column dead time (min)
t_R	retention time in isocratic elution (min)
V	volume of mobile phase at some time after the start of the gradient (eqn. 2)
V_D	dwell volume of gradient HPLC system; equal to $t_D F$ (ml)
V_G	gradient volume, equal to Ft_G (ml)
V_M	mixing volume of gradient HPLC system (ml)
$\delta\%$	change in %B at beginning of gradient due to rounding (see Fig. 2b)
Δt_g	difference in t_g values for two adjacent bands; proportional to resolution R_s (min)
Δt_R	difference in t_R values for two adjacent bands; proportional to resolution R_s (min)
σ_{ec}	extra-column band broadening of gradient system (ml)

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