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Initial experiments in high-performance liquid chromatographic method development
I. Use of a starting gradient run

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Abstract

A single gradient elution run with acetonitrile (B)–water (A) as mobile phase can be used to estimate preferred conditions for subsequent method development experiments based on reversed-phase high-performance liquid chromatography. For a broad range of sample types, that includes both very hydrophilic and hydrophobic compounds, it was found that isocratic retention is given by $\log k \approx \log k_w - 4.2\varphi$, where $\varphi = 0.01$ %B. An initial gradient run allows values of $\log k_w$ to be estimated for each compound in the sample, which then permits compound retention to be approximated as a function of either isocratic or gradient experimental conditions.

The use of an initial gradient run in this way provides a rational basis for the subsequent development of a final HPLC method. Predictions based on this initial run can be used to (a) select between isocratic or gradient elution for further experiments and the final method, (b) choose a value of %B to achieve a desired value of k for the initial or final band in isocratic separation and/or (c) choose values of %B for the initial and final mobile phase in gradient elution. The present approach is based on a wide range of sample types and different reversed-phase columns; for this reason it is expected to be reasonably general and accurate. Errors in subsequent predictions based on an initial gradient run can also be estimated as a function of experimental conditions, allowing the selection of conditions for minimum error.

Keywords: Method development; Gradient elution; Mobile-phase composition; Anxiolytics; Pesticides; Benzenes; Phenazines; Triazines; Polynuclear aromatic hydrocarbons

1. Introduction

Reversed-phase HPLC method development is often best begun with a separation by gradient elution, which can be used to determine whether isocratic or gradient elution is more appropriate for a given sample [1,2]. If isocratic elution is preferred, the gradient run allows an estimate of the best mobile-phase strength (value of %-organic or %B) for isocratic separation. If gradient elution is required, estimates of the best gradient range (initial and final %B) are possible. Different procedures have been used to estimate an optimum %B from a starting gradient run [1–7]; one of the more well thought out approaches is that of de Galan and co-workers [2,6]. The latter

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procedure begins with a methanol–water gradient, which then allows the prediction of the best %B value for isocratic separation with any of the three commonly used organic solvents: methanol, acetonitrile or tetrahydrofuran (THF).

Reversed-phase HPLC method development is better started (and, if possible, completed) with acetonitrile as solvent, rather than methanol. Acetonitrile–water solutions are generally less viscous and allow detection in the low UV (<210 nm). This preference for acetonitrile–water mobile phases prompted us to re-examine the de Galan approach for application to an initial separation based on an acetonitrile–water gradient (instead of methanol–water). This in turn led to a better understanding of the factors involved in accurate predictions from an initial gradient run.

2. Theory

2.1. Procedure of de Galan and co-workers [2,6]

This use of an initial gradient run for HPLC method development can be summarized as follows. Solute retention as a function of mobile phase composition (%B) is approximated as

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

where k is the retention factor, φ is the volume fraction of organic in the mobile phase ($\varphi = 0.01$ %B), k_w is the value of k for water as mobile phase ($\varphi = 0$), and S is a constant for a given solute and organic solvent (e.g., methanol). Values of k_w and S for each solute in a sample can be estimated from an initial gradient run via the following Eqs. 2–4:

$$b = V_m \Delta\varphi S / t_G F \quad (2)$$

where b is gradient steepness, V_m is the column dead volume (ml), $\Delta\varphi$ is the change in φ during the gradient, t_G is gradient time (min), and F is flow-rate (ml/min);

$$t_R = (t_o/b)\log(2.3k_o b + 1) + t_o + t_D \quad (3)$$

where t_R is solute retention time (min) in the initial gradient run, t_o is column dead time, k_o is the value of k at the beginning of the gradient (for $\varphi = \varphi_o$), and t_D is the dwell time (equal to V_D/F, where V_D is the equipment dwell volume);

$$S = p + q(\log k_w) \quad (4)$$

For methanol as solvent and different solutes (other HPLC conditions the same), p and q are assumed constant. When the experimental conditions (t_G, F, V_m, φ_o, V_D) are known, an experimental value of t_R then defines values of S and k_w. This in turn allows predictions of isocratic retention from Eq. 1 or gradient retention from Eq. 3.

2.2. Possible errors in the de Galan procedure

Generality of Eq. 4

Herman et al. [6] and others [5] have commented on the fact that Eq. 4 is assumed to apply to any sample, yet the values of p and q assumed in the de Galan procedure are based on only a limited number of compounds that cluster within a narrow retention range in the initial gradient run. Schoenmakers et al. [8] reported that Eq. 4 describes mobile phases that contain methanol or THF, but not acetonitrile. Other studies (see Ref. [9] for a review) find that Eq. 4 can be applicable for all three of these organic solvents, but values of p and q show considerable variation among different studies and samples: (methanol) $0.2 < p < 1.5$, $0.3 < q < 3.0$; (acetonitrile) $0.4 < p < 2.4$, $0.7 < q < 1.5$. Another study [10] found different values of p and q for 20 C_{18} columns, which required a redetermination of these parameters for each column. These observations raise questions concerning the use of Eq. 4 with fixed values of p and q for predictions of retention from a single gradient run. While Schoenmakers et al. [7] note that predictive accuracy is not much affected by the assumption of different values of p and q, possible variations in p or q of five- to ten-fold are a cause for concern.

Equipment dwell volume

The de Galan procedure [2,6] ignores the effects of equipment hold-up volume (dwell

volume V_D) on sample retention. Commercial HPLC systems can have values of V_D that vary from 0.5 to 8 ml [11]. For the recommended gradient time of Ref. [4] ($t_G = 30$ min) and customary flow-rates (e.g., 1 ml/min), uncertainty in values of V_D can create large errors in estimates of isocratic retention using the de Galan method. Herman et al. [6] recommend delaying sample injection until the time the gradient reaches the sample loop (in which case the dwell volume can be ignored), but this procedure is inconvenient for many HPLC systems. In some cases, the value of V_D for the HPLC system may not be known. A procedure that minimizes the effect of dwell volume on gradient retention would therefore be more useful.

2.2. Basis of the present procedure: Use of acetonitrile in the initial gradient run

The data of Schoenmakers et al. [8] show a poor correlation ($r = -0.06$) with Eq. 4 for acetonitrile as solvent, but values of S in the latter study are relatively constant (±10%, 1 R.S.D.). In the present study, where we have examined a larger number of compounds taken from a more diverse range of sample types (Table 1), we also observed for acetonitrile as solvent and C_8 or C_{18} columns that values of S fall within a relatively narrow range: $S = 4.2 \pm 0.8$ (1 S.D.). This suggested the following approach to the use of an initial acetonitrile/water gradient run for use in HPLC method development.

For solutes that elute early in the gradient, the dwell volume V_D can result in pre-elution of the solute and a shorter retention time t_R than is predicted by Eq. 3. It is possible to correct for this effect [16] and to calculate a more accurate value of t_R as follows. The fractional migration through the column during pre-elution is $x = t_D/(t_o k_o)$, and solute retention is given as

$$t_R = (t_o/b)\log[2.3k_o b(1-x)+1] + t_o + t_D \qquad (5)$$

Beginning with Eqs. 2 and 5, assuming that $S = 4.2$, and given the experimental conditions for the initial gradient run, a value of k_w can be estimated from an experimental value of t_R. If we define $T_R = t_R - t_o - t_D$ and $c = b/t_o$, then

$$k_o = \frac{10^{cT_R} + 2.3ct_D - 1}{2.3ct_o} \qquad (6)$$

Given the initial value of φ for the gradient (φ_o), k_w can be calculated as

$$\log k_w = \log k_o + S\varphi_o = \log k_o + 4.2\varphi_o \qquad (7)$$

In this way, a retention time from the initial gradient run can be used to estimate values of k_w for any band in that chromatogram. Values of $S = 4.2$ and k_w then permit estimates of the retention time of that compound in isocratic elution as a function of %B (φ, Eq. 1), or in gradient elution (t_R from Eq. 5) as a function of column dimensions (V_m), flow-rate, and gradient conditions.

Table 1
Samples used in the present study for C_8 or C_{18} column and acetonitrile–water as mobile phase

Sample	No. of compounds	Range in φ for $k = 5$	Ref.
Anxiolytic drug and impurities	6	0.05–0.20	[13]
Herbicides	15	0.14–0.63	[12]
Substituted benzenes	11	0.23–0.44	[14]
Mixture of phenazines and triazines	16	0.31–0.76	[15]
Polycyclic aromatic hydrocarbons (PAHs)	16	0.43–0.84	[12]
Total	64	0.05–0.84	

The original experimental data were collected using either isocratic or gradient elution; two runs with either %B or t_G varying were used to determine values of k_w and S. Values of k varied 2.5–3-fold between the two runs.

3. Experimental

Values of k_w and S used in the present investigation were taken from the literature or are reported in the following paper [14]. Table 1 summarizes the samples used (C_8 or C_{18} column and acetonitrile–water as mobile phase). These particular test compounds were chosen to cover a wide range in compound structure and retention properties (5–84%B for $k = 5$). In each case, prior studies allowed values of S and k_w to be determined for these sample compounds. It was then possible to calculate retention times for either isocratic or gradient elution as a function of different experimental conditions (column size, flow-rate, %B, gradient conditions, etc.). The use of computer simulation (DryLab/Windows software, LC Resources, Walnut Creek, CA, USA) made these calculations convenient [17] and also permitted us to examine many different experimental possibilities, without the need for additional laboratory work.

3. Results and discussion

Our procedure was to assume a single (arbitrary) value of the equipment dwell volume, then use computer simulation to predict retention for different experimental conditions (V_m, t_G, F). The latter retention data were then used to facilitate method development as follows:

1. determine whether isocratic or gradient elution is preferred;
2. if isocratic elution is preferred, estimate the value of %B that will provide some desired value of k for a given compound;
3. if gradient elution is preferred, estimate the best values of %B to start and finish the gradient.

3.1. Evaluation of the accuracy of isocratic predictions based on an initial gradient run

Values of S and k_w for the 64 compounds of Table 1 were used with Eq. 6 to calculate t_R for all compounds in two gradient runs differing only in gradient time t_G. These retention time data and gradient conditions were then made the basis for further computer simulations, assuming the same column type (either C_8 or C_{18} in Table 1) and organic solvent (acetonitrile). All calculations assumed $V_D = 4$ ml and a 5–100% B gradient.

These computer simulations provided values of t_R for each compound in gradient runs where gradient time, column length, and flow-rate were varied. Isocratic values of %B for elution of each compound with different values of k ($k = 0.5$, 5, 20) were also calculated, from Eq. 1, using experimental values of k_w and S. Eqs. 6 and 7 next allowed estimates of k_w from the initial gradient run for use in Eq. 1 to predict values of %B [$(\%B)_{est}$] for elution of each compound with some value of k ($k = 0.5$, 5, 20). The errors in the estimated values of $(\%B)_{est}$ were then obtained by comparison with accurate values of %B derived from Eq. 1 using known values of S and k_w (as opposed to approximate values based on $S = 4.2$). This is illustrated in Table 2 for a 25 × 0.46 cm column, a 5–100% acetonitrile–water gradient at 1 ml/min, in a gradient time of 60 min. Values of %B for isocratic elution of each compound with $k = 5$ are compared with estimates of %B [$(\%B)_{est}$] from the gradient run. Generally, good agreement (±0.6% R.S.D.) is observed for this example.

Table 3 summarizes the results of similar computer simulations (as in Table 2). Elsewhere [17–19] it has been shown that two gradient runs (same conditions except t_G or F) can be used to predict isocratic or gradient retention quite accurately, because two such runs allow the calculation (not estimation) of values of S and k_w for each compound in the sample. Present estimates of isocratic retention on the basis of a single initial gradient run are therefore limited mainly by the assumption of constant S ($S = 4.2$). The magnitude of this error can be assessed in terms of the observed variability of values of S (Table 1; $\delta S = \pm 0.8$ units, 1 S.D.). As derived in Appendix A, the use of a gradient run to predict an isocratic value of φ for some value of k for a given solute will be in error by an amount $\delta\varphi$, where

$$\delta\varphi = (1/4.2)\log(k/k^*)\{1 - (4.2/[4.2 + \delta S])\} \quad (8)$$

Table 2
Predicted versus actual values of %B for $k = 5$

Compounds	$(\%B)_{est}$	%B	Compounds	$(\%B)_{est}$	%B
Anxiolytic drugs			*Phenazines, triazines*		
1	2.5	4.9	1	31.0	31.3
2	5.1	7.8	2	38.4	38.5
3	9.6	11.0	3	42.7	43.3
4	9.9	11.3	4	45.2	46.0
5	11.1	12.2	5	47.6	44.7
6	19.0	19.7	6	50.6	50.9
			7	50.5	50.8
Herbicides			8	53.3	53.5
1	14.2	13.9	9	54.2	54.4
2	17.5	17.2	10	58.1	58.1
3	18.3	18.0	11	59.6	59.5
4	19.5	19.2	12	63.4	62.6
5	28.7	28.2	13	66.4	65.9
6	32.6	32.3	14	73.3	72.6
7	32.3	31.8	15	74.0	73.0
8	33.9	33.3	16	76.3	75.7
9	34.4	33.9			
10	35.4	34.8	*PAHs*		
11	36.0	35.5	1	43.3	43.0
12	40.0	39.7	2	46.4	46.1
13	42.9	42.5	3	50.8	50.6
14	47.3	46.9	4	51.5	51.2
15	63.6	63.4	5	53.5	53.3
			6	55.9	55.7
Benzenes			7	58.2	58.0
1	21.8	22.6	8	59.9	59.8
2	28.4	29.7	9	66.0	66.0
3	28.7	30.0	10	67.2	67.3
4	28.7	29.7	11	72.0	72.3
5	35.1	36.4	12	74.5	74.9
6	38.0	38.2	13	76.3	76.9
7	39.1	39.7	14	80.3	80.8
8	40.3	40.8	15	81.2	82.1
9	41.5	42.3	16	83.3	84.2
10	43.8	43.4			
11	44.3	44.4			

Compounds of Table 1, 5–100% acetonitrile gradient in 60 min, 25 × 0.46 cm column, 1 ml/min, $V_D = 4$ ($k^* = 5.1$).

The quantity k^* is the effective value of k during gradient elution and is given as

$$k^* = 1/1.15b = 0.85t_G F/(V_m \Delta\varphi S) \approx 0.20t_G F/(V_m \Delta\varphi) \quad (9)$$

Eq. 8 provides an estimate of these errors in %B as a function of k/k^* for comparison with the data of Table 3. Fig. 1 summarizes this comparison; the solid curve is from Eq. 8 using the observed value of $\delta S = 0.8$ for the compounds of Table 1. The open circles in Fig. 1 are the standard deviation values from Table 3. As expected, there is reasonable agreement between Eq. 8 and these observed errors in $(\%B)_{est}$. There was no consistent trend in these errors as a function of %B, suggesting that $q \approx 0$ in Eq. 4 for acetonitrile as solvent.

According to Eq. 8, estimated values of %B in Table 3 should be most accurate when $k = k^*$.

Table 3
Error in prediction of isocratic retention from an initial gradient run as a function of column length and flow-rate

Conditions[a]		k^*[b]	Error in $(\%B)_{est}$ for different k (1 S.D.)[c]		
L (cm)	F (ml/min)		$k = 0.5$ (%B)	$k = 5.0$ (%B)	$k = 20$ (%B)
5	2	51	8.4	3.7	1.3
5	1	26	7.6	2.9	0.7
15	2	17	6.8	2.1	0.9
15	1	8.5	5.8	1.2	1.8
25	2	10	6.0	1.4	1.6
25	1	5.1	4.8	0.6	2.7

Compounds and conditions of Table 1, column I.D. is 0.46 cm, initial run has $V_D = 4$ ml and gradient is 5–100% B in 60 min. See text for details.
[a] Column length L and flow-rate F.
[b] Eqs. 2, 8; assumes $S = 4.2$ (also Eq. 10).
[c] Predicted %B for elution with indicated value of k.

For example, $k^* = 17$ for a 15-cm column and 2 ml/min, and the error in this case of estimated values of %B for $k = 20$ is small (±0.9 %B). Likewise, a 25-cm column and 1 ml/min has $k^* = 5.1$ and the error in %B for $k = 5$ is only ± 0.6 %B. The use of experimental data for widely different samples from different laboratories (and different columns) in the present study provides some assurance that our findings will prove applicable for any sample and any column. Additional data not reported here further support that conclusion. More important, the present study provides a basis for minimizing any errors (due to values of S differing from 4.2) by an appropriate choice of experimental conditions so that k^* is close to the value of k intended for isocratic separation.

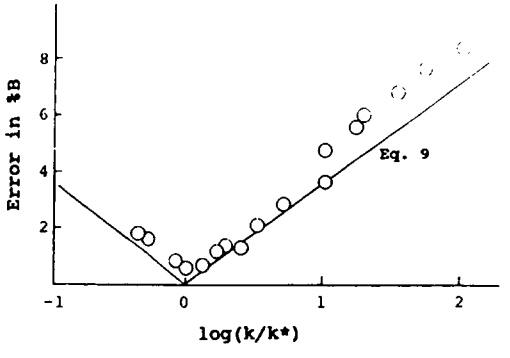


Fig. 1. Error in isocratic predictions of retention based on an initial gradient run. Data of Table 3 plotted versus the absolute value of $\log(k/k^*)$. Solid curve predicted by Eq. 9.

Predictions for other experimental conditions

The compounds of Table 2 have molecular masses (M_r) that are <300 u. It is known that larger molecules have larger values of S [20–22], and an approximate relationship is [20]

$$S = 0.48M_r^{0.44}. \quad (10)$$

Values of S calculated from Eq. 10 should be used in place of $S = 4.2$ for sample molecular masses >500 u.

Values of S for the same compound can vary with column type. Data from several studies summarized in Ref. [9] suggest that phenyl or cyano columns will exhibit S values that are 10–20% lower than values found with C_8 or C_{18} columns. Additional unpublished data from our laboratory confirm this behavior and suggest that $S = 3.6$ is a better approximation for phenyl or cyano columns; this should provide predictions of $(\%B)_{est}$ for phenyl or cyano columns that are comparable in accuracy to those obtained with a C_8 or C_{18} column.

3.2. Application of a starting gradient run in HPLC method development

On the basis of the preceding discussion and other considerations (see the following paper, Ref. [14]), we recommend beginning method development using reversed-phase conditions with a 15 × 0.46 cm, 5-μm C_8 or C_{18} column and acetonitrile–water as the mobile phase (plus buffer if acidic or basic compounds are to be separated). Eqs. 1, 2, 5–7 allow estimates of k_w for the first and last bands in the chromatogram and subsequent calculations of t_R for these compounds in either isocratic or gradient elution as an aid to method development. This approach requires the use of a computer program to be practical. Alternatively, the interpretation of the initial gradient run can be carried out manually (Tables 4–6, discussed below.

Choice of isocratic versus gradient elution

If a maximum range in isocratic k-values is assumed, e.g., $1 < k < 10$, then the initial gradient run allows a test of whether isocratic or gradient elution is preferable. This is illustrated in Fig. 2. The retention times t_R for the first (a) and last (z) bands of the gradient run (Fig. 2a) can be used to determine the k-values (k_a and k_z) for the first and last bands in an isocratic separation (Fig. 2b). If S is constant, the ratio k_z/k_a does not vary with %B, and this ratio can be estimated as a function of retention times t_{Ra} and t_{Rz} from the initial gradient run (Appendix B). For isocratic elution to be possible,

$$(t_{Rz} - t_{Ra})/t_G = \Delta t_R/t_G < 0.25 \log[(k_z/k_a)_{max}] \quad (11)$$

Here, $(k_z/k_a)_{max}$ is the maximum allowed value of the ratio k_z/k_a. The quantity $\Delta t_R/t_G$ is seen in Fig. 2a to represent the fraction of the gradient chromatogram that is occupied by peaks. Eq. 11 is an acceptable approximation for bands that elute later in the chromatogram. Table 4 (top) provides more accurate data for both early and late eluting bands, for values of (k_z/k_a) equal 10 ($1 < k < 10$) or 40 ($0.5 < k < 20$). The uncertainty

Table 4
Determining whether isocratic separation is possible, based on an initial gradient run (see Fig. 2a)

t_{Ra} (min)	Allowable values of t_{Rz} for indicated k-range	
	$1 < k < 10$	$0.5 < k < 20$
<1.5	–[a]	–[a]
2	8	17
3	12	21
4	14	24
5	16	26
7	19	29
10	23	33
15	29	38
20	35	44
25	40	49
30	45	54
35	50	59
40	55	64
>40	–[b]	–[b]
Uncertainty[c]	± 3 min	± 5 min

Conditions: 15 × 0.46 cm column, 5–100% ACN–water gradient in 60 min, 2 ml/min.

[a] Sample may not be sufficiently retained for reversed-phase separation; see following paper (Ref. [14], discussion of Fig. 2).

[b] Sample may be retained too strongly for reversed-phase separation; see following paper (Ref. [14], discussion of Fig. 2).

[c] Estimated uncertainty in these values as derived from data of Table 1.

(±3–5 min, 1 S.D.) in the estimates of Table 4 should be noted.

The value of $(k_z/k_a)_{max}$ selected is the choice of the individual chromatographer. A maximum range in isocratic k-values is probably $0.5 < k < 20$, corresponding to $(k_z/k_a)_{max} = 40$. From Table 4, this corresponds to $\Delta t_R/t_G < 0.40$. A restricted range in k (e.g., $1 < k < 5$) might be selected if it is desired to vary selectivity by further changes in %B; see discussion of Ref. [23]. A maximum range in k (e.g., $0.5 < k < 20$) will be acceptable when it is important to avoid a final gradient method; e.g., when gradient equipment is not available. Fig. 2 illustrates this procedure, where it was desired that $0.5 < k < 20$ in an isocratic separation. Observed values of t_R

Table 5
Estimation of %B for the first isocratic run, based on the retention time t_{Rz} of the last peak in the gradient run

t_{Rz}	$(\%B)_{est}$		
(min)	$k = 5$ (% ACN)	$k = 10$ (% ACN)	$k = 20$ (% ACN)
5	6	0	–
10	19	12	5
15	29	22	14
20	37	30	22
25	45	38	30
30	53	46	38
35	61	54	46
40	69	62	54
45	77	70	62
50	85	78	70
55	93	86	78
60	100	94	86
65	–	100	94

Conditions: 15 × 0.46 cm column, 5–100% ACN in 60 min, 2 ml/min.

Table 6
Estimation of initial and final %B for gradient elution, based on retention time t_R for first (a) and last (z) band in the initial gradient run (see Appendix C)

t_{Ra}, t_{Rz} [a] (min)	Initial %B (%)	Final %B [b] (%)
5	3	14
10	11	22
15	19	30
20	27	38
25	35	46
30	43	54
35	51	60
40	59	68
45	67	76
50	75	84
55	83	100
60 [c]	–	–

Conditions for initial gradient run as in Table 5.
[a] Retention time for first peak a (for initial %B) or last peak z (for final %B).
[b] For steeper gradients, %B(final) must be increased by as much as 36% (Appendix C).
[c] Normal-phase or non-aqueous reversed-phase HPLC may be required.

in the gradient run of Fig. 2a are 9.5 min for the first band (a) and 24.1 min for the last band (z). From Table 4, for $0.5 < k < 20$, the maximum allowable value of t_{Rz} is 32 min. Therefore, isocratic separation is possible, as seen for this sample in Fig. 2b (29% B, $3 < k < 24$).

For higher-molecular-mass samples, gradient elution will often be required in any case. Assuming a maximum k-range, i.e., $0.5 < k < 20$, maximum allowable values of $\Delta t_R/t_G$ are as follows (Eq. 10): $M_r < 500$, 0.40; $M_r = 1000$, 0.17; $M_r = 10\,000$, 0.06.

Choice of isocratic conditions

If on the basis of Table 4 or Eq. 11 it is determined that isocratic separation is preferable, then the initial gradient run can be used to estimate the best %B for the next isocratic run. The data of Table 5 for otherwise preferred gradient conditions (15-cm column, 2 ml/min, 60 min gradient; see Ref. [14]) are most reliable for $10 < k < 20$, since $k^* \approx 17$. It is therefore better to estimate %B for a desired k-value of the last band (k_z) equal to 10–20. This is illustrated for the sample of Fig. 2. From the gradient run of Fig. 2a, t_R for the last band is 24 min. From Table 5, for $k = 20$, a mobile phase of 29% acetonitrile should be selected. This run is shown in Fig. 2b ($3 < k < 24$), and it is seen that the actual retention for the last band ($k = 24$ min) is close to the intended value ($k = 20$ min).

Choice of gradient conditions

If further method development runs are carried out in a gradient mode, it is advisable to first reduce the gradient range (change in %B during the gradient) so as to reduce run time [1]. As described in Appendix C, estimates of initial and final %B-values can be obtained from retention values t_{Ra} and t_{Rz} for the first and last bands in the initial chromatogram (see Fig. 2). These estimates are summarized in Table 6. Note that the recommended value for %B(final) depends on gradient steepness (Table 6 and Appendix C). For this reason, it may be best to maintain 100% B for the end of the gradient until the final gradient steepness is selected.

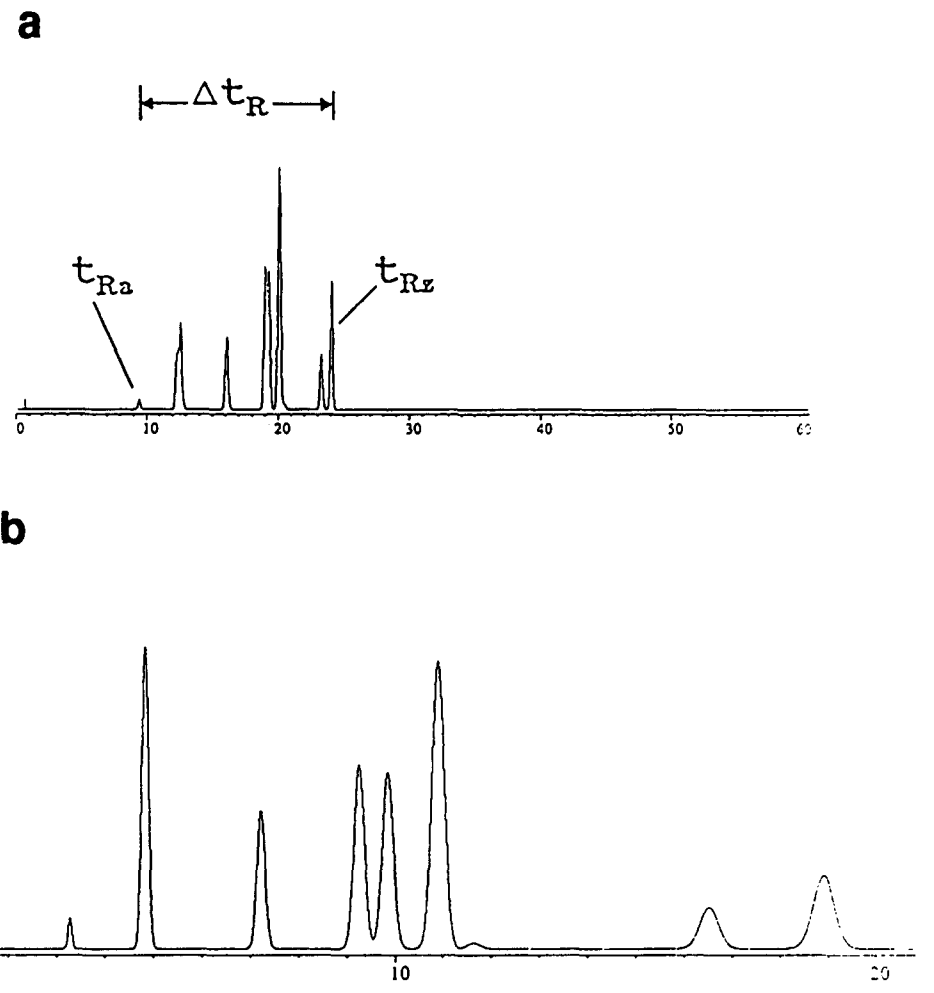


Fig. 2. Illustration of present procedure (see text for details). (a) Initial separation of substituted-benzene sample of Table 1: 5–100% acetonitrile–water in 60 min, 15 × 0.46 cm C_8 column; 2 ml/min; (b) isocratic separation with 29% B, other conditions the same.

Predictions for ionized sample compounds

Values of S have been reported to be significantly lower for ionized bases [24]. The use of Tables 4 and 5 (based on $S = 4.2$) might therefore be expected to lead to predictive errors for samples that contain compounds of this type. This now appears not to be the case. Unpublished data [25] have been provided to us for the reversed-phase retention of 48 drug-related compounds which include numerous protonated strong bases under the conditions of separation (pH 2.5). There was no indication that protonated bases which are significantly retained ($k >$ 1) have lower S values compared to other, neutral compounds in this data set. The conclusion above based on Ref. [24] refers mainly to compounds that have $k \ll 1$ for the totally protonated species, which is of limited interest for HPLC method development.

4. Conclusions

The use of a gradient elution separation to begin HPLC method development is now well established [1]. Previously it has been assumed

that a methanol–water gradient run would be used for this purpose, whereas acetonitrile–water is usually preferred. The present study provides a model for the quantitative interpretation of an initial acetonitrile–water gradient run for use in selecting conditions for subsequent HPLC method development experiments.

An initial gradient run can be used to arrive at several conclusions regarding the course of method development: (1) Is isocratic or gradient elution preferred for further experiments (Table 4)? (2) If isocratic elution is preferable, what %-acetonitrile should be used for the next experiment (Table 5)? (3) If gradient elution is preferred, what %-acetonitrile values should be used to start and finish the gradient (Table 6)? Acceptable accuracy for these predictions (using the present model) was demonstrated for several representative samples from different laboratories. The following paper (Part II, Ref. [14]) shows that the accuracy of predictions based on an initial gradient run can be further improved by selecting appropriate experimental conditions for this run.

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Appendix A. Error in the prediction of isocratic retention from an initial gradient run as a function of the difference between assumed and actual values of S

The hypothetical plots of Fig. 3 will be helpful in this connection. In Fig. 3, isocratic values of log k are plotted versus values of φ. According to Eq. 1, such plots should be linear, as illustrated by curve S for some compound separated in the initial gradient run. For this gradient run, there will be an average value of k, shown as k^* in Fig. 3. The quantity k^* can be related to the conditions of the initial gradient run as [3,26]

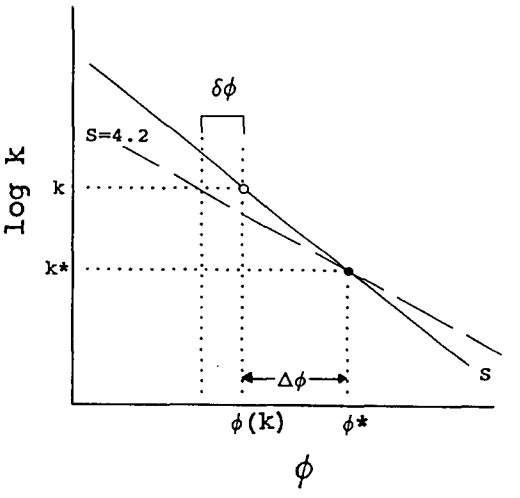


Fig. 3. Origin of errors in isocratic predictions of retention based on an initial gradient run; full line, actual retention plot; dashed line, retention plot assuming $S = 4.2$. See text for details.

$$k^* = 1/1.15b. \quad \text{(A.1)}$$

If a value of S is assumed (4.2) that is different from the actual value of S, then the predicted (approximate) dependence of log k on φ will be described by the curve in Fig. 3 labeled S = 4.2. For $k = k^*$, the two curves must give the same value of $\varphi = \varphi^*$; i.e., the curves intersect at the point k^*,φ^*. In practical terms, if the conditions used for the gradient run determine some value $k = k^*$, and if it is desired to predict an isocratic value of φ $[\varphi(k)]$ such that an isocratic value $k = k^*$ results, there will be no error in this prediction due to our assumption $S = 4.2$. However, for the prediction of values of φ that correspond to other values of k, an error $\delta\varphi$ will result (as shown in Fig. 3).

The magnitude of this error $\delta\varphi$ can be determined as follows. First, define $\Delta\varphi = \varphi^* - \varphi$. From Fig. 1, it can be seen that

$$\log(k/k^*) = S\,\Delta\varphi = 4.2(\Delta\varphi + \delta\varphi)$$

or

$$\delta\varphi = (1/4.2)\log(k/k^*)\{1 - (4.2/[4.2 + \delta S])\}, \quad \text{(A.2)}$$

i.e. Eq. 8. Here, δS is the error in the estimate

of S; i.e., $\delta S = S - 4.2$. Note also that there is an error in log k (δ log k) which is proportional to $\Delta\varphi$.

Appendix B. Use of an initial gradient run to determine whether isocratic elution of the sample is feasible

If the initial band a is not eluted early in the initial gradient run, then Eq. 3 simplifies to

$$t_R = (t_o/b)\log(2.3k_o b) + t_o + t_D. \quad \text{(B.1)}$$

For peaks a and z in the initial run, the following relationship can then be derived:

$$t_{Rz} - t_{Ra} = \Delta t_R = (t_G/\Delta\varphi\, S)\log(k_z/k_a). \quad \text{(B.2)}$$

For $S = 4.2$, 5–100% B, and some maximum range in isocratic k-values, expressed by $(k_z/k_a)_{max}$, the maximum value of Δt_G (for isocratic separation) can be expressed as a fraction of the gradient time t_G:

$$\Delta t_R/t_G < (1/\Delta\varphi\, S)\log[(k_z/k_a)_{max}] \quad \text{(B.3)}$$

or, for $S = 4.2$,

$$\Delta t_R/t_G < 0.25\log[(k_z/k_a)_{max}], \quad \text{(B.4)}$$

i.e. Eq. 11.

Appendix C. Estimating initial and final %B values for gradient elution from an initial 5–100% B gradient run

The mobile-phase composition for elution of the first (a) and last (z) peaks in the initial gradient run is given by

$$\varphi_i = ([t_R - t_o - t_D]/t_G)\Delta\varphi + \varphi_o, \quad \text{(C.1)}$$

where t_R is the retention time of band a or z. For the recommended conditions for the first run (5–100% B in 60 min, 2 ml/min, 15 × 0.46 cm column) and an estimated dwell volume $V_D = 4$ ml, Eq. C.1 becomes

$$\varphi_i = 0.016(t_R - 2.75) + 0.05. \quad \text{(C.2)}$$

The value of k at elution is $k^*/2$, or for these conditions $k \approx 8$. If the gradient steepness is not changed, values of φ_a and φ_z from Eq. C.2 can be used to define the gradient range required for the sample in question. To allow for error in these estimates of φ_i, the value of φ_a can be reduced by 0.05 and the value of φ_z increased by 0.05.

If the gradient steepness is changed, the values of φ_a and φ_z at elution will change. Because it is desirable for $1 < k^* < 20$, and $k^* \approx 17$ for the initial run [23], any change in gradient steepness will normally be toward a steeper gradient. For steeper gradients (larger b), values of φ_i will increase. Therefore, the value of φ_z should be increased if gradient steepness is increased. This change can be estimated as follows. From Eqs. 2, 3 and C.1, and recognizing that for the last band z $2.3k_o b \gg 1$, we have

$$\varphi_i = (1/S)\log(2.3k_o b). \quad \text{(C.3)}$$

The increase in φ_z required for an increase in b is then

$$\Delta\varphi_z = (1/S)\log(b_2/b_1), \quad \text{(C.4)}$$

where b_1 refers to the value of b for the initial gradient run and b_2 to that for a gradient run with a change in steepness. For a maximum change in gradient steepness (from $k^* = 17$ to $k^* = 0.5$, or $b_2/b_1 = 34$), the increase in φ_z for the steeper gradient would be ca. 0.36. Therefore, it is recommended to delay selecting the final %B in the gradient until the gradient steepness has been chosen (maintaining a value of 100% B is the safest procedure).

List of symbols

A	water
ACN	acetonitrile
b	gradient steepness parameter (Eq. 2)
b_1, b_2	different values of b; Eq. C.4
B	organic solvent in mobile phase
c	Eq. 6; $c = b/t_o$
F	flow-rate (ml/min)
k	solute retention factor equal to $(t_R - t_o)/t_o$
k_a, k_z	k-value of first (a) and last (z) bands

	in an isocratic separation; see Fig. 2b
k_o	value of k at the beginning of gradient elution (for $\varphi = \varphi_o$)
k_w	value of k for water as mobile phase ($\varphi = 0$)
$(k_z/k_a)_{max}$	maximum allowable value of the ratio k_z/k_a
k^*	effective value of k in gradient elution (Eq. 9)
L	column length (cm)
MeOH	methanol
p, q	constants in Eq. 4
P	pressure drop across column (p.s.i.)
P_{max}	maximum allowable value of P
S	constant in Eq. 2; equal to d(log k)/dφ
t_D	gradient equipment dwell time (min)
t_G	gradient time (min)
THF	tetrahydrofuran
t_o	column dead time (min)
t_R	solute retention time (min)
t_{Ra}, t_{Rz}	values of t_R for first (a) and last (z) bands in a gradient run; see Fig. 2a
T_R	Eq. 6; $T_R = t_R - t_o - t_D$
V_D	gradient equipment dwell volume (ml)
V_m	column dead volume (ml); equal to t_o/F
x	parameter in Eq. 5; $x = t_D/(t_o k)$
δS	error in assumed value of S, equal to $S - 4.2$
Δt_R	$t_{Rz} - t_{Ra}$; see Fig. 2a
$\Delta\varphi_i$	difference in φ_i values for a solute as a result of a change in gradient steepness b (Eq. C.4)
$\Delta\varphi$	change in φ during the gradient δ_{t_R}
φ	volume fraction of organic in mobile phase; equal to 0.01 %B
φ_i	value of φ for a band i at elution (gradient run)
φ_o	value of φ at start of gradient
η	mobile phase viscosity (cPoise)
$(\%B)_{est}$	estimated value of %B to obtain a given value of k for some solute (based on initial gradient run).

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