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Maintaining fixed band spacing when changing column dimensions in gradient elution

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Abstract

In gradient elution separations, it may be required to change either column length (to increase resolution or shorten run time) or column diameter (for an increase in sensitivity or for preparative separations). In either of these changes of column dimensions, it is usually desired to maintain the same relative band spacing (selectivity), so as to increase resolution in proportion to (column plate number)$^{1/2}$ when increasing column length, or to maintain constant resolution when changing column diameter. A general rule for avoiding changes in band spacing in these situations is to maintain the quantity [(gradient time)×(flow-rate)/(column volume)] constant, while holding the initial and final gradient mobile phase compositions (%B) fixed. This rule is only valid as long as the equipment hold-up volume (dwell volume) is negligible, or if all sample components are strongly retained at the start of the gradient. When neither of the latter conditions apply, then significant changes in band spacing may result when changing column size. Rules are presented for recognizing this potential problem for a given sample/HPLC-equipment combination, and adjustments in separation conditions that can avoid this problem are discussed. Changes in band spacing as a result of change in column size are of special concern when developing procedures for preparative chromatography under gradient conditions. © 1998 Elsevier Science B.V.

Keywords: Gradient elution; Computer simulation; Column dimensions; Band spacing; Selectivity

1. Introduction

A systematic approach to method development for gradient elution should begin by adjusting gradient conditions and optimizing band spacing [1]. If resolution needs to be improved further, or run time shortened, this can be achieved by subsequent changes in column length L and/or flow-rate F. It may also be desired to vary the column diameter d_c, in order to either increase detection sensitivity (smaller d_c) or increase column loadability (larger d_c). However, once band spacing has been optimized as discussed in [1], it is important to maintain the same spacing of sample bands when changing flow-rate or column dimensions. A general rule can be applied to this situation [1–3]: relative retention will not change when L, d_c and/or flow-rate F are varied, providing that the quantity $(t_G F)/(V_m \Delta\varphi)$ is held constant. Here, t_G is the gradient time, V_m is the column dead volume (proportional to Ld_c^2), and $\Delta\varphi$ is the change in the volume fraction φ (φ=0.01× %B) of the B-solvent during the gradient. Prior to changing L, d_c or F, it is desirable to select a specific value of $\Delta\varphi$, which then requires that $Q=[(t_G F)/Ld_c^2]$ be held constant. If F, L or d_c are changed, it is convenient to simultaneously vary t_G so as to

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maintain constant Q and obtain the same (presumably optimized) band spacing.

We have encountered experimental situations where the above rule (when changing column size, hold Q constant for unchanged band spacing) has been found unreliable. Specifically, when column length was increased to improve the resolution of a 'critical' (i.e., most overlapped) band pair while holding Q constant, band spacing changed, and unanticipated changes in resolution were observed. A similar situation was encountered when column diameter was changed. This problem is illustrated in Fig. 1 for the separation of a mixture of pharmaceutical compounds. In Fig. 1a, with a 15-cm column and a gradient time of 20 min, the critical band pair is 7/8 (noted by *), for which resolution $R_s=1.0$. Since baseline resolution (i.e., $R_s \geq 1.5$) of each band-pair is preferred [1], one option is to increase column length while holding Q constant. By increasing column length from 15 to 30 cm (so that N increases by 2-fold, and resolution by $2^{1/2}$-fold) and gradient time from 20 to 40 min (to hold Q constant, other conditions the same), the minimum resolution was expected to increase to about $R_s=1.4$ (near-baseline separation) for bands 7/8, with the resolution of other bands also increasing by a ratio of 1.4 (with relative retention staying constant). The actual separation for $L=30$ cm and $t_G=40$ min was somewhat surprising (Fig. 1b), in that there is a significant change in relative retention; as a result, the resolution of band-pair 7/8 has increased to $R_s=2.7$ (vs. 1.4 expected), while the resolution of

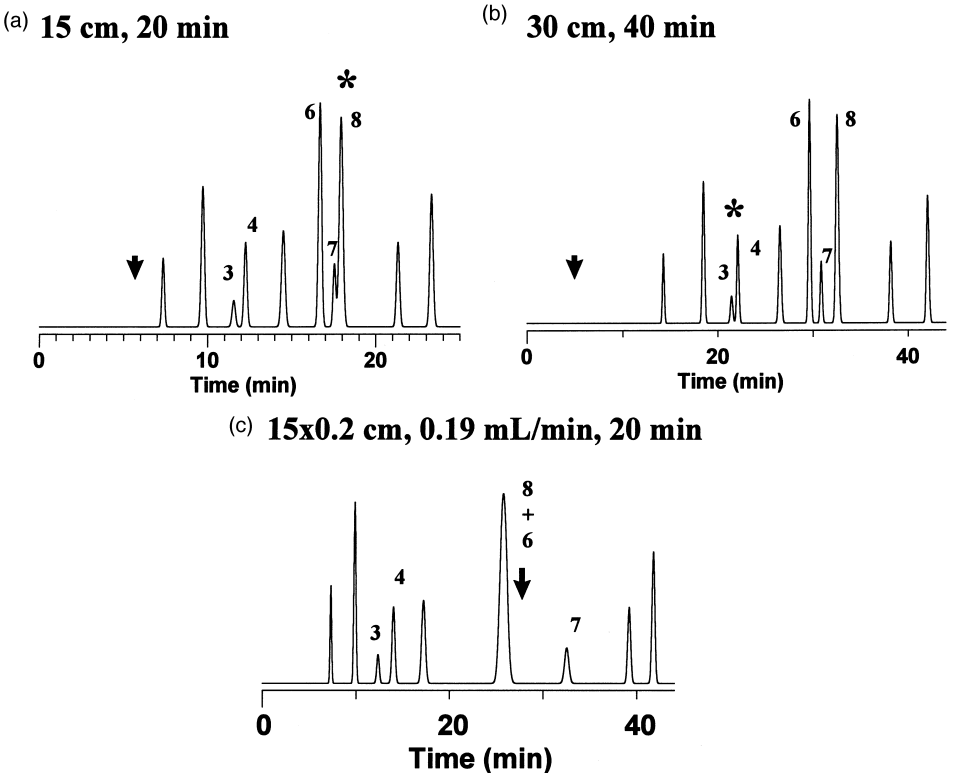


Fig. 1. Separation of 10-component drug sample with change in column dimensions. Sample: 1, phenylpropanolamine; 2, tranylcypromine; 3, amphetamine; 4, tripelennamine; 5, methamphetamine; 6, codeine; 7, *N*-acetylprocainamide; 8, phentermine; 9, β-hydroxytheophylline; 10, ethylmorphine. Conditions: (a) 15×0.46 cm C_{18}, 5-μm particle column; gradient from 0–15% acetonitrile in buffer in 20 min; flow-rate, 1 ml/min; temperature, 30°C; dwell volume, 5.2 ml. (b) Same as (a), except 30-cm column and 40-min gradient; (c) same as (a), except 15×0.20 cm column, 0.19 ml/min and 20-min gradient. Computer simulations based on data reported in [5]; $N=11\,200$ in (a,c) and 22 600 in (b). Arrows indicate arrival of gradient at column inlet in each run, and * indicates critical (least resolved) band pair.

band-pair 3/4 has been reduced from $R_s = 1.9$ to $R_s = 1.4$! In other cases which we have encountered, the critical-band-pair resolution has actually decreased when L is increased with Q held constant by a similar increase in gradient time. The problem arises in this case from a significant hold-up ('dwell') volume $V_D = 5.2$ ml for the gradient system, combined with only moderate retention of critical sample bands at the beginning of the gradient [the sample of Fig. 1 shows appreciable elution when aqueous buffer (0%B) is used as the mobile phase]. As a result, the actual gradient does not begin until 5.2 min after the sample is injected (noted by arrows in Fig. 1a,b) and during this time, early bands in the sample undergo isocratic migration through the column. It should be noted that the arrows in Figs. 1–6 define the time of arrival of the gradient at the column inlet. The arrival of the gradient at the column outlet is more relevant to the following discussion, and this occurs at a time t_0 later than indicated by the arrows (1.5 min in Figs. 1 and 6; 0.75 min in Figs. 2–5).

An even more pronounced change in relative retention and separation resulted for this sample when column diameter was reduced from 0.46 cm to 0.20 cm, while holding Q constant. When reducing column diameter, it is usually necessary to reduce flow-rate (by the ratio of d_c^2 values) in order to maintain constant pressure, which should then give the same separation if other conditions (including the gradient) are kept unchanged; note that this combined change in column diameter and flow-rate maintains both Q and the column plate number constant (assuming no extra-column band broadening). For the present 0.2-cm I.D. example, the new flow-rate was therefore set at 0.19 ml/min so that pressure (and Q) remained the same. The resulting separation, which should be compared with that of Fig. 1a, is shown in Fig. 1c. In this case, because of the large dwell volume (5.2 ml) and low flow-rate (0.19 ml/min), the gradient does not reach the column inlet for 28 min (arrow in Fig. 1c), which means that bands 1–5 are eluted isocratically before the gradient starts. The resulting effect on relative retention and resolution is profound. Bands 8/6 now coelute ($R_s = 0.3$), and the elution order of bands 6–8 has changed from $6<7<8$ in Fig. 1a to $8\approx6<7$ in Fig. 1c.

The following discussion will allow the problem illustrated in Fig. 1 to be anticipated for different samples and separations. We will also show how this problem can be avoided in practice.

2. Theory

The major application of HPLC under gradient conditions is for reversed-phase (RPLC) systems, which will be assumed in the following discussion. However, the general conclusions we will draw are also applicable to other HPLC modes (normal-phase, ion-exchange, etc.). For RPLC separation, solute retention factors k can be approximately related to the volume-fraction of organic solvent B in a mobile phase φ as:

$$\log k = \log k_w - S\varphi \tag{1}$$

where k_w is the (extrapolated) value of k for water as mobile phase ($\varphi = 0$), and S is a characteristic constant for each solute in the sample. Retention time in a linear-gradient separation can then be derived [4] as:

$$t_R = (t_0/b)\log(2.3k_0b + 1) + t_0 + t_d \tag{2}$$

Here, t_0 is the column dead-time, k_0 is the value of k at the start of the gradient, and b is a gradient steepness parameter given by:

$$b = t_0\Delta\varphi S/t_G = V_m\Delta\varphi S/t_G F = (\Delta\varphi/t_G)(V_m S/F) \tag{3}$$

where $\Delta\varphi$ is the change in the volume-fraction of B during the gradient ($\varphi = 0.01$%B), V_m is the column dead-volume, and F is the flow-rate. The quantity t_d is the equipment hold-up or 'dwell' time, equal to the hold-up or 'dwell' volume V_D divided by flow-rate. The equipment dwell volume is often assumed to be negligible ($V_D = 0$), which leads to the problem addressed by the present paper.

Relative retention t_R/t_0 can be defined, and from Eq. (2) (assuming $t_d = 0$):

$$t_R/t_0 = (1/b)\log(2.3k_0b + 1) + 1 \tag{4}$$

If column dimensions or flow-rate are changed, $t_0 = V_m/F$ will change. However, as long as Q is held constant (by varying t_G), b and t_R/t_0 remain constant

for each solute, and relative retention will not change. Eqs. (2) and (4) are actually only approximations when $V_D > 0$ [4], but it is still possible to calculate exact values of t_R as a function of k_0, t_0, b and V_D. Resolution in gradient elution can be expressed in terms that are similar to isocratic separation [1,4]:

$$R_s = (1/4)(\alpha - 1)N^{1/2}(k^*/[k^* + 1]) \quad (5)$$

where k^* (the average retention factor k during gradient elution) is given as:

$$k^* = 1/[1.15b + (1/k_0)] \quad (6)$$

For peaks that elute later in the gradient, and for which $k_0 \gg 1.15b$,

$$k^* = 1/1.5b \quad (6a)$$

2.1. Dwell-volume effects

Eq. (4), which predicts unchanging relative retention as long as b (and Q) are constant, assumes that $V_D = 0$. This situation changes when there is a significant dwell volume V_D. During the initial elution of the solute after injection, the gradient is delayed by a dwell time $t_D = V_D/F$, so that elution of the solute occurs (for a time t_D) under isocratic conditions with $k = k_0$. This is equivalent to a value of b that is smaller than given by Eq. (5) for the subsequent gradient ($b = 0$ for isocratic elution), so that the composite value of b (b'; i.e. effective gradient steepness) for a separation with sample preelution is therefore smaller. The effective gradient steepness b' is intended as a qualitative concept that merely reflects the obvious consequence of initial elution with $b = 0$, followed by elution with b given by Eq. (3).

Because the effect of preelution on b' will be greatest for early-eluting bands that undergo the most migration during preelution, b'/b will approach 1 for later-eluting bands, and become progressively smaller as t_R becomes smaller. This composite gradient steepness parameter b' will depend both on k_0 and the ratio of dwell volume to column volume (V_D/V_m). Unless both Q and (V_D/V_m) remain constant when changing column dimensions, b'/b will vary for different sample bands, and changes in relative retention are then possible. If V_D/V_m is held constant while changing column dimensions, relative retention (t_R/t_0) should remain unchanged.

3. Experimental

All 'experiments' described here are the result of computer simulation using DryLab for Windows software (Version 1.97, LC Resources). This software assumes that Eq. (1) is valid, but it is otherwise based on rigorous chromatographic theory [1,4], including the effect of gradient dwell volume on separation. Comparisons of actual gradient separations with predictions from DryLab usually show agreement of t_R values to better than 1% [1,4,7]. Separations of one sample used in the present studies (Figs. 1 and 6) correspond to 'real' experiments that are based on experimental data reported in [5]. Two hypothetical samples (Figs. 2–5) were also created for further evaluation and illustration of the effects of a significant dwell volume combined with low sample retention at the start of the gradient (Table 1). Sample A assumes $S = 4$ for every component of

Table 1
Sample characteristics and separation conditions assumed in present study (Figs. 2–5)[a]

Solute	Sample A		Sample B	
	Log k_w	S	Log k_w	S
1	0.00	4.0	0.01	4.4
2	0.02	4.0	0.03	3.6
3	0.30	4.0	0.31	4.4
4	0.32	4.0	0.33	3.6
5	1.00	4.0	1.04	4.4
6	1.02	4.0	1.00	3.6
7	1.30	4.0	1.57	4.4
8	1.32	4.0	1.47	3.6
9	2.00	4.0	2.13	4.4
10	2.02	4.0	1.93	3.6

[a] Separation conditions: 0–100% B gradient; 20°C; column length varies and column I.D. = 0.46 cm; N equal 10 000 for 15-cm column; N is adjusted ('corrected') to $N = 10\,000$ in some cases for a change in column length, resulting in corrected resolution values R_s.

the sample, while sample B is composed of closely adjacent pairs of compounds (1/2, 3/4, etc.) which differ in their values of S by 20%. The following discussion relates to the relative retention and resolution of these adjacent band-pairs. The assumption of an average value of $S=4$ for both samples is based on the observation that $S\approx4$ for samples with molecular weights of masses 150–400 [6]. The variation of individual solute S-values within a given sample can exceed $\pm20\%$ but is usually less [7,8]. We will see that as b is varied (e.g., by change in t_G), relative retention varies little for sample A of Table 1; conversely, relative retention for sample B changes markedly with b. Actual samples typically show an intermediate behavior between that of samples A and B. Finally, the samples of Table 1 were also selected for initial retention values that varied from low to intermediate (small values of k_0), because samples of this kind are most subject to dwell-volume effects. See the further discussion of [8,9].

4. Results and discussion

4.1. Sample differences

The samples A and B were chosen to illustrate two limiting (but not uncommon) cases: sample A, for which relative retention does not change with gradient time, and sample B, whose relative retention varies with gradient time. This is illustrated in Fig. 2 for two different gradient times (20 and 120 min, other conditions the same). A zero dwell volume ($V_D=0$) is assumed in this example. In both cases, the overall sample resolution (or peak capacity) is increased for the longer (120-min) gradient, but only sample B (Fig. 2c,d) shows changes in relative retention (band reversals of 5/6, 7/8 and 9/10) when t_G is changed. Note that band reversals are only an extreme example of changes in band spacing; smaller changes in band spacing (two peaks moving together) can result in a loss in resolution (cf. bands 1/2 and 3/4 in Fig. 2d vs. 2c). The sample

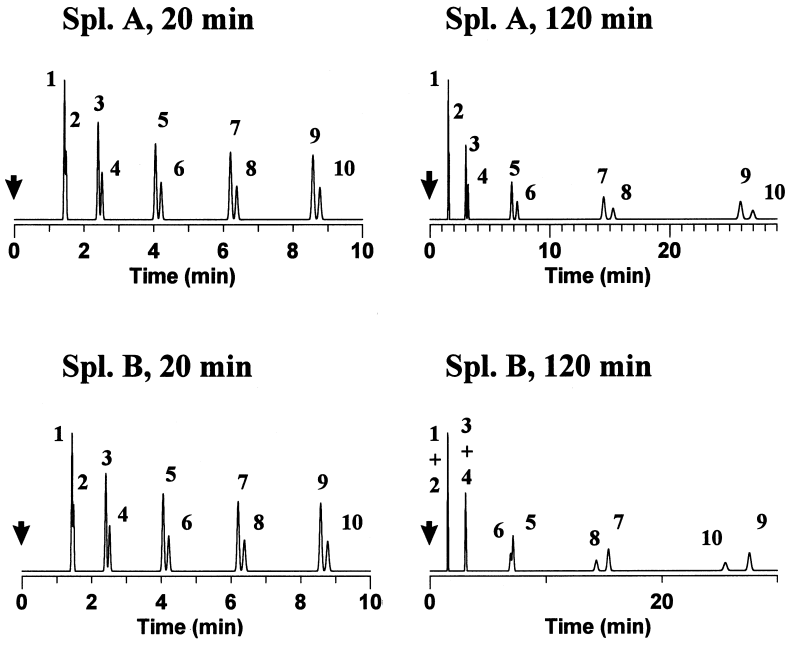


Fig. 2. Computer simulations of hypothetical samples A and B of Table 1 with gradient time varied. Conditions: column, 15×0.46 cm C_{18} column; 0–100% acetonitrile in buffer gradient; flow-rate, 2.0 ml/min; temperature, 20°C; $N=10\,000$; dwell volume, 0.0 ml. (a) Sample A, 20-min gradient time; (b) sample A, 120-min gradient; (c) sample B, 20-min gradient; (d) sample B, 120-min gradient. Arrows indicate start of gradient.

differences illustrated in Fig. 2 are described quantitatively in Table 2, where the resolution of peak-pairs 1/2, 3/4, etc. are listed for each sample and gradient time.

The data of Table 2 merit attention, as this will (a) further clarify the reasons for our choice of these two samples and (b) provide some useful background for the following discussion. Samples A and B were selected to give a similar separation of 'critical' peak-pairs 1/2, 3/4, etc. for a gradient time of 20 min. The isocratic separation factor α is constant and equal to 1.05 for sample A, while values of α for sample B vary with %B or gradient time.

As gradient time increases for sample B, the first band in each adjacent band-pair (1/2, 3/4, etc.) moves toward the second band; e.g., band-1 moves toward band-2, band-3 toward band-4, etc. The relative movement of the first adjacent band (Nos. 1,3,5,...) toward the second band (Nos. 2,4,6,...) is less for smaller values of k_0 (early bands), because this change in relative retention increases for a larger change in k^* between the two runs (20- and 120-min gradients). From Eq. (6) we see that a change in gradient time and b results in less change in k^* for less-retained bands having a smaller value of k_0. The changes in relative retention for sample B in Fig. 2d vs. 2c are the result of different S-values for each pair of adjacent bands (1/2, 3/4, etc.; see 8,9). For band-pairs 1/2 and 3/4, the first band moves toward the second band, but not by enough to cause band inversion (as in later pairs 5/6, 7/8 and 9/10).

When the gradient time is increased from 20 to 120 min for sample A (thereby decreasing b), selectivity does not change, but k^* values increase (Eq. (6)). This increase in k^* accounts for the small increases in R_s (+0.2–0.4; see Eq. (5)) for the adjacent bands of sample A in the 120-min vs. 20-min runs of Table 2. A change in gradient time for sample B results in much more pronounced changes in resolution (Table 2), as a result of the different S-values for each adjacent peak-pair. Such changes in selectivity with change in gradient time are fairly common [8,9], which allows band spacing to be improved by a suitable choice of gradient time. Changes in relative retention as gradient time is varied (as in Fig. 2c,d) are indicative of a sample whose relative retention can change when changing column dimensions while holding Q constant (because $V_D > 0$).

4.2. Column dimension and flow-rate effects: zero dwell volume

An examination of this case will provide a basis for discussing the effect of a nonzero dwell volume on relative retention when L, d_c or F is varied, holding Q constant. Our intention is to examine possible changes in selectivity for this situation and to quantify these changes in terms of changes in resolution (as in Table 2). When L is changed, the plate number N will also change (also affecting resolution), but we prefer to ignore this effect in the discussion of following examples (Figs. 3–5) based on computer simulations for samples A and B of Table 1. We have therefore adjusted N to a value of 10 000 for each simulation, regardless of changes in L. Reported values of R_s (Table 3) are also adjusted for $N = 10\,000$, so as to eliminate the effect on

Table 2
Resolution of overlapping peak-pairs in Fig. 2 for samples A and B; 15×0.46-cm column, $F = 2.0$ ml/min, $V_D = 0$, $N = 10\,000$

Peak-pair	Resolution R_s				Change in R_s[a]		% change in R_s	
	$t_G = 20$ min		$t_G = 120$ min					
	A	B	A	B	A	B[a]	A	B
1/2	0.84	0.84	1.04	0.60	0.20	−0.24	+24%	−28%
3/4	1.29	1.29	1.49	0.47	0.20	−0.81	+16	−63
5/6	1.47	1.47	1.82	−0.86[b]	0.25	−2.33	+24	−158
7/8	1.48	1.47	1.91	−2.54[b]	0.42	−4.01	+29	−210
9/10	1.58	1.58	1.97	−4.02[b]	0.39	−5.60	+25	−284

[a] R_s calculated as $2(t_2 - t_1)/W$, where t_1 is the value for bands 1,3,5,7,9 and t_2 is the value for bands 2,4,6,8,10; this provides a measure of migration of the first band toward the second.
[b] Band reversal.

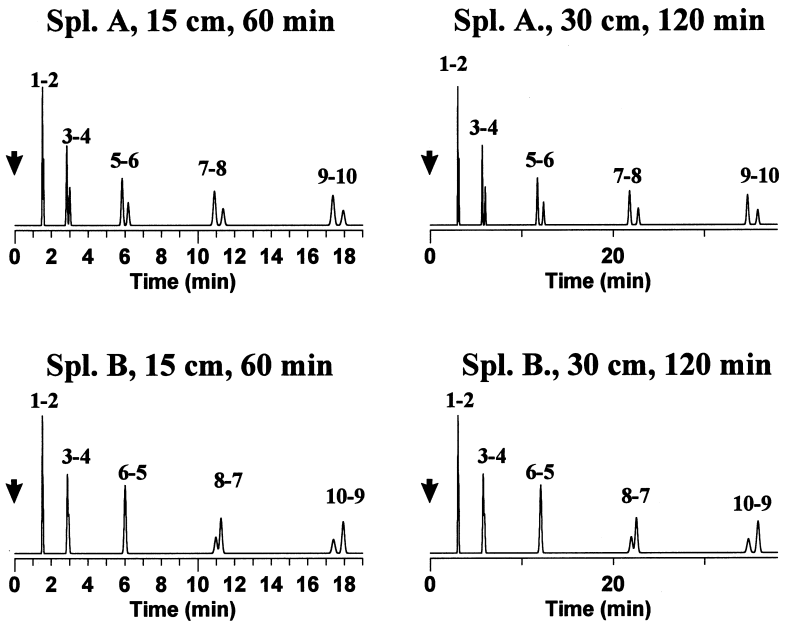


Fig. 3. Computer simulations of hypothetical samples A and B of Table 1 with column length varied while holding Q constant (zero dwell volume). Conditions as in Fig. 2, except where noted otherwise. (a) Sample A, 15-cm column, 60-min gradient; (b) sample A, 30-cm column, 120-min gradient; (c) sample B, 15-cm column, 60-min gradient; (d) sample B, 30-cm column, 120-min gradient. Arrows indicate start of gradient. Column plate number is fixed at $N = 10\,000$, regardless of column length.

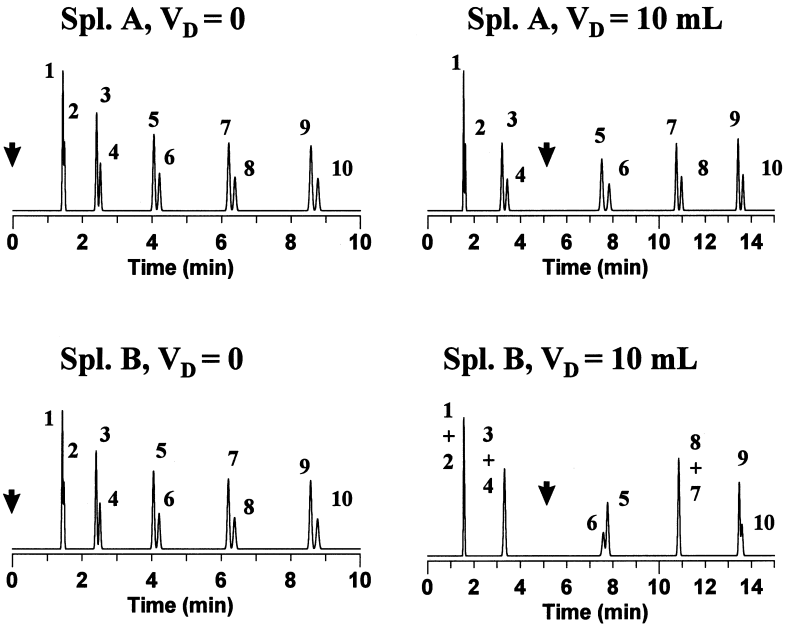


Fig. 4. Computer simulations of hypothetical samples A and B of Table 1 with change in dwell volume. Conditions as in Fig. 2a,c, except for change in dwell volume. (a) Sample A, 15-cm column, 20-min gradient; dwell volume = 0; (b) same as (a), except 10-ml dwell volume; (c) sample B, other conditions as in (a); (d) sample B, other conditions as in (b). Arrows indicate start of gradient.

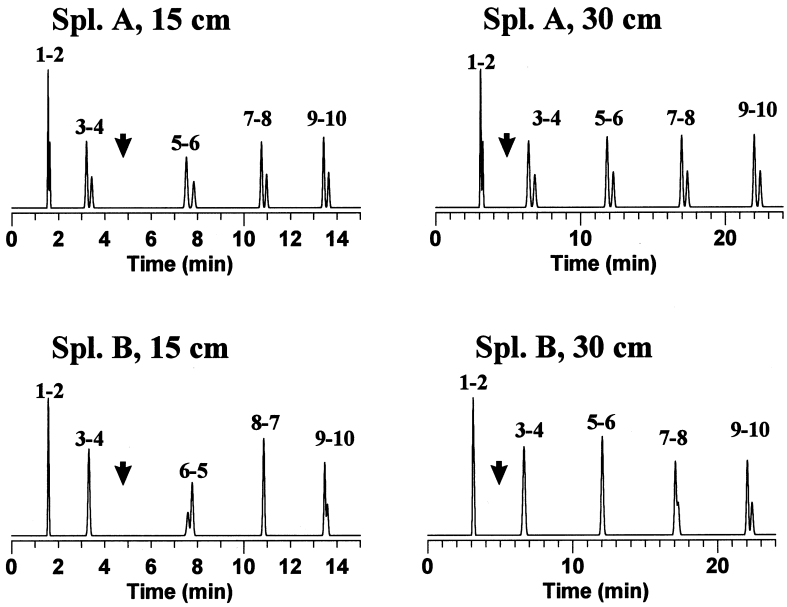


Fig. 5. Computer simulations of hypothetical samples A and B of Table 1 with change of column length holding Q constant (dwell volume = 10 ml). Conditions as in Fig. 4b,d, except for change in column length. (a) Sample A, 15-cm column, 20-min gradient; (b) sample A, 30-cm column, 40-min gradient; (c) sample B, 15-cm column, 20-min gradient; (d) sample B, 30-cm column, 40-min gradient. Column plate number is fixed at $N = 10\,000$, regardless of column length.

Table 3
Resolution R_s (for $N = 10\,000$[a]) of adjacent peak-pairs for samples A and B (V_D varies, $d_c = 0.46$ cm, Q held constant)

Peak-pair	Resolution R_s ($t_G = 20$ min)/(change in R_s)[b]					
	(Fig. 4a, c) $V_D = 0$ ml $L = 15$ cm $t_G = 20$ min		(Fig. 4b, d) $V_D = 10$ ml $L = 15$ cm $t_G = 20$ min		(Fig. 5b, d) $V_D = 10$ ml $L = 30$ cm $t_G = 40$ min	
	A	B	A	B	A	B
1/2	0.84	0.84	1.09 (0.25)	0.54 (−0.30)	1.09 (0.25)	0.54 (−0.30)
3/4	1.29	1.29	1.54 (0.25)	0.18 (−1.11)	1.51 (0.22)	0.18 (−0.63)
5/6	1.47	1.47	1.76 (0.29)	1.03[c] (−2.50)	1.60 (0.13)	0.15 (−1.32)
7/8	1.48	1.47	1.57 (0.09)	0.03[c] (−1.50)	1.52 (0.04)	0.73 (−0.74)
9/10	1.58	1.58	1.60 (0.02)	0.87 (−0.71)	1.59 (0.01)	1.23 (−0.35)

[a] Resolution values based on $N = 10\,000$, regardless of column length.
[b] Values in parentheses are change in R_s vs. values of R_s.
[c] Band reversal.

resolution of changing N, and simplify the interpretation of changes in relative retention when changing column length.

Fig. 3 illustrates the consequences of changing L while holding Q constant for each sample ($V_D = 0$). In each case, relative retention and resolution are unchanged as a result of changing L, as expected for equipment having zero dwell-up volume. We will contrast this behavior for $V_D = 0$ with following examples which involve a dwell volume $V_D = 10$ ml (an upper limit on values of V_D for most HPLC systems [1,10]).

4.3. Effect of changes in dwell volume (other conditions constant)

Before examining changes in column size, it is useful first to compare the effects of change in dwell volume vs. change in gradient time. In Fig. 2, it was seen that a change in gradient time leads to changes in relative retention for sample B (because of

differences in the *S*-values of adjacent band-pairs 1/2, 3/4, etc.). Similar changes in relative retention can also occur when the dwell volume is changed while holding other conditions constant [10,11], as illustrated in Fig. 4 and Table 3. In Fig. 4a,b, it is seen that a change in dwell volume has little effect on the relative retention of Sample A. As shown in Table 3, the change in resolution for this 10-ml increase in dwell volume for sample A is relatively small (0.02–0.29 units of R_s), and can be attributed to a small decrease in values of b' for each band as V_D increases (similar to the case where gradient time was varied; Fig. 2a,b and Table 2).

For the same change in dwell volume for sample B (Fig. 4c,d), significant changes in relative retention occur, accompanied by major changes in resolution (0.30–2.50 units of R_s). This is similar to the case where gradient time was varied (Fig. 2c,d and Table 2) and is the result of the same sample characteristic: different values of *S* for adjacent band-pairs. Thus, samples that show changes in relative retention as t_G is varied are likely to show changes in relative retention as the dwell volume is varied. This latter behavior is of considerable practical interest, because it can lead to failure of a routine gradient method when transferred to an instrument with a different dwell volume. However, this dependence of relative retention on dwell volume has been discussed previously (10,11) and need not concern us further here.

4.4. Column dimension and flow-rate effects: nonzero dwell volume

In Fig. 5, we continue these simulated examples for the case of varying column length (holding *Q* constant) and equipment having a dwell volume of 10 ml. For sample A, a change in *L* from 15 to 30 cm (a,b) does not lead to significant changes in either relative retention or resolution (Table 3; 0.01–0.25 units change in R_s). For sample B, the same change in *L* leads to major changes in relative retention (Fig. 5c,d) and resolution (Table 3; 0.30–1.32 units change in R_s).

As seen in Table 3 for the above separations (Fig. 5c,d) of sample B, where column length is varied and the dwell volume is significant, the change in resolution is relatively small for early adjacent bands (1/2, 3/4), is largest for intermediate bands (1.3 units of R_s for 5/6), and then decreases for later bands (0.7 and 0.3 units of R_s for 7/8 and 9/10, respectively). This pattern is typical of what can be expected in other examples that involve samples whose component *S*-values vary and where $V_D \gg 0$. Early bands are less retained and therefore less affected by dwell-volume effects, just as in the example of Fig. 2d (where gradient time was changed) and for the same reasons. Late-eluting bands are held at the column inlet during isocratic preelution by the held-up mobile phase, and therefore the separation of these bands is also less affected by differences in dwell volume. Intermediate bands therefore show the largest changes in relative retention and resolution.

4.5. Correcting for dwell-volume effects when changing column dimensions

In the Section 2, it was concluded that changes in relative retention can be avoided when changing column dimensions while holding *Q* constant, providing that the ratio V_D/V_m is held fixed. Therefore, if V_m is to change (as a result of change in *L* or d_c), we must adjust V_D to meet this condition. If there is an increase in V_m (as when increasing column length), it is easy to increase the equivalent dwell volume V'_D, simply by adding an isocratic hold at the beginning of the gradient so as to make the sum of V_D plus this isocratic-hold volume (V_{iso}, equal to the isocratic-holdup time$\times$flow-rate) equal to V'_D. In the case where the column volume V_m can decrease, either the dwell volume must be decreased, or an isocratic hold must be used for the initial separation (prior to optimizing band spacing and changing column size), so as to increase V'_D in the separation with the first column and allow its subsequent reduction for the second column (by reducing or eliminating V_{iso}).

We can illustrate this for the drug sample of Fig. 1. Fig. 6a repeats the separation of Fig. 1a for a 0–15% B gradient in 20 min (15$\times$0.46 cm column, 1 ml/min). The column length is to be increased to 30 cm, and in Fig. 1b this led to changes in relative retention and resolution (*Q* held constant by increasing t_G to 40 min). Since the column volume is doubled, the equivalent dwell volume V'_D must be doubled (V'_D/V_m held constant) to avoid these changes in separation. This can be achieved by an

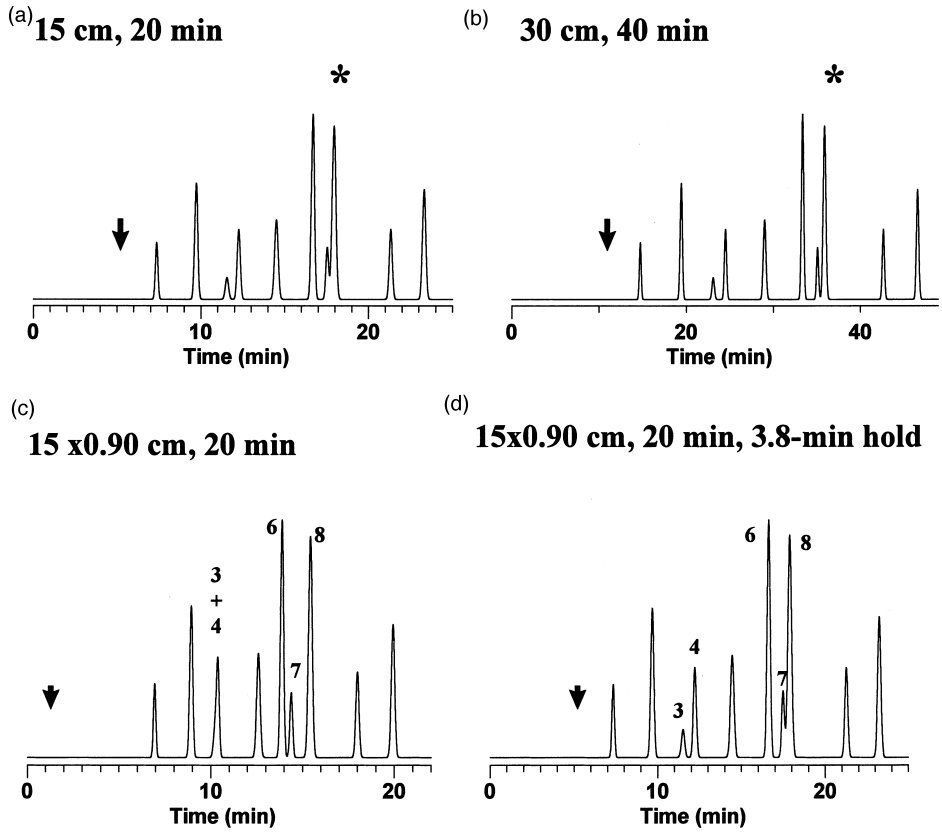


Fig. 6. Separation of sample of Fig. 1 where column length or diameter is increased (Q constant) and the gradient is delayed so as to maintain the ratio of dwell volume to column volume (V_D/V_m) constant. (a) Same separation as in Fig. 1a (15-cm column, 20-min gradient); (b) column length increased to 30 cm, gradient time increased to 40 min, and gradient delayed by a time V_{iso}/F so as to maintain V'_D/V_m and relative retention constant. $N=11\,200$ in (a) and 22 600 in (b); (c) Separation as in Fig. 1a, except column diameter increased from 0.46 to 0.90 cm; flow-rate increased to 3.8 ml/min to maintain pressure constant; $V'_D=V_D$, so V_D/V_m changes vs. the separation of (a); (d) separation as in (c), except with an initial gradient hold (0/0/15%B in 0/3.8/23.8 min) to maintain V'_D/V_m and band spacing constant.

initial isocratic delay, so that the dwell volume of $V_D=5.2$ ml for the 15-cm column is increased to $V'_D=10.4$ ml for the 30-cm column; i.e., by an isocratic hold of $V_{iso}=5.2$ ml, or a gradient delay of 5.2 min (at 1 ml/min). Fig. 6b shows the resulting separation, which is seen to give the same relative retention as in Fig. 6a. Because the column plate number has been doubled, the resolution of all band pairs is increased by a factor of $2^{1/2}\approx 1.4$.

A second example involving the same sample is shown in Fig. 6c,d. It was desired to scale up this separation, by increasing the column diameter from 0.46 to 0.90 cm. To maintain pressure constant, the flow-rate was increased by $(0.90/0.46)^2=3.8$ ml/min; other conditions were kept the same. If this separation had been carried out on a zero-dwell-volume instrument, or if the sample had more closely resembled sample A (similar S-values for all components), little change in relative retention would be expected. Since neither of these conditions holds for this example, it is not surprising to see significant changes in relative retention for the separation of Fig. 6c vs. that of Fig. 6a. In Fig. 6c, band-pair 3/4, which was previously separated to baseline (Fig. 6a), is now completely unresolved. Similarly, band 7 has moved relative to bands 6 and 8.

Again, the problem of Fig. 6c can be resolved by increasing the equivalent dwell volume so as to make

V'_D/V_m the same in both runs (where column diameter is increased). Since the column volume is increased by 3.8-fold for the 0.90-cm column, the equivalent dwell volume must also be increased by this ratio: $3.8 \times 5.2 = 19.9$ ml. The net increase in V_D equals V_{iso} which is then $19.9 - 5.2 = 14.7$ ml; for a flow-rate of 3.8 ml/min, this corresponds to an initial gradient delay of $14.7/3.8 = 3.9$ min. Fig. 6d shows the resulting separation with this isocratic hold: 0/0/15%B in 0/3.9/23.9 min. An equivalent separation is obtained as in Fig. 6a, which is the desired result.

It can be seen in the latter example (Fig. 6) that changes in relative retention and resolution are smaller than observed in Fig. 5 for sample B and similar changes in column dimensions. Sample B was chosen to maximize dwell volume effects so as to more clearly see changes in relative retention and resolution. The drug sample of Fig. 6 is more typical of what can be expected when column dimensions are changed for typical samples and equipment dwell volumes. It should be emphasized that anticipating dwell volume effects and changing conditions to minimize changes in relative retention can be achieved as long as we know the dwell volume of the equipment. Computer simulation, while useful for optimizing other separation conditions, is not required for the above adjustments in effective dwell volume.

An alternative approach to adjusting the equivalent dwell volume V'_D, when changing column size, is to eliminate the effect of dwell volume on separation at the beginning of method development. Several such procedures have been described (pp. 390–92 of Ref. [1]), but not all of these are applicable and convenient for a particular HPLC instrument.

4.6. Situations that can promote changes in relative retention when column dimensions are varied

The likelihood that a change in column size will lead to unwanted changes in relative retention can be assessed to some extent from a knowledge of the sample and an inspection of the initial chromatogram (prior to a change of column). The magnitude of any such change in relative retention will increase with the value of V_D.

4.6.1. Changes in relative retention with gradient time

As illustrated in Figs. 2 and 5, samples such as B which exhibit important changes in relative retention as gradient time is varied can also show changes in relative retention when column size is changed. Band-pairs that elute early in the gradient, but after the arrival of the gradient at the column inlet (arrows in each figure), will be most affected (Table 3). Therefore, if any of these intermediate band pairs are 'critical', a change in relative retention may result in loss of resolution. So, if critical band-pairs elute just after the arrival of the gradient at the column inlet and show changed relative retention when gradient time is changed (as in Fig. 2), there is a likelihood that relative retention will change (with possible loss of resolution) when the column size is changed (Q constant). If two such bands move together when gradient time is increased, they will move apart when column volume V_m is increased (note examples for sample B in Fig. 2 vs. 5), and vice versa (this rule only applies for band-pairs which do not reverse their relative retention when changing t_G or V_m).

4.6.2. Effect of sample molecular mass

Higher molecular mass samples will be less subject to changes in relative retention with column size, other factors equal. The reason is that these samples generally have larger S-values, as shown by the approximate relationship [12,13]:

$$S \approx 0.5(M_r)^{1/2} \quad (7)$$

Thus, protein samples with molecular masses $M_r >$ 10 000 will have S-values in excess of 50, and in most cases these compounds will either be strongly retained at the beginning of the gradient (with large k_0, and therefore unaffected by the dwell volume), or they will elute at the beginning of the chromatogram (with small k_0, and again unaffected by the dwell volume). The relative retention of samples having molecular masses of 1000–10 000 (e.g., peptides, small proteins) can be affected by dwell-volume effects, but to a lesser extent than small solutes of the kind examined in this study.

4.7. Practical application

4.7.1. Method development

When developing a gradient method, we start with a particular HPLC instrument and column, so that V_D and V_m are initially determined. The next step is to adjust gradient conditions (initial and final %B, gradient time), followed by changes in selectivity or band spacing if necessary [1]. Further improvement of the separation depends on whether resolution and/or run time are acceptable at this point. If an increase in R_s is desired, this can be achieved by increasing column length and/or decreasing flow-rate. If resolution is more than adequate, a reduction of run time can be achieved by shortening the column and/or increasing flow-rate. The preceding discussion suggests that we may need to either increase or decrease V'_D as part of any final adjustment of column length.

If a possible reduction in column length is anticipated, it is suggested that V'_D be increased during method development on the initially-used column. This approach seems worth considering, if a short run time is an important criterion for the final method. Alternatively, we have often found that changes in relative retention due to change in column length (as in Fig. 1b) can be compensated to some extent by reoptimizing gradient time after the change to the second (longer or shorter) column. It is not possible in this way to achieve the same relative retention (for the entire chromatogram) as for the initial column, but often a satisfactory resolution of the critical band-pair(s) can be achieved. The reason is that the effect on relative retention of a change in V_D/V_m is similar to the effect of a change in b (by changing t_G).

4.7.2. Method scale-up

An attractive approach to developing a preparative-scale separation is to first optimize conditions (including sample size) for a smaller-diameter column (e.g., 0.46 cm I.D.; see Ch. 13 of [1]). Then, if the same packing material is available in a larger-diameter column of the same length, it is possible to obtain the identical separation on the latter column by maintaining the same conditions, except for an increase in flow-rate and sample size by a factor equal to the ratio of column volumes. Method development experiments on the larger column are undesirable, because of the large amounts of sample and solvent involved. For this and other reasons, it is important to consider the possible effects of instrument dwell volume on scale-up carried out in this way. While dwell volume effects may not be critical for the separation of a given sample, there is no reason to risk possible changes in separation when repeating the small-column separation on the larger column. An isocratic hold at the beginning of the large-column gradient, so as to maintain V'_D/V_m constant, will guarantee exact comparability of the two separations (large vs. small column).

4.7.3. Change to a smaller-diameter column

The possible need for an initial dwell volume that can be reduced upon reducing column diameter has been discussed. Other considerations suggest a need for smaller-dwell-volume equipment when column diameter is reduced by a large factor.

5. Conclusions

A change in column dimensions or flow-rate in gradient elution, if accompanied by a calculable change in gradient time, can allow relative retention to remain constant. This is desirable when separation selectivity has first been optimized, and it is then required to (a) change column length for better resolution or shorter run time, or (b) change column diameter for either micro or preparative application. However, this approach may not work for some samples, when the instrument hold-up ('dwell') volume is significant. Instead of predictable separation after a change in column size, relative retention may change significantly, and the observed separation with the new column may be disappointing.

The likelihood of adverse changes in relative retention and separation (as above) increases with the following factors:

A larger hold-up volume for the HPLC system.

Samples which show larger changes in relative retention when gradient time is changed.

Samples where the most overlapped ('critical') band-pair elutes shortly after the gradient reaches the column inlet (i.e., after the isocratic elution of early peaks before the gradient).

Lower molecular mass samples.

When undesired changes in relative retention are expected for a change in column size, it is possible to maintain the same relative retention and resolution by adjusting the equivalent dwell volume V'_D. V'_D is equal to the equipment dwell volume V_D plus the volume (V_{iso}) of mobile phase in an isocratic hold at the beginning of the gradient. When column size is increased, V_{iso} should be increased so that V'_D/V_m is held constant (V_m is the column dead volume, proportional to column volume). If column size is to be decreased, it is necessary to make $V'_D < V_D$. This can be achieved by returning to the separation on the original column, adding an initial isocratic hold so as to increase V_D at this point in method development, optimizing relative retention and resolution, then reducing V'_D for the smaller (second) column by decreasing the time for the isocratic hold.

In our experience, dwell-volume-related problems in maintaining acceptable resolution during a change in column length are infrequent and can often be compensated for by small changes in gradient time. Alternatively, and much simpler, the effect of equipment dwell volume on such separations can be eliminated by late injection of the sample for both columns, so that the gradient arrives at the column inlet at the same time the sample is injected (however, this alternative is not available for all HPLC systems). Changes in selectivity when going from a small- to a large-diameter column for preparative separation are a more common and serious possibility. In the latter case, we recommend routinely adding an isocratic hold for the large-column separation so as to eliminate changes in relative retention.

6. Glossary of terms

b	Gradient steepness parameter (Eq. (3))
b'	An effective gradient steepness parameter, which is less than b from Eq. (3) because of initial migration of the solute band under isocratic conditions (for which b=0)
B	B-solvent (organic) for the gradient; %B refers to vol.%
d_c	Column internal diameter (cm)
F	Flow-rate (ml/min)
I.D.	Column internal diameter (cm); equal to d_c
k	Retention factor
k_w	Extrapolated value of k for $\varphi=0$ (Eq. (1))
k^*	Average or effective value of k for a solute during gradient elution (Eqs. (6) and (7))
L	Column length (cm)
N	Column plate number
Q	Gradient parameter equal to $(t_G F)/(L d_c)$
R_s	Resolution of two adjacent bands
S	Solute parameter defined by Eq. (1)
t_G	Gradient time (min)
t_0	Column dead time (min)
t_R	Retention time (min); (Eq. (2))
V_D	Instrumental hold-up or 'dwell' volume (ml)
V'_D	Equivalent dwell volume, equal to V_D+V_{iso} (ml)
V_{iso}	The total volume of mobile phase in an initial isocratic hold (ml)
V_m	Column dead-volume (ml)
α	Separation factor for two adjacent bands
φ	Volume-fraction of B solvent in the mobile phase ($\varphi=0.01\times$%B)
$\Delta\varphi$	Change in φ during the gradient

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