

CHROM. 16,396

MEASUREMENT AND USE OF RETENTION DATA FROM HIGH-PERFORMANCE GRADIENT ELUTION

CORRECTION FOR "NON-IDEAL" PROCESSES ORIGINATING WITHIN THE COLUMN

M. A. QUARRY*, R. L. GROB* and L. R. SNYDER**.*

Biomedical Products Department, E. I. du Pont de Nemours & Co., Concord Plaza, Wilmington, DE 19898 (U.S.A.)

(Received October 25th, 1983)

SUMMARY

Under "ideal" conditions it is possible to model retention in gradient elution so as to be able to calculate retention times, t_g , as a function of isocratic retention in corresponding liquid chromatographic systems. In this paper we consider various "non-ideal" processes that lead to errors in calculated values of t_g . The more important of these are solvent demixing due to uptake of one mobile phase component by the column packing, non-linear plots of $\log k'$ vs. gradient time or mobile phase composition and changes in column dead-time, t_0 , due to changes in mobile phase composition and flow-rate. Expressions are derived to correct for these various "non-ideal" effects, including equipment limitations discussed in the preceding paper. Calculated values of t_g for reversed-phase gradient elution systems then agree with experimental values to within $\pm 1\%$ (1 standard deviation) of the total gradient time, t_G . These results should prove useful in (a) improving the precision of retention in gradient elution (which should be comparable to that in isocratic elution), (b) using gradient elution for more efficient method development in isocratic procedures and (c) better understanding gradient separations of macromolecules such as proteins.

INTRODUCTION

In the preceding paper¹ we reviewed some of the applications of gradient elution, including its use for convenient and rapid method development for isocratic separation. The latter approach can be applied in various ways, including optimization of solvent strength and selectivity, optimization of temperature effects and optimization of pH effects and band shape. In each of these cases it is desired to use retention data from one or more gradient elution runs to predict retention times in

* Chemistry Department, Villanova University, Villanova, PA 19085, U.S.A.

** Lloyd R. Snyder, Inc., 2281 William Court, Yorktown Heights, NY 10598, U.S.A.

corresponding isocratic systems. Theoretical models or numerical procedures exist which allow this interconversion of gradient and isocratic data (e.g., ref. 2), but prior models assume "ideal" conditions, with no complications introduced by the equipment or by processes occurring within the column. Often these latter effects are of minor importance, and many studies have reported reasonable agreement between measured and calculated retention times in gradient elution, based on separate measurements of isocratic retention in the same high-performance liquid chromatographic (HPLC) systems (for review see, e.g., refs. 2-8). In certain cases, however, it is required that the calculation of gradient data from isocratic measurements (and *vice versa*) be as accurate as possible. In other cases, e.g., the separation of macromolecular species⁹, small errors in the gradient measurements appear to translate into large errors in derived isocratic parameters. Then it is necessary to minimize effects from gradient "non-ideality" and/or to correct for retention contributions from these effects. For these reasons we studied various contributions to gradient non-ideality. In the preceding paper¹ we examined non-ideal effects arising from the gradient equipment. In this paper we consider non-ideal effects that originate within the column.

Our approach is to combine theoretical analysis with experimental confirmation from a model liquid chromatographic (LC) system: the five C₁-C₅ *n*-dialkyl phthalates as solutes, acetonitrile-water as mobile phase and various C₁₈ reversed-phase columns. We believe the results can be readily extrapolated to other LC systems, particularly those involving reversed-phase separations. The gradient shapes specifically studied correspond to the generally optimal linear solvent strength (LSS) gradients². For reversed-phase systems, this means gradients where mobile phase composition (or volume fraction of organic solvent, ϕ) increases linearly with time. The extension of our findings to other gradient shapes is possible, but more complicated.

The immediate objective of this study was to accumulate gradient retention data over a wide range of conditions for several solutes, and to compare these retention times, t_g , with values calculated from isocratic retention times, t_R , as indicated in refs. 1 and 2. Differences in experimental *vs.* calculated t_g values ($\delta\phi$) were then analyzed in terms of various theoretical contributions ($\delta\phi_i$) to error in the calculated t_g value (which ignores gradient "non-ideality"). Our aim was to account quantitatively for these experimental $\delta\phi$ values, so as to permit correction for gradient non-ideality in any gradient run.

THEORY

In the preceding paper¹ we reviewed certain fundamental equations that we shall use in this paper:

$$\log k' = \log k_0 - b(t/t_0) \quad (1)$$

$$\log k' = \log k_w - S\phi \quad (2)$$

$$b = S\Delta\phi t_0/t_G \quad (3)$$

$$t_g = (t_0/b) \log[2.3k_0b(t_{\text{sec}}/t_0) + 1] + t_{\text{sec}} + t_D \quad (4)$$

For details on these relationships see refs. 1 and 2 and the glossary at the end of this paper, which lists all symbols used in this and the preceding paper¹.

Eqn. 4 expresses retention time, t_g , in gradient elution as a function of certain parameters that can be measured in corresponding isocratic systems (eqns. 1-3). These relationships (eqns. 1-4) are based on certain assumptions:

- (1) absence of gradient "nonideality" at the column inlet:
 - (a) perfect mixing and proportioning of gradient solvents as required by the selected gradient shape; constant flow of gradient mobile phase during separation;
 - (b) no delay in the arrival of the gradient at the column inlet, following injection of the sample (eqn. 4 does recognize the gradient delay time, t_D);
 - (c) no distortion of the gradient shape due to its dispersion in the gradient mixer, connecting tubing, etc.;
- (2) retention data obtained in both isocratic and gradient systems are adequately precise and reproducible; the precisions of these data are known;
- (3) variations in t_0 and t_{sec} with experimental conditions (e.g., refs. 9-12) are taken into account;
- (4) Eqn. 2 describes the isocratic retention data (linear plots of $\log k'$ vs. ϕ);
- (5) solvent demixing as a result of preferential retention of a mobile phase component within the stationary phase does not occur;
- (6) the stationary phase is at equilibrium with the mobile phase at any time during the gradient and at any position within the column;
- (7) k' is not a function of column pressure;
- (8) the column is adequately equilibrated with starting mobile phase before each gradient run;
- (9) the column for both isocratic and gradient runs is adequately thermostated, mobile phase entering the column is at the temperature of the column and frictional heating of the column by the mobile phase has no significant effect on retention;
- (10) all other requirements for valid retention time measurements are met: small sample size for linear isotherm data, sample dissolved in weak solvent, etc. (e.g., ref. 13).

These various effects are discussed below.

Gradient "non-ideality"

The preceding paper¹ dealt with these issues in detail. Eqn. 4 includes the effect of gradient delay (t_D).

Reproducibility of gradient and isocratic retention data

Reproducibility of retention data depends on holding many separation parameters constant: temperature, flow-rate, mobile phase composition, etc.^{13,14}. In the following section it will be seen that the reproducibility of retention data as measured by us was generally better than the agreement between isocratic and gradient data. Therefore, reproducibility *per se* was of less interest in this study. We did, however, examine two cases of general interest to gradient/isocratic correlations.

First, consider all the contributions from system variability that affect k' in an isocratic separation. These include variations in temperature, mobile phase composition, the condition of the column, etc. What will be the effect of imprecision in k'

(isocratic) on gradient retention? Typically, under so-called gradient conditions², the value of k_0 (eqn. 1) for a given solute in a given gradient separation will be large. Under these conditions, eqn. 4 can be approximated by

$$t_g = (t_0/b)[\log 2.3 b(t_{sec}/t_0) + \log k_0] + t_{sec} \quad (5)$$

and an error in k_0 of δk_0 yields an error in t_g (δt_g) of

$$\delta t_g = (t_0/2.3b) (\delta k_0/k_0) \quad (6)$$

or

$$\delta \varphi_k = (\delta t_g/t_g) \Delta \varphi = (t_0 \Delta \varphi / 2.3 b t_g) (\delta k_0/k_0)$$

and

$$\delta \varphi_k = (\delta k_0/k_0) / 2.3 S \approx (\delta k'/k') / 2.3 S \quad (7)$$

Second, modern HPLC pumps which employ low-pressure mixing of the gradient solvents often experience a timing delay in starting the gradient. In the case of the DuPont Model 8800 HPLC system, there is an uncertainty of 0–6 sec in the initiation of the gradient after a command by the operator or microprocessor, as a result of the cycle time for the solenoid valves that feed the gradient mixing chamber. This in turn yield an imprecision in t_g of $6/\sqrt{12} = 1.7$ sec (*e.g.*, ref. 14).

Variation in t_0 and t_{sec} with experimental conditions

Eqn. 4 expresses gradient retention time, t_g , as a function of the column dead-time t_0 (retention time for a small, unretained solute molecule such as water) and t_{sec} . The quantity t_{sec} is the retention time of the solute of interest if it is unretained, or of a molecule of equivalent size (so far as size-exclusion retention) that is not retained by the alkyl-silica surface (see discussion in ref. 9). Several workers have discussed the variation of t_0 with mobile phase composition in reversed-phase systems^{10–12}. In practice, it is convenient to measure t_0 in these systems from the retention time for D₂O as the sample. It can be assumed that values of t_{sec} likewise vary with mobile phase composition, but the direct measurement of t_{sec} for solutes that are retained ($k' \neq 0$) in a given mobile phase is not possible. Further complicating the application of eqn. 4 is the fact that mobile phase composition varies during a gradient separation, as do values of t_0 and t_{sec} .

Fortunately, values of t_g from eqn. 4 are not strongly dependent on the values of t_0 and t_{sec} assumed for a given system. This can be seen as follows. The isocratic value of k' is given⁹ by

$$k' = (t_R - t_{sec})/t_{sec} \quad (8)$$

for solutes where $t_0 \neq t_{sec}$. The value of k_0 (eqn. 1) will normally be large, so we can write

$$k_0 \approx C_1/t_{sec} \quad (9)$$

where C_1 , to a first approximation, is not a function of t_{sec} (because the retention time $t_R \gg t_{\text{sec}}$). Likewise, it can be seen from eqn. 3 that the lumped-parameter term b/t_0 equals $S\Delta\phi/t_G$ and is also not a function of t_0 or t_{sec} . Eqn. 4 can now be rewritten, using eqns. 3 and 9, to give

$$\begin{aligned} t_g &= (t_0/b) \log[2.3(b/t_0)(k_0 t_{\text{sec}}) + 1] + t_{\text{sec}} \\ &= (t_G/\Delta\phi S) \log[2.3(\Delta\phi S/t_G)C_1 + 1] + t_{\text{sec}} \\ &= f(t_G, \Delta\phi, S, C_1) + t_{\text{sec}} \end{aligned} \quad (10)$$

The term $f(t_G, \dots)$ in eqn. 10 is not a function of t_0 or t_{sec} , so that the functional dependence of t_g on t_0 and t_{sec} is contained in the final term of eqn. 10, *i.e.*, t_g depends on t_{sec} but not on t_0 . Uncertainty in the value of t_{sec} will normally be small compared with the value of t_g , meaning that errors in calculated values of t_g should not be large owing to errors in the value of t_{sec} assumed.

Because the calculated value of t_g (eqn. 4) is not strongly dependent on errors in the estimate of t_{sec} , we are justified in an approximate treatment which allows us to estimate t_{sec} as a function of mobile phase composition. We can then estimate the average value of t_{sec} during band migration in gradient elution, by taking the value of t_{sec} corresponding to the average mobile phase composition ϕ during migration¹. Our approach to the calculation of t_{sec} values for C_1 - C_5 dialkyl phthalates in acetonitrile-water mobile phases with different ϕ values was briefly as follows. First, a size-exclusion chromatography calibration plot was determined for the column of interest, tetrahydrofuran as mobile phase and various polystyrenes as solutes (Table II in ref. 9). Second, the various C_1 - C_5 dialkyl phthalates were run as solutes in this system to determine their retention times, t_{sec} . Third, the ratio t_{sec}/t_0 was assumed to remain constant for a given solute as ϕ is varied. For the phthalates as solutes, this meant that $t_{\text{sec}} \approx 0.93 t_0$. The values of t_{sec} given in Table I are from a more sophisticated approach, based on a consideration of the fractional pore volume accessible to the solute, and assuming that differences in t_0 arise from changes in the volume of mobile phase within the packing pores that is accessible to small solutes. Details of the latter analysis are available from the authors.

Non-linear plots of log k' vs. ϕ

It is usually observed for reversed-phase LC systems (*e.g.*, ref. 15) that plots of log k' vs. ϕ are linear, within experimental error. The use of linear ϕ vs. time (t) gradients as in this study then gives adherence to eqn. 1, which in turn is the basis for calculations of t_g values via eqn. 4. For the precise calculation of values of t_g , however, even slight non-linearity of log k' vs. ϕ can result in unacceptable errors if eqns. 1-4 are used without modification.

Other workers (*e.g.*, ref. 16) have noted that plots of log k' vs. ϕ often exhibit slight curvature, particularly when data are collected over a wide range of ϕ . We found this to be the case for the dialkyl phthalates studied here, as shown in Fig. 1 for one of the two columns studied by us. Isocratic retention data for both columns and all solutes are given in Table II. Our approach to the complication of non-linear log k' vs. ϕ plots was as follows. First, experimental tabulations of log k' vs. ϕ for

TABLE I

EXPERIMENTAL VALUES OF t_0 AND CALCULATED VALUES OF t_{sec} FOR DIALKYL PHTHALATE SOLUTES IN THE PRESENT LC SYSTEMS

Temperature, 35°C; flow-rate, 2.0 ml/min.

Column	ϕ	t_0^* (min)	t_{sec}^{**} (min)	
			C_1^{***}	C_5^{***}
6-nm pore	0.00	1.23	1.13	1.11
	0.10	1.18	1.08	1.07
	0.20	1.15	1.06	1.04
	0.30	1.12	1.03	1.02
	0.40	1.09	1.01	0.99
	0.50	1.07	0.99	0.98
	0.60	1.09	1.01	0.99
	0.70	1.15	1.06	1.04
	0.80	1.17	1.08	1.06
	0.90	1.26	1.15	1.13
	1.00	1.35	1.23	1.21
15-nm pore	0.00	1.37	1.31	1.29
	0.20	1.27	1.22	1.20
	0.40	1.22	1.17	1.15
	0.60	1.24	1.19	1.17
	0.80	1.30	1.24	1.23
	1.00	1.40	1.34	1.32

* Measured with deuterium oxide as in refs. 10 and 11.

** Calculated from experimental t_0 values as described in the text.*** Dimethyl (C_1) and dipentyl (C_5) phthalates.

each solute were fitted to a polynomial (see Table II). Enough terms in the polynomial were included to provide a fit of experimental points to the curve within ± 0.01 unit in $\log k'$. Second, a value of $\bar{\phi}$ is estimated, and the tangent $d(\log k')/d\phi$ to the $\log k'$ vs. ϕ curve at this value of $\bar{\phi}$ is determined. The tangent equation is then taken as an approximation to eqn. 2, values of b and k_0 are calculated from eqns. 2 and 3 and eqn. 4 is used to calculate t_g . A new value of $\bar{\phi}$ is then recalculated from eqns. 8 and 9 of the preceding paper¹. If the latter value of $\bar{\phi}$ differs significantly from the original estimate of $\bar{\phi}$, the new value of $\bar{\phi}$ is used to determine a new tangent curve to the $\log k'$ vs. ϕ isocratic plot, and the calculation of b , k_0 , t_g and $\bar{\phi}$ is repeated. This iterative approach to t_g is continued until the estimated and calculated values of $\bar{\phi}$ agree, or until successive values of t_g do not change.

The success of the latter procedure in providing accurate t_g values via eqn. 4 has been checked^{15,16} by numerical integration of the fundamental equation for retention in gradient elution (eqn. A1 in the preceding paper¹). The error in the resulting t_g values is generally less than 1%, except for much more severe curvature of $\log k'$ vs. ϕ plots than was observed by us. For 10–100% organic/water gradients as used by us, this corresponds to an error in t_g of $\delta\phi < 0.005$ (see ref. 1 and Glossary for the significance of values of $\delta\phi$).

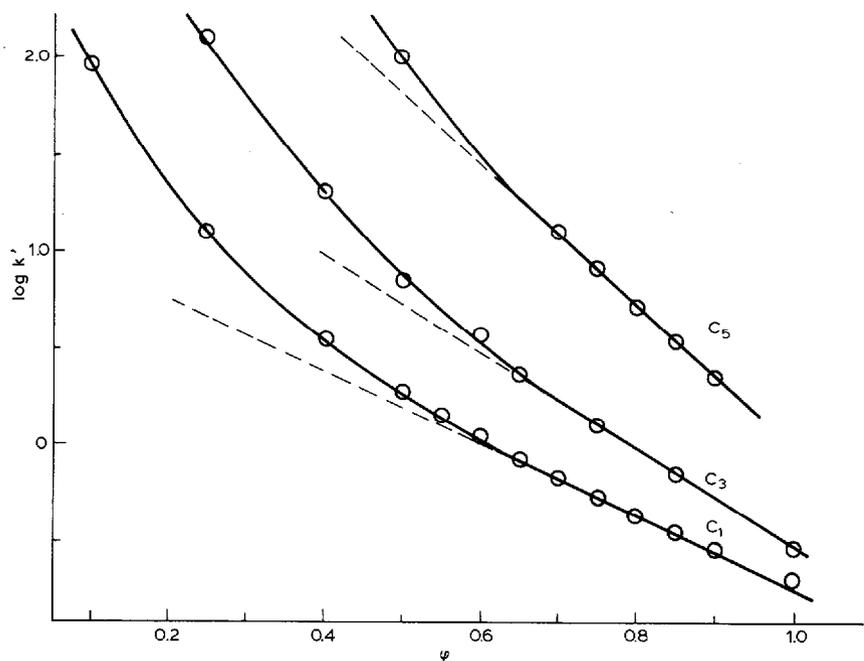


Fig. 1. Dependence of capacity factor ($\log k'$) on mobile phase composition (ϕ) for C_1 , C_3 and C_5 dialkyl phthalates as solutes. Isocratic data at 35°C , acetonitrile-water as mobile phase, 6-nm pore C_{18} column and flow-rate 2 ml/min.

TABLE II

ISOCRATIC RETENTION DATA (k') FOR C_1 - C_5 DIALKYL PHTHALATES IN THE PRESENT LC SYSTEMS

Column	T ($^\circ\text{C}$)	ϕ	k'				
			C_1	C_2	C_3	C_4	C_5
6-nm pore	35	0.10	98.4				
		0.20	23.1	107.4			
		0.25	13.62	55.2	133.4		
		0.40	4.01	11.11	20.3	120.0	
		0.50	2.25	5.19	8.20	35.7	95.6
		0.55	1.69	3.63	5.42	22.0	54.5
		0.60	1.32	2.66	3.73	14.09	32.6
		0.65	1.00	1.94	2.59	9.18	20.1
		0.70	0.79	1.46		6.23	12.95
		0.75	0.61	1.10	1.35	4.21	8.31
		0.80	0.48	0.82		2.88	5.40
		0.85	0.36	0.60	0.68	1.93	3.43
0.90	0.25	0.43		1.25	2.11		

(Continued on p. 26)

Solvent demixing during gradient elution

If a portion of alkyl-silica reversed-phase packing material is equilibrated with mobile phase of composition φ , it is observed^{10,11} that the value of φ in the mobile phase decreases, corresponding to uptake of organic solvent by the stationary phase. In gradient elution with systems where such sorption of organic from the mobile phase can occur, it would be expected that the value of φ for a volume element of the gradient moving through the column would be decreased as a result of similar sorption of organic solvent by the stationary phase. This process is illustrated schematically in Fig. 2. In Fig. 2a a gradient is observed within the bed, across two adjacent particles, 1 and 2. On the left in Fig. 2a, the gradient is portrayed at some time t during the separation. On the right in Fig. 2a the gradient is shown at a later time $(t + dt)$. Volume elements of concentration $(\varphi - d\varphi)$, φ and $(\varphi + d\varphi)$ have advanced a distance equal to one particle diameter. In Fig. 2a, no sorption of organic solvent by the particles is assumed. In Fig. 2b the same process is shown again, but with depletion of the mobile phase by sorption of organic solvent. A change in concentration due to sorption is observed, equal to $-d\varphi_1$ from sorption on to particle 1, and $-d\varphi_2$ from sorption on to particle 2. The net effect of such sorption of organic solvent by the packing material will be a change in the gradient (φ vs. t) relative to that predicted in the absence of sorption effects.

We can model the depletion of the mobile phase and resulting distortion of the gradient as follows (Fig. 3). A column initially equilibrated with mobile phase of composition φ_0 is assumed (Fig. 3a). The stationary phase concentration will then be ψ_0 ($\mu\text{l}/\text{m}^2$ of particle surface). The first differential volume of the gradient will have concentration $\varphi + d\varphi$, and this now moves into the column to give the new mobile phase composition profile shown in Fig. 3b. The cells (numbered 1, 2,...) in Fig. 3 correspond to differential column lengths which are just large enough to accommodate the differential volume of mobile phase transferred to the column in Fig. 3b. Each cell is divided into mobile phase and stationary phase compartments (m and s). Equilibration of mobile and stationary phase compartments occurs in the next step, yielding the concentration profile of Fig. 3c. Finally, another movement of mobile phase occurs (Fig. 3d), and the process is continued iteratively. A knowledge of the sorption isotherm allows the calculation of equilibrium concentrations after each differential transfer of mobile phase, and continuation of the process

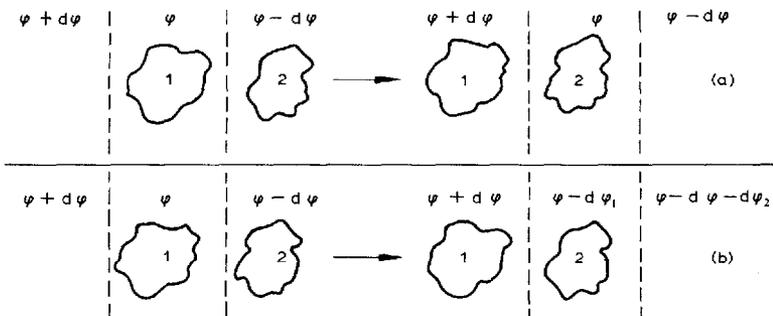


Fig. 2. Origin of solvent demixing during gradient elution. (a) Representation of the mobile phase gradient across particles 1 and 2 within the column at times t and $t + dt$ (no solvent sorption); (b) same but at a later time $t + dt$, showing decrease in φ as a result of sorption of organic solvent by particles 1 and 2.

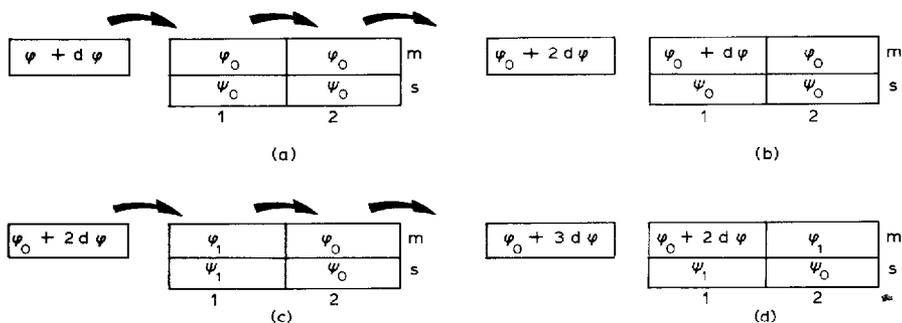


Fig. 3. Model for calculating organic-solvent sorption during gradient elution. (a) Beginning of gradient, column equilibrated with mobile phase of $\varphi = \varphi_0$; (b) first volume element of gradient enters column; (c) new mobile phase and column inlet are equilibrated, with change in mobile phase (φ) and surface (ψ) concentrations; (d) second volume element of gradient enters column.

through the end of the gradient in turn yields the perturbation of the original gradient as a result of sorption of organic solvent.

Calculation of the change in mobile phase composition after each transfer as in Fig. 3 is based on conservation of mass. Thus, assume a differential volume dV of mobile phase has an initial composition (before equilibration) of φ_i , and assume the surface area of the associated stationary phase is ds , with surface concentration ψ_i . Let the concentrations after equilibration be φ_{i+1} and ψ_{i+1} , respectively. The amount of solute in the differential system is $(\varphi_i dV + \psi_i ds)$ initially, which must equal the final amount of solute: $\varphi_{i+1} dV + \psi_{i+1} ds$. The quantity ψ at equilibrium is related to φ by

$$\psi = K_s \varphi \quad (11)$$

which then yields

$$\varphi_{i+1} = (\varphi_i dV + \psi_i ds) / (dV + K_s ds) \quad (11a)$$

Values of ψ as a function of φ (sorption isotherms) can be calculated from the data in refs. 10 and 11 for the mobile phases water-methanol, water-acetonitrile and water-tetrahydrofuran at temperatures in the range 20–40°C (the isotherms are not very temperature dependent). The data in refs. 10 and 11 are given as excess of sorbed material, ψ^* vs. φ , and the saturation uptake by the packing is equal to about 1.3 $\mu\text{l}/\text{m}^2$ for both acetonitrile and tetrahydrofuran. Values of ψ are then related to values of ψ^* by

$$\psi = \psi^* + 1.3\varphi \quad (12)$$

The values obtained for ψ vs. φ at 40°C for acetonitrile-water are given in Table III (data from ref. 11).

The effect on t_g of a change in the original gradient composition, φ , at any point in the gradient can be determined as follows. First, the average effect over the

TABLE III

TOTAL UPTAKE BY ALKYL-SILICA OF ACETONITRILE FROM WATER-ACETONITRILE MIXTURES AT 40°C

Calculated from data of ref. 11 using eqn. 12.

φ	ψ ($\mu\text{l}/\text{m}^2$)	φ	ψ
0	0	0.6	1.10*
0.1	0.38	0.8	1.21*
0.2	0.63	1.0	1.3*
0.3	0.81		
0.4	0.95		
0.5	1.03		

* Fitted to saturation uptake of 1.3 $\mu\text{l}/\text{m}^2$.

entire gradient is given approximately by the change in φ at $\bar{\varphi}$, which we shall define as $\delta\varphi_s$. That is, $\delta\varphi_s$ is the change in φ at the column midpoint. As for the similar case of gradient mixer-related changes in φ , $\delta\varphi_m^1$, we expect that the contribution of $\delta\varphi_s$ to $\delta\varphi$ will be about

$$\delta\varphi = -\delta\varphi_s \quad (13)$$

Actually, modeling of this situation in terms of the fundamental equation of gradient retention (eqn. A1 in ref. 1) shows that

$$\delta\varphi = -1.1\delta\varphi_s \quad (13a)$$

which is the same result obtained for the relationship of $\delta\varphi$ to $\delta\varphi_m$ (eqn. 34 in ref. 1). That is, in each instance the contribution to $\delta\varphi$ is about 1.1-fold greater than that estimated from the change in φ at the column midpoint.

The next step in determining values of $\delta\varphi_s$ is to use the model described in Figs. 2 and 3 with the isotherm data for acetonitrile-water in Table III. The results of such a calculation for given values of s and V_G are given in Fig. 4, where $\delta\varphi_s V_G/s$ is plotted against $\bar{\varphi}$ for various gradients: 10–100% acetonitrile-water (as used in the present studies), 30–100% and 50–100%. These plots show that $\delta\varphi_s$ is small for solutes eluting under gradient conditions near the beginning or end of the chromatogram, and reaches a maximum value near the middle of the chromatogram. Errors in calculated values of t_g (eqn. 4) due to solvent demixing during gradient elution are therefore largest for solutes with intermediate values of t_g . These errors also increase with increasing surface area of the column, and decrease with increasing values of V_G . That is, a larger value of s results in a greater uptake of the organic solvent by the column (as uptake is proportional to s ; see ref. 11). Similarly, the effect of solvent uptake by the column is greatest when the total volume of mobile phase passing through the column (V_G) is smaller, as then the concentration change in the mobile phase is greater.

The general results in Fig. 4 apply exactly only for sufficiently large values of V_G/s , because as V_G/s becomes small the gradient is distorted in a major way and values of $\delta\varphi_s V_G/s$ then become dependent on the value of V_G/s . For reversed-phase

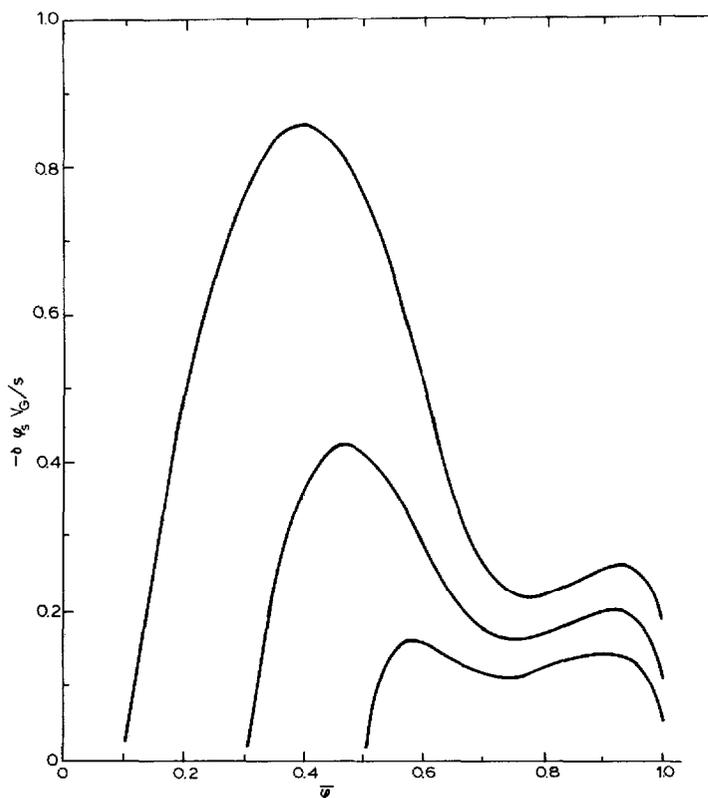


Fig. 4. Change in mobile-phase composition ($\delta\phi_s$) during gradient elution with a reversed-phase column as a result of uptake of organic solvent by packing. Acetonitrile-water gradients for various starting compositions ϕ_0 . Calculated according to the scheme in Fig. 3.

columns and common organic solvents, it appears that this effect is generally unimportant, as long as $V_G/s \geq 20 \mu\text{l}/\text{m}^2$. Likewise, the curves in Fig. 4 depend on the plate number of the column used. However, this effect is small when the value of N is large, as will generally be the case in LC separations.

Stationary phase non-equilibrium

Two types of stationary phase non-equilibrium can be distinguished. First, the above discussion and Table III indicate that a variable amount of organic solvent will be "sorbed" on to the stationary phase at equilibrium, depending on the value of ϕ or time during the gradient. Presumably values of k' are affected by the relative amount of sorbed organic solvent, apart from the value of ϕ in the mobile phase. During gradient elution it is conceivable that complete equilibration of the stationary and mobile phases is not achieved at each point within the column and at every time during the gradient. This might then result in some variation of k' values from the equilibrium values predicted by eqn. 1. This in turn would result in deviations of experimental t_g values from values predicted by eqn. 4. In a later section we shall show that experimental values of $\delta\phi_s$ are in good agreement with values predicted by the model in Figs. 2 and 3, which assumes instantaneous equilibrium of the sta-

tionary and mobile phases throughout the gradient (relative to uptake of organic solvent by the column packing). This suggests that equilibrium is in fact rapid, and the effect of phase non-equilibrium is therefore insignificant.

A second type of stationary phase non-equilibrium is also possible, having no direct connection with the amount of organic solvent sorbed on to the packing. Several studies have shown that changes in φ or temperature for bonded-phase LC systems can lead to slow changes in k' from the original to final conditions¹⁷⁻²¹. It is believed that these effects are due to slow configurational changes in the alkyl groups bonded to the packing surface. Available experimental evidence suggests that such effects are more important for pronounced changes in conditions (temperature jump, change from one organic solvent to another, etc.) and for mobile phases composed of pure water. Therefore, gradient elution runs in which the starting value of φ (φ_0) is not equal to zero (as is common practice) should be less sensitive to slow changes in alkyl-group configuration. This study provided no indication that this effect is important in affecting values of t_g .

Pressure dependence of k'

In principle, values of k' can be pressure dependent, which suggests another source of deviation from eqn. 4 at higher flow-rates and column pressures. One study¹⁰ has shown that t_0 in reversed-phase systems can vary markedly with column pressure, particularly for lower values of φ . We observed similar effects in this study, as discussed in a later section. However, the preceding analysis (eqn. 10) suggests that variation in t_0 (or t_{sec}) by itself will have only a minor effect on calculated values of t_g .

Equilibration of the column before gradient elution is begun

Following a gradient run, the column must be flushed with the starting mobile phase ($\varphi = \varphi_0$) prior to initiating the next gradient separation. Sufficient flushing must occur so that the column is at equilibrium with mobile phase of composition φ_0 , as confirmed by constant t_R values for repeated injection of a solute under isocratic conditions (before starting the gradient). This requirement for repeatable retention times, t_g , in gradient elution is well known (*e.g.*, refs. 2 and 13) and was adhered to in this study. In general it was found by us that a minimum of 15 column volumes of initial solvent ($\varphi = \varphi_0$) were required in order to wash the column between completion of one gradient run and initiation of the next gradient run.

Other requirements

These are well known (*e.g.*, ref. 13) and were adhered to in this study. Systematic errors in column temperature or mobile phase composition lead to predictable trends in t_R and t_g values for a series of related solutes of varying retention (*e.g.*, C₁-C₅ alkyl phthalates as in this study)²². The variation in apparent error in t_g (experimental vs. calculated values) as a function of solute structure can therefore be used to test for specific causes of experimental error.

EXPERIMENTAL

Equipment

The liquid chromatograph used for these experiments was described in the preceding paper¹. A refractive index detector (DuPont, Wilmington, DE, U.S.A.) was used for t_0 measurements. Retention data were recorded with a Model 3380A integrator (Hewlett-Packard, San Diego, CA, U.S.A.).

Reagents

HPLC-grade solvents were as described in ref. 1. Phthalate esters were obtained from Chem Service (West Chester, PA, U.S.A.): dimethyl (C_1), diethyl (C_2), diallyl (C_3), di-*n*-butyl (C_4) and di-*n*-pentyl (C_5).

Columns

Columns were 25×0.46 cm I.D., packed with nominal 6- μ m particles based on Zorbax-SIL (DuPont) and bonded with dimethyloctadecylchlorosilane (C_{18}). Particles referred to as "6-nm pore" were provided as commercial columns of Zorbax-ODS, and columns of "15-nm pore" particles were prepared from non-commercial materials.

Chromatographic conditions

Isocratic and gradient retention data for a mixture of five phthalate esters (C_1 - C_5) were obtained in triplicate (within-day runs). Only linear gradients were used (10-100% acetonitrile-water). Isocratic k' values were calculated from the equation $k' = (t_R - t_{sec})/t_{sec}$, using values of t_{sec} from Table I (t_R is retention time). Values of t_0 (Table I) were determined by injecting mobile phase supplemented with deuterium oxide (as in ref. 10). Values of t_{sec} were calculated for each solute from t_0 values as described in the preceding section.

The reproducibility of both isocratic and gradient retention data was studied over a 60-day interval for the five solutes. For isocratic runs, ϕ was equal to 0.75 and the flow-rate was 2.0 ml/min (6-nm pore column, 35°C). For the gradient runs the same conditions were used, except ϕ was varied from 0.1 to 1.00 during the gradient.

RESULTS AND DISCUSSION

Isocratic and gradient retention data were collected for five solutes (C_1 - C_5 dialkyl phthalates) with acetonitrile-water mobile phases. A broad range of separation conditions was examined, in order to assess adequately the various sources of error discussed under Theory. For isocratic systems these included two columns (6- and 15-nm pore diameters), two temperatures (35 and 60°C) and mobile phase compositions with $0.10 \leq \phi \leq 1.00$. For gradient systems, the same columns and temperatures were used, and the gradient conditions covered the following ranges: $5 \leq V_G \leq 640$ ml, $2.5 \leq t_G \leq 320$ min, $0.01 \leq b \leq 2.2$ and $0.2 \leq F \leq 4$ ml/min. The resulting values of k' (isocratic) and t_g (gradient) are summarized in Tables II and IV, respectively.

As discussed in the preceding paper¹, it is convenient to express the error in

TABLE IV

EXPERIMENTAL VS. CALCULATED (EQN. 4) VALUES OF GRADIENT RETENTION t_g AND COMPARISON OF DIFFERENCES IN t_g (EXPRESSED AS $\delta\phi$) WITH GRADIENT DISTORTION FROM DISPERSION ($\delta\phi_m$) AND SORPTION OF ORGANIC SOLVENT ($\delta\phi_s$)

Gradient: 10-100% acetonitrile-water.

Column	T (°C)*	t_e (min)**	F (ml/min)***	V_M/V_C^\S	Solute ^{§§}	t_g (min)	$\bar{\phi}^{\S\S\S}$	$\delta\phi^\dagger$	$-(\delta\phi_m + \delta\phi_s)^{\dagger\dagger}$	Residual ^{†††}	
											Exptl.
6-mm pore, 25 cm	35	5	2.0	0.20	C ₁	6.88	6.79	0.016	0.019	0.003	
					C ₂	7.66	7.56	0.018	0.008	0.010	
					C ₃	7.94	7.87	0.013	0.008	0.005	
					C ₄	9.07	8.94	0.023	0.039	-0.016	
					C ₅	9.75	9.32	1.00	0.077	-0.003	
	80	80	0.2	0.125	C ₁	81.4	79.5	0.46	0.021	0.016	0.005
					C ₂	93.5	91.2	0.60	0.025	0.010	0.015
					C ₃	98.2	98.2	0.67	0.000	0.007	-0.007
					C ₄	113.9	112.5	0.86	0.016	0.007	0.009
					C ₅	122.0	120.0	0.95	0.022	0.022	0.00
	15	10	2.0	0.10	C ₁	8.82	8.64	0.43	0.016	0.015	0.001
					C ₂	10.31	10.06	0.57	0.022	0.011	0.011
					C ₃	10.92	10.73	0.63	0.017	0.009	0.008
					C ₄	12.83	12.76	0.83	0.006	0.004	0.002
					C ₅	13.74	13.73	0.92	0.001	0.008	-0.007
160	15	2.0	0.07	C ₁	10.44	10.17	0.38	0.016	0.010	0.006	
				C ₂	12.60	12.25	0.52	0.021	0.008	0.013	
				C ₃	13.56	13.22	0.58	0.020	0.007	0.013	
				C ₄	16.42	16.23	0.77	0.011	0.003	0.008	
				C ₅	17.79	17.70	0.87	0.005	0.002	0.003	
20	20	2.0	0.06	C ₁	106.3	104.5	0.38	0.010	0.009	0.001	
				C ₂	130.3	126.6	0.51	0.021	0.008	0.013	
				C ₃	140.7	137.1	0.58	0.020	0.007	0.010	
				C ₄	171.3	169.0	0.76	0.013	0.003	0.007	
				C ₅	186.5	184.9	0.86	0.009	0.002	0.004	
	20	2.0	0.05	C ₁	11.78	11.53	0.35	0.011	0.007	0.001	
				C ₂	14.64	14.26	0.48	0.017	0.007	0.001	
				C ₃	15.94	15.62	0.55	0.014	0.006	0.008	
				C ₄	19.72	19.45	0.72	0.012	0.003	0.009	

(Continued on p.34)

TABLE IV (continued)

Column	T (°C)*	t _G (min)**	F (ml/min)***	V _M /V _G [§]	Solute ^{§§}	t _g (min)		φ ^{§§§}	δφ [†]	-(δφ _m + δφ _{s})^{††}}	Residual ^{†††}
						Exptl.	Calc.				
C ₅						21.64	21.51	0.83	0.006	0.002	0.004
C ₁		80	0.5	0.05		47.3	46.1	0.35	0.013	0.007	0.006
C ₂						58.6	57.0	0.48	0.018	0.007	0.011
C ₃						63.8	62.5	0.55	0.014	0.006	0.008
C ₄						78.9	77.7	0.72	0.012	0.003	0.008
C ₅						86.5	86.1	0.83	0.005	0.002	0.003
C ₁	30		2.0	0.03		14.22	13.89	0.32	0.010	0.005	0.005
C ₂						18.42	17.94	0.44	0.014	0.005	0.009
C ₃						20.43	19.89	0.51	0.016	0.004	0.012
C ₄						26.00	25.46	0.67	0.016	0.002	0.014
C ₅						28.97	28.65	0.78	0.010	0.002	0.008
C ₁	320		0.2	0.03		148.2	143.1	0.31	0.014	0.004	0.010
C ₂						192.5	186.3	0.44	0.017	0.005	0.012
C ₃						213.9	207.1	0.50	0.019	0.004	0.015
C ₄						272.7	266.3	0.66	0.018	0.002	0.016
C ₅						304.7	300.5	0.77	0.012	0.002	0.010
C ₁	40		2.0	0.02		16.20	15.90	0.29	0.007	0.003	0.004
C ₂						21.72	21.83	0.42	0.002	0.004	0.004
C ₃						24.45	23.89	0.48	0.013	0.003	0.010
C ₄						31.76	31.10	0.64	0.015	0.002	0.013
C ₅						35.80	35.42	0.74	0.009	0.001	0.008
C ₁	160		0.5	0.02		65.4	63.6	0.29	0.010	0.003	0.007
C ₂						87.3	87.3	0.42	0.000	0.004	-0.004
C ₃						98.2	96.5	0.48	0.009	0.003	0.006
C ₄						127.2	124.4	0.64	0.016	0.002	0.014
C ₅						143.4	141.7	0.74	0.009	0.001	0.008
C ₁	40		4.0	0.01		11.46	11.18	0.24	0.006	0.001	0.005
C ₂						16.73	16.36	0.36	0.008	0.002	0.006
C ₃						19.52	19.06	0.42	0.010	0.002	0.008
C ₄						26.52	25.78	0.57	0.017	0.001	0.015
C ₅						30.54	29.98	0.67	0.012	0.001	0.011
C ₁	80		2.0	0.01		22.86	22.36	0.24	0.006	0.001	0.005
C ₂						33.35	32.71	0.36	0.007	0.002	0.005
C ₃						38.92	38.10	0.42	0.009	0.002	0.007
C ₄						52.85	51.57	0.57	0.011	0.001	0.010

80	4.0	0.006	C ₅	60.9	60.0	0.67	0.010	0.001	0.009
			C ₁	15.97	15.74	0.25	0.002	0.000	0.002
			C ₂	25.88	25.44	0.30	0.005	0.001	0.004
			C ₃	31.52	30.86	0.37	0.007	0.001	0.007
			C ₄	45.0	43.6	0.51	0.016	0.001	0.015
			C ₅	52.9	51.7	0.60	0.013	0.001	0.012
160	2.0	0.006	C ₁	31.99	31.48	0.19	0.003	0.000	0.003
			C ₂	51.7	50.9	0.30	0.004	0.001	0.003
			C ₃	62.9	61.7	0.37	0.007	0.001	0.006
			C ₄	89.7	87.2	0.51	0.014	0.001	0.013
			C ₅	105.4	103.4	0.60	0.011	0.001	0.010
160	4.0	0.003	C ₁	22.00	21.80	0.15	0.001	0.001	0.001
			C ₂	40.2	39.5	0.26	0.004	0.001	0.003
			C ₃	51.4	50.1	0.31	0.007	0.001	0.006
			C ₄	77.5	75.1	0.46	0.013	0.001	0.012
			C ₅	92.7	90.1	0.54	0.015	0.001	0.014
320	2.0	0.003	C ₁	43.8	43.6	0.15	0.001	0.000	0.000
			C ₂	79.8	79.0	0.26	0.002	0.001	0.001
			C ₃	102.1	100.2	0.31	0.005	0.001	0.004
			C ₄	153.5	150.3	0.46	0.009	0.001	0.008
			C ₅	183.6	180.3	0.54	0.009	0.001	0.009
6-nm pore, 50 cm	2.5	0.40	C ₁	7.31	7.21	0.80	0.036	0.063	-0.027
			C ₂	7.94	7.62	0.92	0.115	0.132	-0.017
			C ₃	8.12	7.76	0.96	0.130	0.164	-0.034
			C ₄	9.45	8.14	1.11	0.472	- ^a	- ^a
			C ₅	10.37	8.25	1.15	0.76	- ^a	- ^a
5.0	2.0	0.20	C ₁	8.74	8.60	0.66	0.025	0.018	0.007
			C ₂	9.56	9.35	0.80	0.038	0.018	0.020
			C ₃	9.80	9.64	0.85	0.029	0.025	0.004
			C ₄	11.24	10.64	1.02	0.11	- ^a	- ^a
			C ₅	12.19	10.92	1.07	0.23	- ^a	- ^a
10	2.0	0.10	C ₁	11.20	10.83	0.52	0.033	0.025	0.008
			C ₂	12.70	12.37	0.68	0.030	0.010	0.020
			C ₃	13.22	12.99	0.74	0.021	0.008	0.013
			C ₄	15.17	15.13	0.92	0.004	0.013	-0.009
			C ₅	16.26	15.89	0.99	0.033	0.039	-0.006
20	2.0	0.05	C ₁	15.07	14.53	0.44	0.024	0.015	0.009
			C ₂	17.97	17.37	0.58	0.027	0.011	0.016

(Continued on p. 36)

TABLE IV (continued)

Column	T (°C)*	t _G (min)**	F (ml/min)***	V _M /V _G [§]	Solute ^{§§}	t _g (min)		φ ^{§§§}	δφ [†]	-(δφ _m + δφ _{s})^{††}}	Residual ^{†††}
						Exptl.	Calc.				
15-nm pore, 25 cm	35	40	2.0	0.02	C ₃	19.17	18.71	0.64	0.021	0.008	0.013
					C ₄	22.98	22.77	0.82	0.009	0.004	0.005
					C ₅	24.69	24.71	0.91	0.001	0.004	-0.005
					C ₁	21.08	20.31	0.36	0.017	0.007	0.010
					C ₂	26.74	25.77	0.50	0.022	0.007	0.015
	5		2.0	0.20	C ₃	29.33	28.49	0.55	0.019	0.006	0.013
					C ₄	36.91	36.15	0.73	0.017	0.003	0.014
					C ₅	40.71	40.27	0.83	0.010	0.002	0.008
					C ₁	6.66	6.65	0.47	0.002	0.021	-0.019
					C ₂	7.42	7.34	0.60	0.014	0.014	0.00
	10		2.0	0.10	C ₃	7.72	7.67	0.67	0.009	0.009	0.00
					C ₄	8.71	8.56	0.83	0.027	0.014	0.013
					C ₅	9.27	9.02	0.91	0.045	0.031	-0.014
					C ₁	8.35	8.23	0.38	0.011	0.014	-0.003
					C ₂	9.79	9.59	0.51	0.018	0.014	0.004
	20		2.0	0.05	C ₃	10.42	10.23	0.58	0.017	0.011	0.006
					C ₄	12.24	12.08	0.74	0.014	0.004	0.010
					C ₅	13.13	13.02	0.83	0.010	0.004	0.006
					C ₁	10.88	10.68	0.31	0.009	0.008	0.001
					C ₂	13.65	13.32	0.44	0.002	0.008	0.006
80		2.0	0.01	C ₃	14.98	14.64	0.50	0.015	0.007	0.008	
				C ₄	18.50	18.20	0.66	0.014	0.004	0.010	
				C ₅	20.40	20.15	0.75	0.011	0.003	0.008	
				C ₁	19.46	19.44	0.20	0.000	0.001	-0.001	
				C ₂	29.58	29.10	0.32	0.005	0.002	0.003	
		2.0		C ₃	35.17	34.35	0.38	0.009	0.002	0.007	
				C ₄	48.35	47.12	0.52	0.014	0.002	0.012	
				C ₅	55.91	55.06	0.61	0.010	0.001	0.009	

6-nm pore, 25 cm	60	5	2.0	0.20	C ₁	6.63	6.55	0.48	0.014	0.020	-0.006
					C ₂	7.43	7.34	0.63	0.016	0.010	0.006
					C ₃	7.69	7.62	0.69	0.013	0.008	0.005
					C ₄	8.68	8.57	0.87	0.020	0.023	-0.003
					C ₅	9.22	9.00	0.94	0.040	0.038	0.002
	10		2.0	0.10	C ₁	8.37	8.23	0.39	0.013	0.012	0.00
					C ₂	9.89	9.67	0.53	0.020	0.013	0.008
					C ₃	10.47	10.27	0.59	0.018	0.009	0.009
					C ₄	12.28	12.20	0.78	0.007	0.004	0.003
					C ₅	13.11	13.04	0.86	0.006	0.004	0.002
	20		2.0	0.05	C ₁	10.96	10.79	0.32	0.008	0.007	0.001
					C ₂	13.88	13.52	0.45	0.016	0.007	0.009
					C ₃	15.12	14.73	0.51	0.018	0.007	0.011
					C ₄	18.74	18.53	0.69	0.009	0.003	0.006
					C ₅	20.53	20.40	0.78	0.006	0.002	0.004
	40		2.0	0.02	C ₁	14.69	14.53	0.26	0.004	0.003	0.001
					C ₂	20.28	19.72	0.38	0.013	0.004	0.009
					C ₃	22.86	22.28	0.44	0.013	0.004	0.009
					C ₄	29.98	29.34	0.60	0.014	0.003	0.011
					C ₅	33.70	33.41	0.70	0.006	0.002	0.004

* Temperature (°C).

** Gradient time (min).

*** Flow-rate (ml/min).

§ $V_M = 2.0$; V_G in ml.

§§ Refers to carbon number of each alkyl chain in dialkyl phthalate solute (C₁, dimethyl phthalate).

§§§ Calculated value.

† Actual difference in t_g (exptl. - calc.).

†† Sum of corrections for gradient dispersion and solvent demixing. Note that factor of 1.1 in eqn. 34 is cancelled exactly by $\Delta\phi$ factor = 0.9.

††† $\delta\phi + (\delta\phi_m + \delta\phi_s)$.

^a $\phi > 1$ (elution outside of gradient).

calculated values of t_g (eqn. 4) in terms of values of $\delta\phi$ equal to $[(t_g)_{\text{expt}} - (t_g)_{\text{calc}}]\Delta\phi/t_G$. It is further assumed that $\delta\phi$ is the sum of specific error contributions, $\delta\phi_i$, from each error source i (e.g., $\delta\phi_m$ and $\delta\phi_s$, which have already been discussed). We shall define several such error sources as we proceed, based on the discussion in the Theory section.

Reproducibility of retention

A total of 30 isocratic and 30 gradient runs were made over a 2-month period in order to assess the reproducibility of retention (300 data points). The results are summarized in Table V. It can be seen that the overall variation in isocratic values of k' was $\pm 1.2\%$ (1 standard deviation, S.D.). According to eqn. 7, this should result in a variation in t_g values of $\delta\phi = 0.012/2.3 \times 3 = 0.0017$ (assuming an average value of $S = 3$ for the present system and solutes). The corresponding value of the standard deviation in t_g values is then $0.0017 (t_g/\Delta\phi) = 0.02$ min. The cycle time of the solenoid valves that service the gradient mixer contributes an additional 1.7 sec to uncertainty in t_g (or 0.03 min, 1 S.D.), so that the predicted variation in t_g values is then $(0.02^2 + 0.03^2)^{1/2} = 0.04$ min (1 S.D.). This is in rough agreement with the observed variation of t_g values in Table V (-0.02 min).

More limited data were obtained for the reproducibility of t_g values for other values of t_G : $t_G = 5$ min (± 0.01 min), $t_G = 20$ (± 0.04 min) and $t_G = 40$ (± 0.16 min). Eqn. 7 predicts that $\delta\phi$ should be independent of t_G , or that variation in t_g should be proportional to t_G . This is approximately the case, as shown in Table VI.

Gradient "non-ideality"

The preceding paper¹ provides guidelines for predicting errors in experimental t_g values due to distortion of the gradient via dispersion in the gradient system. Values of $\delta\phi_m$ (gradient error due to the system) could be obtained for each t_g value in Table

TABLE V
REPRODUCIBILITY OF ISOCRATIC AND GRADIENT RETENTION DATA

Thirty replicates each procedure over a 2-month period; isocratic conditions, 2.0 ml/min, one 6-nm C₁₈ column, 35°C, $\phi = 0.75$; gradient conditions, same except gradient from $\phi = 0.10$ to $\phi = 1.00$, $t_G = 10$ min.

Solute*	t_R (min)**	k' **	CV (%)***	t_g (min)§
C ₁	1.74 ± 0.01	0.58	1.8	8.84 ± 0.03
C ₂	2.26 ± 0.01	1.05	1.0	10.32 ± 0.02
C ₃	2.53 ± 0.015	1.30	1.1	10.92 ± 0.02
C ₄	5.56 ± 0.045	4.05	1.0	12.84 ± 0.02
C ₅	9.90 ± 0.11	8.00	1.2	13.75 ± 0.02
Average			± 1.2 ^{§§}	± 0.02 min

* Di-*n*-alkyl phthalates (methyl to pentyl).

** Isocratic separation.

*** Coefficient of variation in k' .

§ Gradient elution ($t_0 = 1.0$ min, $b = 0.3$).

§§ The within-day precision was much better ($\pm 0.2\%$).

TABLE VI
REPRODUCIBILITY OF t_g or $\bar{\phi}$ VALUES

t_G (min)	$\delta\phi$	t_g (min)
5	± 0.002	± 0.01
10	± 0.002	± 0.02
20	± 0.002	± 0.04
40	± 0.004	± 0.16

IV from Table I in ref. 1. The contribution of these gradient errors to t_g was then given as $\delta\phi = -1.1\delta\phi_m$, as in ref. 1.

Flow-rate errors due to compressibility effects were analyzed in the preceding paper¹. For the present acetonitrile-water systems, with their lower column pressures, errors from this source ($\delta\phi_f$) were insignificant. The delay time, t_D (equal to V_D/F), was determined directly as described in the preceding paper¹: V_D was equal to 5.5 ± 0.1 ml. Solute pre-elution, as discussed previously¹, was also insignificant, except for the case of dimethyl phthalate as the solute with the 15-nm-pore C_{18} column. Data for this case were ignored.

Values of t_0 and t_{sec}

Values of t_0 were measured for each system and used to calculate values of t_{sec} as described in the Theory section and summarized in Table I. For the present system and solutes, errors in the calculation of $t_{sec}F = V_{sec}$ are believed to be no larger than ± 0.02 ml, suggesting a maximum error ($\delta\phi$) of $0.02/V_G$. For $V_G \geq 5$ ml, as in the present study, the contribution to values of $\delta\phi$ from errors in t_{sec} is then less than 0.004 and usually much smaller.

Non-linear plots of $\log k'$ vs. ϕ

Fig. 1 shows plots of $\log k'$ vs. ϕ for a particular system (6-nm pore column, 35°C) and three of the five solutes (C_1 , C_3 , C_5). These plots are linear in the region $0.65 \leq \phi \leq 1.00$, but become significantly curved at lower values of ϕ . In initial work we measured k' values only over the range $0.7 \leq \phi \leq 0.9$ and concluded (incorrectly) that linear plots of $\log k'$ vs. ϕ persisted at lower values of ϕ ($\phi < 0.6$).

TABLE VII

ERROR IN CALCULATED t_g VALUES DUE TO ASSUMPTION OF LINEAR $\log k'$ VS. ϕ PLOTS IN FIG. 4 (DASHED CURVES)

Column, 25 × 0.46 cm I.D., 6-nm pores; temperature, 35°C; flow-rate, 2 ml/min.

Solute	$t_G = 5$ min			$t_G = 40$ min			$t_G = 80$ min			$t_G = 160$ min		
	Exptl.	Calc.	$\bar{\phi}$	Exptl.	Calc.	$\bar{\phi}$	Exptl.	Calc.	$\bar{\phi}$	Exptl.	Calc.	$\bar{\phi}$
C_1	4.4	3.9	0.43	11.5	5.9	0.23	16.0	6.3	0.20	22.0	6.5	0.15
C_3	5.5	5.3	0.64	19.5	14.5	0.39	31.5	19.2	0.31	57.4	23.8	0.24
C_5	6.9	6.8	0.91	30.5	30.2	0.67	52.8	51.3	0.59	92.8	86.8	0.50

This in turn resulted in major errors in calculated values of t_g (eqn. 4), as illustrated in Table VII. Thus, calculated values of t_g for the C_1 and C_3 solutes are grossly in error for t_G values of 40 min or greater, corresponding to $\bar{\varphi}$ values of less than 0.4 (where the $\log k' vs. \varphi$ plots are non-linear). When $\bar{\varphi} \geq 0.6$ (Table VII), experimental and calculated values of t_g are seen to be in reasonable agreement. Use of the tangent approximation in actual $\log k' vs. \varphi$ plots (as described in the Theory section) was used in Table IV to calculate values of t_g from eqn. 4. We shall see that the resulting agreement between experimental and calculated t_g values was then satisfactory. From these results it is seen that agreement between experimental and calculated values of t_g in gradient elution will generally require that isocratic data overlap the mobile phase composition that corresponds to $\bar{\varphi}$ (composition at the column midpoint when the band is half eluted by the gradient).

Solvent demixing during gradient elution

The data in Table IV yield $\delta\varphi$ values as a function of the separation conditions; these deviations of experimental t_g values from calculated values (eqn. 4) can be compared with $\delta\varphi_s$ values calculated as described in the Theory section and plotted against $\bar{\varphi}$ in Fig. 4. However, it is necessary first to recognize other contributions to $\delta\varphi$, specifically the value of $\delta\varphi_m$ due to gradient dispersion by the equipment. If $\delta\varphi_s$ and $\delta\varphi_m$ are the major contributions to $\delta\varphi$, then we can write (see eqn. 34 in ref. 1 and eqn. 13 in this paper)

$$\delta\varphi \approx -1.1(\delta\varphi_m + \delta\varphi_s) \quad (14)$$

Experimental values of $\delta\varphi_s$ can then be obtained from eqn. 14, given experimental values of $\delta\varphi$ and calculated values of $\delta\varphi_m$ from Table I in ref. 1. The quantity $\delta\varphi_s V_G/s$ can in turn be calculated for the data in Table IV, permitting a comparison with values predicted by the plot of this quantity against $\bar{\varphi}$ in Fig. 4. This is shown in Fig. 5. The data points shown in Fig. 5 represent data for which $\delta\varphi_s$ values are most reliable. This involved excluding data for which $V_G/s > 48$, since then values of $\delta\varphi_s$ are small, and uncorrected contributions to $\delta\varphi$ from other sources lead to greater relative errors in $\delta\varphi_s$. Data for $\bar{\varphi} > 0.9$ were also excluded, as for this case values of $\delta\varphi_m$ are larger and values of $\delta\varphi_s$ smaller, again leading to less reliable values of $\delta\varphi_s$ (see eqn. 14).

Before examining the correlation of experimental and calculated values of $\delta\varphi_s V_G/s$ in Fig. 5, it is necessary to discuss values of s for the columns of 6-nm pore packing. The nominal value of s from the surface area of the unbonded silica is 1000 m^2 /column. From Table III, the maximum uptake of acetonitrile by the column would be predicted to be 1.30 ml, but the total pore volume of the column is only 0.82 ml. In this instance, it appears that the small diameter of the pores (6 nm) leads either to a reduced surface after bonding with C_{18} groups, or a maximum surface loading by acetonitrile of less than 1.3 $\mu l/m^2$. It is mathematically convenient to assume that the surface loading remains constant at 1.3 $\mu l/m^2$, and that the surface area for this column is reduced (*i.e.*, $s < 1000 m^2$). The uptake of acetonitrile by the column is related to the value of t_0 using D_2O as solute, and values of $(t_{0w} - t_0)$ per m^2 of surface are given in ref. 11; here, t_{0w} is the value of t_0 for water as mobile phase ($\varphi = 0.00$). Therefore, division of experimental values of $t_{0w} - t_0$ for the

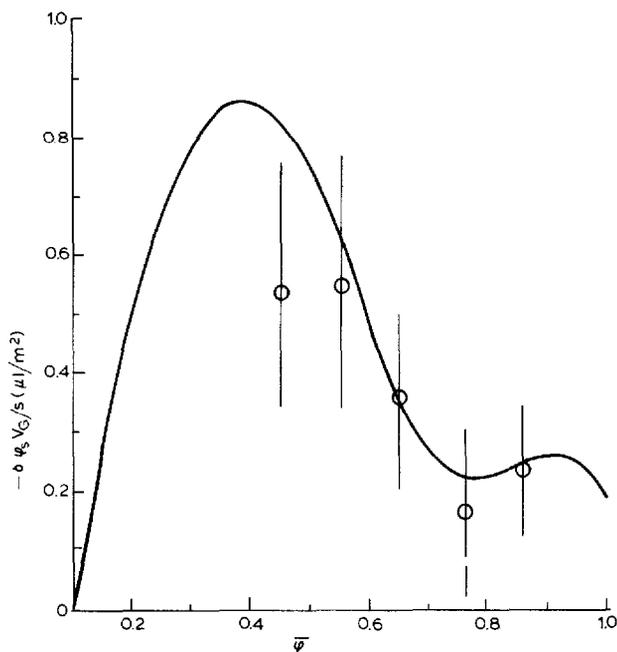


Fig. 5. Comparison of experimental (O) and calculated (—) values of $\delta\phi_s$ as in Fig. 4. See text. Experimental data are averages over increments of 0.05 in ϕ .

present columns by the latter values from ref. 11 yields an effective value of s for the column. The data in Table I were utilized in this fashion for values of ϕ of 0.2, 0.4, 0.6 and 0.8, for both the 6- and 15-nm pore columns. The resulting values of s are given in Table VIII.

For the 6-nm pore silica the resulting value of s (321 m^2) suggests both a lower surface area for this C_{18} -silica and less than $1.3 \mu\text{l}/\text{m}^2$ of acetonitrile uptake at saturation. For the 15-nm pore silica, the value of s is within experimental error of the previous value for the bonded silica. We assume $s = 320$ and 350 (Fig. 5), respectively, for 6- and 15-nm pore packings.

Returning to the correlation in Fig. 5, it is assumed here that the contribution of other (uncorrected) effects to $\delta\phi$ values is about ± 0.005 unit, including the imprecision in $\delta\phi$ of ± 0.002 unit referred to earlier. The vertical lines through each

TABLE VIII

CALCULATED VALUES OF SURFACE AREA

Column	s from value of $t_{0w} - t_0$ (m^2)	Surface area of column (m^2)	
		Unbonded silica	Bonded silica*
6-nm pore	321 ± 10	1000	540
15-nm pore	354 ± 45	420	325

* Surface accessible to retained solutes⁹ (vs. wide-pore bonded silica).

TABLE IX
 VARIATION OF SOLVENT-DEMIXING FUNCTION ($\delta\phi_s V_G/s$) WITH SOLUTE RETENTION, $\bar{\phi}$
 Gradients from ϕ_0 to $\phi = 1.0$.

$\bar{\phi}$	$-\delta\phi_s V_G/s$ ($\mu l/m^2$)		
	$\phi_0 = 0.1$	$\phi_0 = 0.3$	$\phi_0 = 0.5$
0.10	0.00		
0.15	0.26		
0.20	0.49		
0.25	0.66		
0.30	0.77	0.00	
0.40	0.86	0.37	
0.50	0.75	0.41	0.00
0.60	0.50	0.28	0.16
0.70	0.26	0.18	0.12
0.80	0.22	0.18	0.13
0.90	0.26	0.20	0.15
1.00	0.19	0.12	0.05

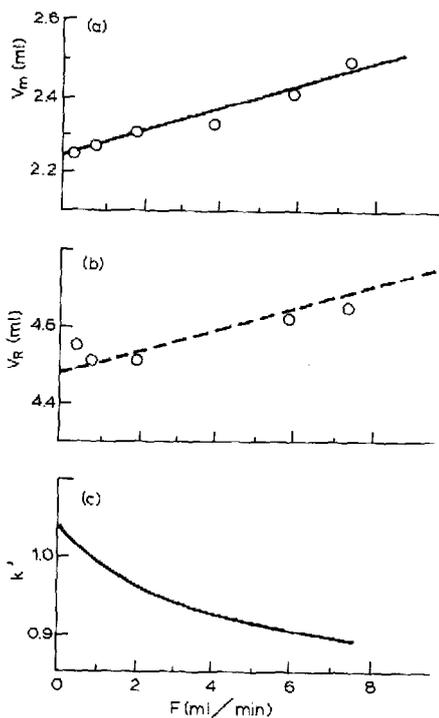


Fig. 6. Variation of column dead-volume ($V_m = Ft_0$) with flow-rate and effect on retention volume, V_R , and capacity factor, k' . C_2 solute, 35°C , $\phi = 0.75$.

data point in Fig. 5 correspond to an uncertainty of ± 0.005 unit in $\delta\phi$ and $\delta\phi_s$. The resulting correlation in Fig. 5 is reasonable and confirms the importance of solvent demixing that was predicted by the model in the Theory section (Fig. 4). The data in Fig. 4 are further tabulated in Table IX. Note that the value of $\delta\phi_s V_G/s$ does not depend on the final value of ϕ in the gradient (ϕ_f).

Stationary phase non-equilibrium

This effect, if it significantly alters t_g values in gradient elution, should be a function of mobile phase flow-rate and/or temperature (see discussion in Theory section). No such effects were observed in this study, as can be seen from the data in Table IV.

Pressure dependence of k'

Previous workers¹⁰ have noted that V_m increases with increasing column pressure (or flow-rate) in reversed-phase systems. We have observed this effect in the present system, as summarized in Fig. 6a. It can be seen that V_m increases by about 10% as the flow-rate is increased from 0 to 7.5 ml/min, corresponding to a range of column inlet pressures of 0–325 atm. This increases retention volumes, V_R , by the same absolute amount, as shown in Fig. 6b (C_2 as solute); the dashed curve in Fig. 6b has the same slope as the solid curve in Fig. 6a. The apparent k' values from Fig. 6a and b are plotted in Fig. 7c.

According to eqn. 10, the effect of variation in V_m and k' (as in Fig. 6) on calculated t_g values (eqn. 4) is simply to add the corresponding variation in t_{sec} (or t_0) to calculated value of t_g . Values of t_{sec} are equal to about 0.93 t_0 (Table I), so from Fig. 6 we have

$$V_{sec} = 2.07 + 0.026 F \quad (15)$$

for $\phi = 0.75$. The corresponding error $\delta\phi_{t_0}$ introduced by variation in t_0 with F is then $0.026\Delta F/V_G$, where ΔF refers to the difference in F values for corresponding gradient and isocratic runs used for comparing experimental vs. calculated t_g values. For most of the data collected here, $\Delta F < 2$ ml/min and $V_G > 10$ ml, so $\delta\phi_{pk} < 0.005$. Errors in the calculated values of t_g due to the effect of Fig. 6 (variation of V_m with F) can be minimized by collecting isocratic data at the same flow-rate as used for the gradient run (or by running at lower flow-rates).

Summary and evaluation of errors in calculated t_g values (eqn. 4)

Table X summarizes the error contributions to eqn. 4 for the calculation of t_g . The major errors encountered were (a) gradient delay (value of t_D), (b) gradient dispersion (value of $\delta\phi_m$), (c) variation of t_{sec} with change in ϕ , (d) solvent demixing (value of $\delta\phi_s$) and (e) the pressure dependence of k' . In each instance these contributions to t_g can be calculated from the experimental conditions. The residual $\delta\phi$ values in Table IV represent the errors in calculated t_g values after correction for effects (a)–(d) above. Appendix I presents a model calculation to show how these corrections are applied, based on the present analysis.

Random errors in t_g or t_R cannot be corrected for. Two such contributions to errors were identified in this study: (a) variable gradient initiation and (b) variable

TABLE X

SUMMARY OF ERRORS CONTRIBUTING TO CALCULATED VALUES OF t_g AS DETERMINED FROM EQN. 4

Effect	Error ($\delta\phi_i/\Delta\phi$)		Maximum error in this study*	
	Correctable	Random	$t_G = 5$	$t_G = 100$
Pumping error (flow-rate $\delta\phi_f$) (eqn. 30 in ref. 1)	$-(\delta F/F)t_0/t_G$	0**	0.004	0.000
Gradient delay, $V_D = Ft_D$:				
Value of t_D (eqn. 10 of in ref. 1)	t_0/t_G		0.55	0.027
Variable gradient initiation		$t_d/\sqrt{12} t_G$	± 0.006	0.000
t_d ($\delta\phi_d$) ¹⁴				
Solute pre-elution ($\delta\phi_p$) (eqn. 20 in ref. 1)	$-V_D/2.3Sk_0V_m$		0.003***	0.003***
Gradient dispersion ($\delta\phi_m$) (Table I in ref. 1)	$-1.1\delta\phi_m$		0.07 [§]	0.004 [§]
Variability of isocratic k' values ($\delta\phi_k$) (eqn. 7)		$(\delta k_0/k_0)/2.3S$	± 0.002 (± 0.003) ^{§§}	± 0.002 (± 0.003) ^{§§}
Variation of t_{sec} with ϕ ($\delta\phi_{sec}$) (eqn. 10, Table I)	$\delta t_{sec}/t_G\phi$		0.056	0.003
Non-linear plots of $\log k'$ vs. ϕ	See discussion of Table VII and Fig. 1			
Solvent demixing ($\delta\phi_s$) (Fig. 4, Table IX)	$-1.1\delta\phi_s$		0.05	0.002
Stationary phase non-equilibrium	Unimportant			
Pressure dependence of k' ($\delta\phi_{pk}$) (eqn. 15)	$-0.026\Delta F/t_G$ ^{§§§}		0.05	0.00

* Flow-rate 2 ml/min, 25×0.46 cm I.D. column (6- or 15-nm pores).** Pumping reproducibility is usually better than $\pm 0.1\%$ for modern LC systems, leading to negligible error in t_g .*** For dimethyl phthalate as solute and 15-nm pore packing, for which $k_0 = 49$ and $S \approx 8$.§ Error varies with $\bar{\phi}$ as in Table I in ref. 1; indicated values are maximum errors for any solute (and any value of $\bar{\phi}$).§§ Recognizes error in both isocratic and gradient measurements, for comparisons based on eqn. 4; equal to $\sqrt{2}$ times error of single isocratic value.§§§ Only for 6-nm pore columns and $\phi = 0.75$; must be determined for other conditions.

k_0 values due to changes in the separation conditions that are common to both gradient and isocratic elution. The data in Table X suggest that random errors contribute a $\delta\phi$ value of ± 0.003 – 0.007 unit, depending on the value of V_G . For most of the data in Table IV, the value of $\delta\phi$ due to random errors should be about ± 0.003 unit. The actual error (± 0.010) for the data in Table IV suggests that we have not accounted for all sources of error in these gradient–isocratic comparisons of retention data, but the following discussion suggests that these remaining errors are not of practical significance.

We did observe that residual errors in experimental t_g values (after correction for various effects) correlate with $\bar{\phi}$, as shown in Table XI. Use of these empirical $\delta\phi$ values (Table XI) reduced the imprecision of calculated t_g values to ± 0.004 (1 S.D.) in ϕ , which is close to the expected value from random error (± 0.003). Errors in the gradient provided by the HPLC system could account for such an effect, but in fact the system was exhaustively tested and found to be free from such errors. No other physical effect of which we are aware can explain the above correlation of errors in t_g with $\bar{\phi}$.

There are two approaches to minimizing errors in calculated values of t_g (or

TABLE XI
CORRELATION OF AVERAGE (UNACCOUNTED FOR) ERRORS WITH $\bar{\phi}$

$\bar{\phi}$	Average error ($\delta\phi$)	$\bar{\phi}$	Average error ($\delta\phi$)
0.1	-0.001	0.7	+0.010
0.3	+0.006	0.9	0.000
0.5	+0.010	1.0	-0.010

errors in isocratic parameters derived from gradient data): (a) use of conditions (large values of V_G) that minimize gradient dispersion and solvent demixing errors, and (b) correction of measured t_g values for these effects. The data in Table IV can be summarized as in Table XII. The use of larger V_G values restricts the choice of k' values for corresponding isocratic conditions, which may be undesirable in some instances. Correction of t_g values is seen to be quite effective, decreasing the variance in t_g by about 86% ($0.027^2-0.010^2$ vs. 0.027^2). The significance of these errors in calculated t_g values will be explored further in the next paper of this series²⁵.

TABLE XII
ERRORS IN t_g AT DIFFERENT V_G VALUES

Value of V_G	Error in t_g ($\delta\phi$)	
	Uncorrected	Corrected
< 20	0.047	0.013
≥ 20	0.020	0.009
All	0.027	0.010

Practical significance of present findings

This study bears on several practical questions that relate to the use of gradient elution:

(1) How reproducible are gradient retention data measured at different times in the same laboratory, or among different laboratories? How does retention reproducibility vary with separation conditions and different LC equipment?

(2) How accurately can isocratic retention data be estimated, based on gradient runs for corresponding LC systems (same solutes, mobile phase, etc.)?

(3) What practical limitations exist in the use of gradient elution to carry out method development for isocratic separation?

The reproducibility of gradient retention data is generally considered to be poorer than that of isocratic data for the same LC system. This study provides theory and data to evaluate this conclusion. Eqn. 7 shows that variability in isocratic retention ($\delta k'/k'$) is equivalent to variability in gradient retention ($\delta\phi$), with a proportionality factor of $(1/2.3S)$. This is illustrated by the data in Table V. In terms of retention times t_R and t_g , it can be seen from Table V that isocratic retention appears

more precise when k' is small ($k' < 2$), whereas gradient retention is more precise when k' is large. For larger solute molecules such as synthetic polymers or proteins, the value of S will be much greater (e.g., refs. 9 and 23), and then values of t_g will generally be much more precise than are values of t_R .

There is further opportunity for variation in gradient retention, owing to its dependence on the LC equipment. Thus, for comparisons of t_g values between different laboratories, the gradient equipment used must either be equivalent in terms of gradient delay (V_D) and gradient dispersion (V_M), or experimental conditions must be chosen to minimize differences in V_D and V_M , e.g., by choosing larger values of V_G . Alternatively, differences in V_D and V_M can be determined for different LC systems, and reported values of t_g can be corrected for these differences as described here. It can then be expected that experimental t_g values will show no more interlaboratory variation than do values of t_R .

The precise calculation of isocratic retention data from gradient runs (in the absence of assumptions such as those in ref. 24) requires two such runs, as described in ref. 9 and further elaborated in ref. 25. The precision of isocratic data obtained in this fashion is directly related to the question examined here: how precisely can gradient retention data be calculated (eqn. 4) from isocratic data? For the present system, an uncertainty in calculated t_g values (eqn. 4) was observed, equal to ± 0.010 unit in $\delta\phi$. For calculation of isocratic retention from gradient runs, an equivalent error can be assumed. This means for the small solute molecules studied here ($S \approx 3$) an error in $\log k'$ of 3×0.010 , or about a 7% error in isocratic k' values. Errors in the separation factor α are much smaller, because of the general correlation of $\delta\phi$ with $\bar{\phi}$ (adjacent bands will have similar $\bar{\phi}$ values) and resulting cancellation of errors in α .

Consider finally the practical limitations on the use of gradient elution for carrying out method development for isocratic separations. Here we have shown that it is possible to calculate gradient retention data from measured isocratic t_R values (and *vice versa*) for small molecules. The optimization of isocratic retention during method development is not very sensitive to absolute values of k' (adjustment of α values is of major concern), and the present analysis suggests that gradient nonideality will not normally be a problem in this respect. A later paper²⁵ will examine this question in greater detail and provide practical examples of the use of gradient elution in isocratic method development. The similar adaptation of the present approach for large-molecule separations requires further study²⁵, as earlier it was observed⁹ that incorrect S values can be derived from gradient runs for the case of molecules of 17,000 daltons and larger.

Although this study is reasonably general, additional data are required for some of the corrections for gradient nonideality. Thus, values of V_D and V_M must be determined (or estimated—see Table II in ref. 1) for a given gradient system. Values of t_0 , t_{sec} , k_0 , etc., must be known as a function of ϕ for the solutes in question. The effective surface area, s , of the column must be known. Data as in Fig. 4 and Table IX for other organic solvents are *not* required, as to a good approximation it appears^{10,11} that resulting values of $\delta\phi_s V_G/s$ are identical for acetonitrile and tetrahydrofuran as organic solvents, and for different column temperatures. Values of $\delta\phi_s V_G/s$ for methanol as the solvent are about half the values in Table IX. Finally, either the pressure dependence of k' must be known, or gradient and isocratic runs must be carried out at low or similar flow-rates.

CONCLUSIONS

This study has provided a theoretical analysis with experimental verification of gradient retention times, t_g , in "non-ideal" systems, *i.e.*, those exhibiting effects not recognized by simple models of gradient elution (as in ref. 2). On the basis of this treatment it is possible to calculate corrections for non-ideal effects, and to calculate values of t_g from retention data for isocratic systems. Calculated and experimental t_g values are in close agreement: $\pm 1.0\%$ of the total gradient time, t_G . This agreement is close to that expected from the random variation of experimental retention data in isocratic and gradient systems as studied by us.

The major non-ideal contributions to gradient retention are as follows: (a) gradient delay or the time required by the mobile phase to pass from the gradient mixer to the column inlet; (b) gradient dispersion or the distortion of gradient shape by the LC equipment; (c) variation of the column dead-time with mobile phase composition and column pressure; and (d) demixing of the mobile phase as a result of uptake by the column packing of strongly sorbed solvent components (organic solvent in the case of reversed-phase systems). Each of these effects was modeled and studied experimentally. A basis now exists for estimating their importance for any gradient elution system.

The practical conclusions of this study include the following:

(1) with proper attention to gradient equipment, retention times in gradient elution should be as reproducible as in isocratic separation;

(2) gradient retention times can be accurately predicted from isocratic data for the same system; isocratic data should similarly be calculatable from corresponding gradient data;

(3) these highly precise relationships between gradient and isocratic retention open the way to a number of more efficient schemes for retention optimization and method development for both gradient and isocratic separations; some of these are discussed elsewhere^{2,5};

(4) a better understanding now exists for gradient separation *per se*, and this paves the way for a new look at the gradient separation of macromolecular species such as proteins.

APPENDIX I

Illustrative calculation of corrected t_g values, starting with isocratic data

The t_g value for diamyl phthalate will be calculated for the following conditions: 25×0.46 cm I.D. Zorbax ODS column, 15-nm pore size, $t_G = 5$ min, $F = 2$ ml/min ($V_G = 10$ ml), $V_M = 2$ ml, $\Delta\phi = 0.9$ (10–100% B), $V_D = 5.5$ ml ($V_M/V_G = 0.2$).

The isocratic data for diamyl phthalate yield (Table II)

$$\log k' = 4.445 - 6.556 \phi + 1.943 \phi^2 \quad (A1)$$

Based on the slope-tangent approach described in this paper, estimate an initial value for $\bar{\phi}$, equal to 0.7. The corresponding values of t_0 and t_{sec} are 1.27 min and 1.20 min, respectively (Table I). The value of $\log \bar{k}$ from eqn. A1 is then 0.808. The slope

of the tangent to the $\log k'$ vs. φ curve at $\bar{\varphi} = 0.7$ is obtained by differentiating eqn. A1:

$$\begin{aligned} S &= -6.556 + 3.886 \varphi \\ &= 3.836 \end{aligned}$$

The value of b for the separation can now be obtained from eqn. 3:

$$b = 3.836 \cdot 0.9 \cdot 1.27/5 = 0.877$$

The value of k_0 is given by

$$\begin{aligned} \log k_0 &= (\bar{\varphi} - \varphi_0)S + \log \bar{k} \\ &= 3.11 \end{aligned}$$

Given values of k_0 , b , t_0 and t_{sec} , a value of t_g can be calculated from eqn. 4 (equal to 6.11 min). This value is based on the initial assumption of $\bar{\varphi} = 0.7$, which must be checked. Calculate a value of \bar{k} from the provisional value of b (see pp. 292-293 in ref. 2):

$$\begin{aligned} \bar{k} &= 1/1.15 b \\ &= 0.99 \end{aligned} \tag{A2}$$

The value of \bar{k} corresponding to our initial estimate of $\bar{\varphi}$ was 6.4 ($\log \bar{k} = 0.808$), so a new value of $\bar{\varphi}$ is estimated based on $\bar{k} = 0.99$ and the whole process repeated. Continuation of this iterative approach eventually yields a final value of $\bar{\varphi} = 0.91$, with the corresponding value of $t_g = 9.02$ min.

Now the latter values of $\bar{\varphi}$ and t_g must be corrected for non-ideal gradient effects, as discussed in this and the preceding paper. The major error contributions are due to gradient dispersion and solvent demixing. To correct for gradient dispersion (see preceding paper) use either eqn. 24, Table I or Fig. 2. In the present example, $V_M/V_G = 0.2$ and $\bar{\varphi} = 0.91$. The value of V/V_G required in Table I in the preceding paper¹ is given by

$$\begin{aligned} V/V_G &= (\bar{\varphi} - \varphi_0)/\Delta\varphi \\ &= 0.9 \end{aligned}$$

from which $\delta\varphi_m/\Delta\varphi = -0.021$. The change in $\bar{\varphi}$ as a result of gradient dispersion is then (eqn. 34 in the preceding paper)¹

$$\delta\varphi = -1.1 (\delta\varphi_m/\Delta\varphi) \cdot 0.9 = 0.021$$

This is equivalent to an increase in t_g of $\delta\varphi t_G/\Delta\varphi = 0.021 \cdot 5/0.9$ or 0.12 min.

The corresponding change in t_g as a result of solvent demixing is calculated as follows. From Fig. 4 (or Table IX) a value of $\delta\varphi_s V_G/s$ can be obtained for $\varphi_0 = 0.1$ and $\bar{\varphi} = 0.91$: $0.26 \cdot 10^{-3}$ ml/m². For the present system, $s = 350$ m² and $V_G = 10$ ml. Therefore, $\delta\varphi_s = -0.010$. The corresponding value of $\delta\varphi$ (eqn. 14) is then

$-1.1(-0.010) = 0.011$ or 0.06 min. The sum of contributions to t_g from gradient dispersion and solvent demixing is $0.06 + 0.12 = 0.18$ min. The calculated value of t_g is then 9.02 min plus this correction, or 9.20 min. The experimental value from Table IV is 9.27 min.

SYMBOLS

(I refers to the preceding paper¹, II to this paper)

A, B, C, D, E	constants in Table II (II);
a	equal to $1 + (2.3k_0b)$;
b	gradient steepness parameter; eqns. 1 and 4 (II);
b_1, b_2	values of b for two different gradient separations, where only the gradient time t_G is varied;
b^x	equal to $(1 - x)b$, corresponding to column of fractional length $1 - x$;
c	equal to $2.3k_0b$;
D_m	average diffusion coefficient of solvents in the mobile phase (cm^2/sec); eqn. 29 (I);
d_t	inner diameter of tubing (cm);
F	mobile phase flow-rate (ml/min);
k'	solute capacity factor;
\bar{k}	value of k' for solute band in gradient elution when it reaches the column midpoint;
k_0	value of k' for solute at beginning of gradient, in mobile phase of composition φ_0 ;
k_w	value of k' for water as mobile phase;
K_s	equilibrium constant ($\mu\text{l}/\text{m}^2$) for sorption of organic solvent from mobile phase on to stationary phase (equal to some function of φ); eqn. 11 (II);
S	slope of plot of $\log k'$ vs. φ ; eqn. 2 (I); also, for non-linear plots, the slope of the tangent to the curve at $\varphi = \bar{\varphi}$ (discussion of Fig. 1, II);
s	effective surface area (m^2) of stationary phase within column, corresponding to uptake of $1.3 \mu\text{l}/\text{m}^2$ of acetonitrile at saturation ($\varphi = 1$); see discussion of Fig. 4, II;
t	time (min) after sample injection or start of gradient;
t_d	delay time (min) between operator initiation of gradient and response of gradient system; 0–0.1 min for Model 8800 system;
t_D	delay time (min) for gradient system; time required for mobile phase to travel through mixing chamber to column inlet; Fig. 1b (I);
t_g	retention time (min) of solute in gradient elution; Eqns. 5 and 6 (I);
t_{g1}, t_{g2}	values of t_g for two gradient runs where only t_G is varied (for $t_G = t_{G1}$ and t_{G2} , respectively);
$(t_g)_{0.5}$	time required for solute band to reach midpoint of column in gradient elution; eqn. 8 (I);

$(t_g)_{\text{calc}}$	value of t_g calculated from isocratic data via eqn. 4 (II);
$(t_g)_{\text{expt}}$	experimental value of t_g ;
t_g^x	value of $(t_g - t_0)$ for column of fractional length $1 - x$; eqn. 13 (I);
t_G	gradient time (min); time from beginning ($\varphi = \varphi_0$) to end ($\varphi = \varphi_f$) of gradient; eqn. 3 (I);
t_0	column dead-time (min); time required for a small, unretained solute molecule to pass through the column;
t_0^x	value of t_0 for column of fractional length $1 - x$;
t_R	solute retention time (min) in isocratic elution;
t_{sec}	time (min) required for an unretained, partially excluded solute molecule to pass through column; see discussion of Table I (II);
V	volume (ml) of mobile phase eluted from column at time t ;
V_a	instantaneous retention volume of solute band (isocratic basis) at some time during gradient elution; eqn. A1 (I);
V_c	volume (ml) contained by connecting tubing between gradient mixer and column inlet;
V_C	contribution to gradient dispersion volume V_M from connecting tubing between mixer and column inlet; eqns. 27 and 29 (I);
V_D	delay volume, equal to total volume of system from gradient mixer to column inlet; eqn. 28 (I);
V_D'	value of V_D corrected for gradient initiation delay; eqn. 32 (I);
V_g	retention volume (ml) of solute in gradient elution; equal to $t_g F$;
V_G	gradient volume (ml); equal to $F t_G$;
V_i	volume (ml) of a component which contributes to V_D or V_M ; eqns. 27 and 28 (I) and see Table II (I);
V_m	volume (ml) of mobile phase within the column; equal to $t_0 F$;
V_M	volume of the gradient mixer (ml); also, dispersion volume of gradient system; eqns. 25-27;
V_{sec}	equal to $t_{\text{sec}} F$;
x	in solute pre-elution, the fractional length of the column traversed by a solute band during elution by the volume V_D of initial mobile phase ($\varphi = \varphi_0$); eqn. 11 (I);
δ	differential (error) in some quantity; e.g., δt_g , δF , etc., represent the error in t_g , F , etc.;
$\Delta\varphi$	change in φ during the gradient, equal to $\varphi_f - \varphi_0$;
φ	volume fraction of organic solvent in mobile phase composed of organic and water;
φ_f, φ_0	final and initial values of φ during gradient;
φ_i	value of φ for mobile phase entering mixer at time t ;
$\bar{\varphi}$	value of φ at the column midpoint at the time the solute band is also at the midpoint;
$\delta\varphi$	difference in experimental and calculated (eqn. 4, II) t_g values, expressed in terms of φ ; eqn. 7 (I);
$\delta\varphi_f$	contribution to $\delta\varphi$ from flow-rate error; eqn. 30 (I);
$\delta\varphi_i$	contribution to $\delta\varphi$ from an error-source i ; eqn. 7a (I);
$\delta\varphi_k$	contribution to $\delta\varphi$ from variation in k' or k_0 ; eqn. 7 (II);

$\delta\varphi_m$	change in φ from ideal gradient as a result of gradient dispersion; Fig. 1d and eqn. 34 (I);
$\delta\varphi_p$	contribution to $\delta\varphi$ as a result of solute pre-elution; eqns. 19 and 20 (I);
$\delta\varphi_s$	change in φ from ideal gradient as a result of solvent demixing; Fig. 4 (II) and eqn. 13a (II);
ψ	surface concentration ($\mu\text{l}/\text{m}^2$) of organic solvent in stationary phase; eqns. 11 and 12 (II);
ψ_0	value of ψ before equilibration of mobile and stationary phases; Fig. 3 (II);
ψ_i	value of ψ after equilibration of mobile and stationary phases; Fig. 3 (II);
ψ^*	surface excess ($\mu\text{l}/\text{m}^2$) of organic solvent in stationary phase (isotherm data reported in refs. 10 and 11 of II).

REFERENCES

- 1 M. A. Quarry, R. L. Grob and L. R. Snyder, *J. Chromatogr.*, 285 (1984) 1.
- 2 L. R. Snyder, in Cs. Horvath (Editor), *High-performance Liquid Chromatography*, Vol. 1, Academic Press, New York, p. 207.
- 3 L. R. Snyder, *Chromatogr. Rev.*, 7 (1965) 1.
- 4 C. Liteanu and S. Gocan, *Gradient Elution Chromatography*, Wiley, New York, 1974, p. 198.
- 5 H. Elgass, *Ph.D. Thesis*, Universität des Saarlandes, Saarbrücken, 1978.
- 6 P. Jandera and J. Churacek, *Advan. Chromatogr.*, 19 (1980) 125.
- 7 P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
- 8 R. A. Hartwick, C. M. Grill and P. R. Brown, *Anal. Chem.*, 51 (1979) 34.
- 9 J. P. Larmann, J. J. DeStefano, A. P. Goldberg, R. W. Stout, L. R. Snyder and M. A. Stadalius, *J. Chromatogr.*, 255 (1983) 163.
- 10 R. M. McCormick and B. L. Karger, *Anal. Chem.*, 52 (1980) 2249.
- 11 N. Le Ha, J. Ungváral and E. sz. Kováts, *Anal. Chem.*, 54 (1982) 2410.
- 12 A. M. Krstulovic, H. Colin and G. Guiochon, *Anal. Chem.*, 54 (1982) 2436.
- 13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979.
- 14 L. R. Snyder and S. van der Wal, *Anal. Chem.*, 53 (1981) 877.
- 15 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
- 16 P. J. Schoenmakers, H. A. H. Billiet and L. De Galan, *J. Chromatogr.*, 185 (1979) 179.
- 17 R. K. Gilpin and J. A. Squires, *J. Chromatogr. Sci.*, 19 (1981) 195.
- 18 R. K. Gilpin, *Amer. Lab.*, (1982) 104.
- 19 R. K. Gilpin and M. H. Gaudet, *J. Chromatogr.*, 248 (1982) 160.
- 20 W. E. Hammers, A. G. M. Theeuwes, W. K. Brederode and C. L. de Ligny, *J. Chromatogr.*, 234 (1982) 321.
- 21 L. R. Snyder and T. C. Schunk, *Anal. Chem.*, 54 (1982) 1764.
- 22 J. R. Gant, J. W. Dolan and L. R. Snyder, *J. Chromatogr.*, 185 (1979) 153.
- 23 M. T. W. Hearne and B. Grego, *J. Chromatogr.*, 255 (1983) 125.
- 24 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 205 (1981) 13.
- 25 M. A. Quarry, R. L. Grob and L. R. Snyder, *Anal. Chem.*, to be submitted.