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CHROMATOGRAPHIC REPRODUCIBILITY IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC GRADIENT ELUTION

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SUMMARY

Normal bonded phase chromatography in a syringe pump gradient system satisfies a basic requirement for high-performance liquid chromatography of retention time reproducibility within half a peak width. Reversed bonded phase chromatography is characterized by a retention time variance in excess of this goal in certain cases. The source of the variance was traced to fluid compressibility effects and ambient thermal fluctuations.

Fluid compressibility effects, due to the change in system backpressure with changing mobile phase viscosity, generated a retention time variation of the order of a peak width for solutes eluting in the 20–50% B portion of a 0–100% acetonitrile (B) in water (A) gradient. However, the retention of solutes eluting beyond 50% acetonitrile was not significantly affected by fluid compressibility. Thermal effects, due to ambient temperature variation, generated retention time variations of the order of a peak width for solutes eluting throughout the gradient program.

A constant backpressure valve was developed which successfully eliminated fluid compressibility effects during gradient elution. Thermal effects were eliminated by use of a water-jacketed column and circulating constant-temperature bath. The dual syringe pump gradient system operating with these modifications reproduced retention times to better than 6 sec out of 1200 sec (σ_{rel} values below 0.5%).

INTRODUCTION

Gradient elution is the most effective technique available for solution of the general elution problem in liquid chromatography. It allows rapid separation of mixtures of solutes having widely different chromatographic behavior and yields symmetric, narrow peaks throughout the chromatogram.

Chromatographic quantitation requires the assignment of response factors to individual peaks. Peak identity is based on elution order and/or retention time. Retention time reproducibility is necessary to avoid erroneous peak identity–response factor assignments. Automated data processing in which peak identities are based on pre-set retention time windows has tightened the requirements on chromatographic reproducibility.

A minimum resolution of 0.5 is required to distinguish two solute maxima separated by a minimum. Differentiation of such peaks on the basis of retention time requires a 95% confidence interval of one half a peak width ($0.5w$). The development of highly efficient microparticle columns yielding small peak widths has thus placed stringent reproducibility requirements on chromatographic instrumentation. For the case in which a systematic variation occurs with successive runs, the confidence interval should be based on the range in retention over a typical sample population rather than on the variance of a series of runs.

Gradient retention time reproducibility has often been considered insufficient for quantitative work. Due to this problem, coupled to the need to reequilibrate the column after each gradient program, many chromatographers have confined their use of gradient elution to a sample scouting technique for defining optimum conditions for isocratic elution.

The development of bonded phase high-performance liquid chromatography (HPLC) columns has significantly reduced column equilibration time between gradients and improved the control of column activity. Gradient reproducibility with bonded phase columns has thus approached that obtained in isocratic elution for many chromatographic systems.

Chromatographers have still observed excessive run-to-run variance in retention time for certain column–mobile phase–solute systems. In some cases, systematic trends in retention time have been observed. A study of the parameters involved in gradient elution was thus conducted in order to improve the reproducibility of the technique. A theoretical description of solute migration in a gradient was developed to aid in studying the effects of gradient parameters on retention time.

EXPERIMENTAL

Gradient elution data were obtained on a dual, constant displacement rate syringe pump liquid chromatograph (Varian 8520 LC). The volume between the mixer and the column was about 500 μl. Injections were made by a six-port high-pressure valve injector (Valco) controlled by an Autosampler™ (Varian 8050). Retention data were collected and stored by a chromatography data system (Varian CDS 101). The syringe pumps were filled (250 ml each) prior to each series of gradient runs. The initial gradient run was always a blank where no sample was injected.

The syringe pump chromatograph was operated with and without a constant backpressure valve (CPV) in the system. The CPV is a low dead volume (100 μl) valve designed to avoid fluid compressibility effects in the pump reservoirs due to a change in column backpressure as mobile phase viscosity changes during the gradient run. The valve operates as a variable resistor, compensating for changes in column backpressure by making a complementary change in its own backpressure. The backpressure on the pump reservoirs is thus maintained constant ($dP/dt = 0$) during gradient elution.

The CPV maintains system pressure to within ± 2 atm out of 400 atm for a wide range of mobile phases and flow-rates. A complete description of the theory, design, and construction of the CPV will be reported elsewhere[1].

System pressure was monitored during gradient runs with a strain gauge pressure transducer located between the A pump outlet and the mixer. The transducer

output was amplified and then displayed on a strip chart recorder. The transducer had been calibrated at pressures of 1 and 540 atm with a reference gauge.

Normal-phase gradient elution studies were run with a microparticulate, polar bonded phase column (Varian MicroPak™ CN-10). Reversed-phase gradient elution studies were run on a microparticulate octadecyl–silica bonded phase column (MicroPak™ CH-10). The octadecyl stationary phase was polymeric, thus exhibiting higher selectivity towards aromatic hydrocarbons than a comparable monomeric stationary phase. The permeability of the polymeric octadecyl column is about 60% of that of a monomeric column, tending to increase compressibility effects.

Both normal- and reversed-phase columns had dimensions of 25 cm × 0.21 cm I.D. and had approximately 500-μl void volumes. Reversed-phase column temperature was either controlled at 35° with a water jacket connected to a circulating constant-temperature bath (Haake F423) or left uncontrolled at ambient temperature. The normal-phase column was run at ambient temperature.

RESULTS AND DISCUSSION

I. Solute migration in gradient elution —Constant flow, constant temperature conditions

Reproducibility of gradient retention time requires precise control of mobile phase composition, column activity, flow-rate, and temperature. A theoretical treatment of solute migration in gradient elution is a prerequisite to understanding the effects of variations in the above parameters on solute retention time.

Consider a gradient program in which the mobile phase composition (C_t) is programmed in increments of 1% B (A, B are the solvents used to form the gradient). The rate at which these steps occur and the gradient limits are set on a programmer. Assume that flow velocity (u) and temperature (T) are constant throughout the runs.

Solute migration can be described in terms of the parameter F_t, the fractional column length traversed during the time interval τ spent at each 1% B step in mobile phase composition C_t. The simplest case to treat is a linear gradient for which τ will be a constant. The more complex case of an exponential gradient is treated in the Appendix.

$$R = \frac{dC_i}{dt}$$

$$\tau = \frac{0.01}{R}$$

where R = gradient rate, sec^{-1}

F_i can be described as

$$F_i = \frac{u_{bi}\,\tau}{L} \tag{1}$$

where

$$u_{bi} = \frac{u}{1 + K_i} = \text{solute band velocity}$$

L = column length
u = mobile phase velocity
K_i = solute capacity factor

and

$$F_t = \frac{u}{(1 + K_t)} \cdot \left(\frac{\tau}{L}\right) = \frac{u}{(1 + K_t)} \cdot \left(\frac{0.01}{RL}\right) \tag{2}$$

K_t is typically a log-linear function of C_t and thus

$$F_t = \frac{0.01u}{RL} \cdot \left(\frac{1}{1 + Ae^{mC_t}}\right) \tag{3}$$

where the constants A and m are the y-intercept and slope, respectively, of the linear plot of ln K_t *versus* C_t. Fig. 1 shows an experimental plot of ln K_t *versus* C_t for a series of polynuclear aromatic hydrocarbon solutes eluted from a reversed-phase column with water–acetonitrile mobile phases.

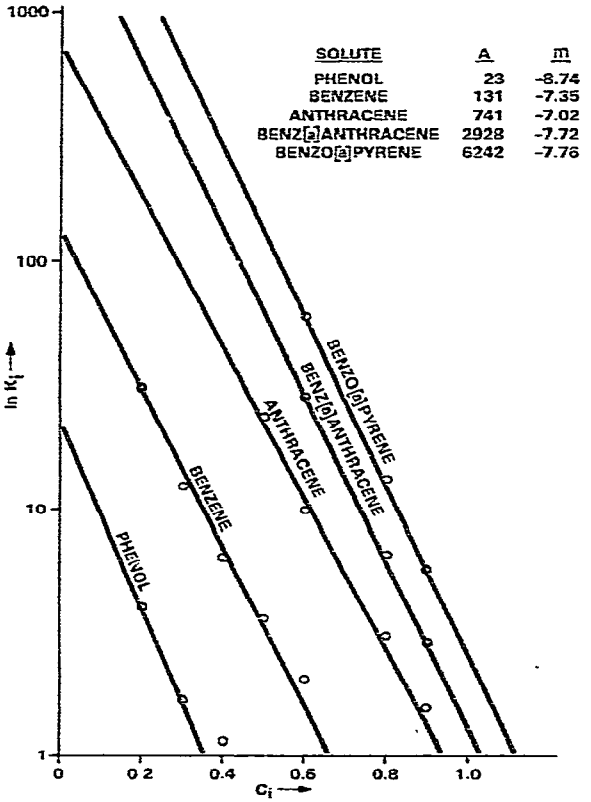


Fig. 1. Plot of ln K_t *versus* C_t for polynuclear aromatic hydrocarbons eluted from a 25 cm × 0.21 cm I.D. MicroPak CH-10 column with water–acetonitrile mobile phases at a temperature of 35°.

The mobile phase composition delivered to the mixer at any time t is

$$C_i^M = C_i^0 + Rt \tag{4}$$

where

C_i^M = mobile phase composition delivered to mixer
C_i^0 = initial gradient composition

The mobile phase composition at the head of the column, C_i^C, will "lag" that in the mixer due to the mobile phase transit time from the mixer to the column.

$$C_i^C = C_i^M - R\tau_{MC} \tag{5}$$

where

τ_{MC} = transit time from mixer to column

From eqns. 4 and 5 it follows that

$$C_i^C = C_i^0 + R(t - \tau_{MC})$$

The mobile phase composition at the solute band, C_i, will "lag" that at the column head as the band migrates further down the column. Thus

$$C_i = C_i^0 + R(t - \tau_{MC} - F_{i-1}\tau_C) \tag{6}$$

where F_{i-1} is the running total of the previous F_i steps and τ_C is the mobile phase transit time through the column.

The lag times τ_{MC} and τ_C can be related to flow terms

$$\tau_{MC} = \frac{V_{MC}}{Q} \tag{7}$$

where

V_{MC} = volume between mixer and column
Q = flow-rate

and

$$\tau_C = \frac{L}{u} \tag{8}$$

To determine the fractional column length traversed in a given time period, one can sum F_i between the period limits t_1 and t_2. For $t_1 = 0$, one obtains the expression

$$F = \frac{0.01u}{RL} \sum_0^t \frac{1}{1 + Ae^{m[C_i^0 + R(t - \tau_{MC} - F_{i-1}\tau_C)]}} \tag{9}$$

F can be determined by numerical evaluation of each F_i value followed by summation. Elution of the solute corresponds to $F = 1.00$. $F(t)$ was evaluated for several polynuclear aromatic hydrocarbon solutes by computer* solution of eqn. 9 with experi-

* Computer programs, written in BASIC, are available upon request for the solution of the equations for solute migration in both linear and exponential gradients.

mentally determined values of A and m and the gradient parameters: A = water; B = acetonitrile; $R = 0.000833\ sec^{-1}$ (5% B/min) from 0 → 100% B; $u = 0.833$ cm/sec (1 ml/min) through a 25 cm × 0.21 cm I.D. MicroPak CH-10 column. The resultant $F(t)$ curves are shown in Fig. 2.

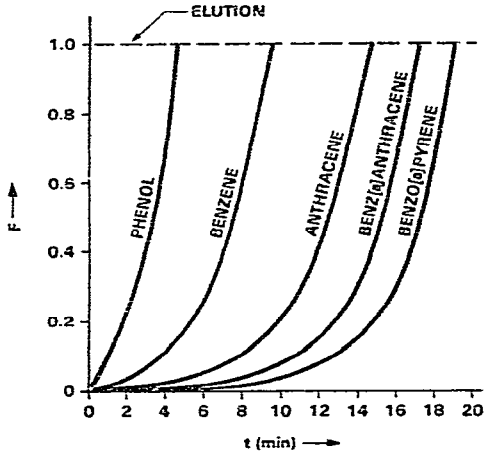


Fig. 2. Theoretical $F(t)$ migration of solutes of polynuclear aromatic hydrocarbons in reversed-phase gradient elution. Column, 25 cm × 0.21 cm I.D., MicroPak CH-10. A = water; B = acetonitrile; gradient, 0 → 100% B at 5% B/min; u = 60 ml/h (0.833 cm/sec). $F(t)$ is calculated from eqn. 9, based on experimentally determined values of A and m for each solute (see Fig. 1).

TABLE I

REPRODUCIBILITY DATA —POLAR BONDED PHASE GRADIENT ELUTION OF STEROIDS

Conditions: Column, 25 cm × 0.21 cm I.D., MicroPak CN-10; 120 ml/h (1.67 cm/sec linear velocity); 25°; 15 → 42% B concave gradient in 6 min; A = *n*-hexane; B = 33% isopropanol in dichloromethane. Pressure varies from 1400–1900 p.s.i. during the run. 254 nm absorbance, 1.0 a.u.f.s. Peak identities: 1 = progesterone; 2 = testosterone; 3 = Reichstein S; 4 = prednisolone acetate; 5 = unknown; 6 = dexamethasone; 7 = prednisolone.

Run	*Pump volume (ml)*		*Peak retention time (sec)*						
	V_A	V_B	*1*	*2*	*3*	*4*	*5*	*6*	*7*
1	170	228	53.0	87.0	183.0	220.2	270.0	329.4	370.2
2			53.0	86.0	181.2	218.4	268.8	328.2	367.2
3			53.0	87.0	181.2	219.0	269.4	327.6	368.4
4			53.4	88.2	184.2	220.2	270.6	329.4	370.2
5			53.4	88.8	186.0	222.6	272.4	330.6	371.4
6			52.2	87.0	183.6	220.2	270.6	330.0	371.4
7			52.2	87.0	184.2	220.8	271.2	329.4	370.2
8			53.0	87.6	185.4	222.6	273.0	331.8	371.4
9	7	175	54.0	89.4	187.8	224.4	274.8	333.6	373.2
$\overline{t_R}$, sec			53.0	87.6	184.1	220.9	271.2	330.0	370.4
σ			0.6	1.1	2.2	1.9	1.9	1.8	1.8
σ_{rel}, %			1.1	1.3	1.1	0.9	0.7	0.5	0.5
95% confidence interval, sec			1.0	1.8	3.0	3.0	3.2	2.6	3.0

II. Gradient reproducibility studies

Chromatographers are often uncertain as to the cause(s) of variation in the reproducibility achievable by gradient elution with different column/mobile phase systems. For example, consider the experimental data of Tables I and II.

Table I demonstrates the reproducibility of nine consecutive gradient separations of a mixture of steroids on a polar bonded phase column (MicroPak CN-10). A dual syringe pump chromatograph (Varian 8520 LC) with hexane in the A pump and a solution of 33% isopropanol in dichloromethane in the B pump was used. A 6-min concave gradient between 15 and 42% B was run at 2 ml/min. Column equilibration after each run was effected by use of a −8% B/min reverse gradient followed by 2 min at 15% B.

The efficient microparticulate column produced 15–30 sec peak widths throughout the chromatogram (Fig. 3). The retention time variations appeared random. The 95% confidence interval for retention time of the seven steroids varied from 1–3 sec and was well within the goal of half a peak width. The data of Table I are representative of those observed for gradient elution with polar bonded-phase microparticulate columns.

The data in Table II demonstrate the reproducibility of nine successive reversed-phase gradient separations of a mixture of polynuclear aromatic hydrocarbons on a MicroPak CH-10 column. The dual syringe pump chromatograph contained water in the A pump and acetonitrile in the B pump. A 20-min linear gradient

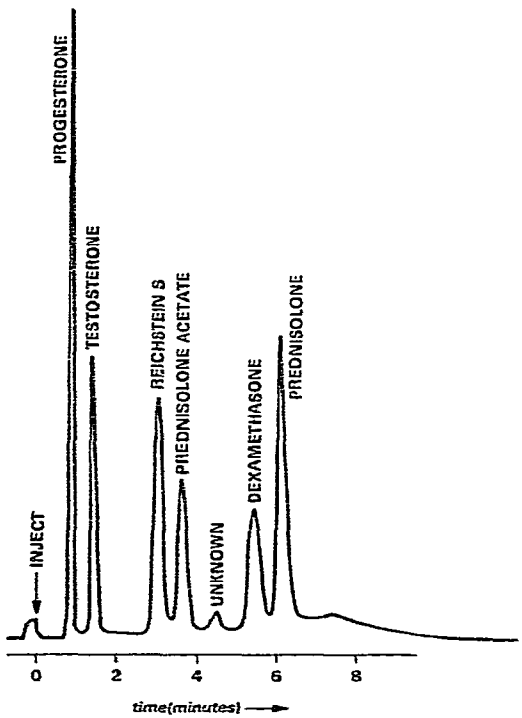


Fig. 3. Normal-phase gradient elution of steroids. For column conditions, see Table I.

TABLE II

REPRODUCIBILITY DATA —C_{18} REVERSED-PHASE GRADIENT ELUTION OF POLYNUCLEAR AROMATIC HYDROCARBONS

Conditions: Column, 25 cm × 0.21 cm I.D., MicroPak CH-10; 60 ml/h; 35°; 0 → 100% B in 20 min at +5% B/min; A = water; B = acetonitrile. Pressure varies between a minimum of ~2000 p.s.i. (at 100% B) and a maximum of ~3200 p.s.i. (at ~45% B) during the gradient run. 254 nm absorbance, 0.32 a.u.f.s. Peak identities: 1 = phenol; 2 = benzene; 3 = anthracene; 4 = benz[*a*]-anthracene; 5 = benzo[*a*]pyrene.

Run	*Pump volume (ml)*		*Peak retention time (sec)*				
	V_A	V_B	*1*	*2*	*3*	*4*	*5*
1	218	218	319.8	642.6	886.8	1015.8	1124.4
2	195	195	295.8	644.4	883.8	1014.0	1123.8
3	173	173	291.0	638.4	882.6	1013.4	1123.2
4	150	150	293.4	633.6	880.2	1012.2	1122.6
5	128	128	297.6	629.4	877.2	1011.0	1121.4
6	105	105	297.6	627.6	877.8	1012.2	1122.0
7	83	83	296.4	624.0	874.8	1011.0	1122.0
8	60	60	295.8	616.8	873.6	1012.2	1122.6
9	38	38	295.8	610.8	873.0	1014.0	1124.4
$\bar{t}_R$, sec			297.9	629.7	878.9	1012.9	1122.9
Range, sec			27.0	31.8	13.8	4.8	3.0
σ			7.8	11.4	4.9	1.6	1.1
σ_{rel}, %			2.6	1.8	0.55	0.16	0.10
95% confidence interval, sec			13.1	range	range	2.7	1.8

between 0 and 100% B was run at 1 ml/min to obtain the separation. The column was water-jacketed and the temperature was maintained at 35°. Column equilibration after each run was achieved by use of a negative 10% B/min reverse gradient followed by 7 min at 0% B.

Peak widths were about 25 sec throughout the chromatogram (Fig. 4). A systematic trend toward decreasing retention time with successive runs was observed for peak 2 (benzene). The retention time range for benzene was about 32 sec or 1.3w, which is in excess of the half-peak-width goal. A similar trend was observed for anthracene, although the retention range was relatively small, at about half a peak width.

A systematic trend in retention time was not observed for peaks 1, 4, or 5 (phenol, benz[*a*]anthracene, and benzo[*a*]pyrene). The 95% confidence intervals for these peaks were equivalent to 0.52, 0.11, and 0.07 peak widths, respectively.

Re-filling the syringe pumps and repeating the series of runs produced similar data in terms of absolute and relative retention times on successive runs. A systematic change in temperature or column activity was thus ruled out as the cause of the trend in retention time of the benzene and anthracene peaks. The latter retention trends did correlate to the gradual decrease in syringe pump volumes with successive runs.

This observation suggests that the retention time trend reflects the effect of fluid compressibility (χ) on the gradient flow-rate and mobile phase composition. Theoretical treatments of compressibility effects in isocratic elution[2,3] and a descrip-

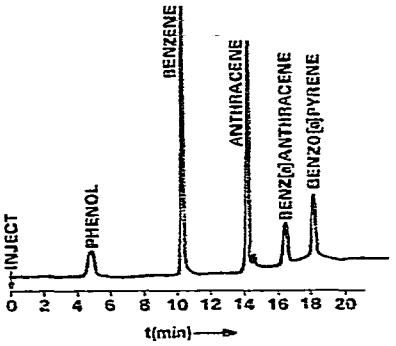


Fig. 4. Reversed-phase gradient elution of polynuclear aromatic hydrocarbons. For column conditions, see Table II.

tion of a commercial syringe pump design[3] which avoids such effects have recently been published. However, neither study extended its treatment to the more complex case of compressibility effects in gradient elution.

III. Effect of fluid compressibility on gradient elution parameters

Consider the operation of a gradient system based on dual, constant displacement syringe pumps. The piston flow-rate of each pump is programmed so as to vary the mobile phase composition delivered to the mixer according to a pre-set schedule, while the total piston flow-rate is kept constant

$$Q_O = Q_{OA} + Q_{OB} = \text{constant} \tag{10}$$

$$C_i^M(t) = \frac{Q_{OB}(t)}{Q_O} \tag{11}$$

where

Q_{OA} = A pump piston flow-rate
Q_{OB} = B pump piston flow-rate
Q_O = total piston flow-rate
$C_i^M(t)$ = mobile phase composition delivered to mixer

During the gradient program, the mobile phase viscosity, η, varies continuously as its composition changes. Since the column backpressure is proportional to mobile phase viscosity, a pressure differential in time, dP/dt is produced. The instantaneous pressure change causes a slight compression or expansion of the solvent into or out of each pump. The fractional change in fluid volume with pressure is the fluid compressibility, χ

$$\chi = \frac{dV}{V dP} \tag{12}$$

The instantaneous flow-rate out of each pump will thus deviate from the programmed piston flow-rate by the quantity $\chi V dP/dt$.

$$Q_A = Q_{OA} - \chi_A V_A \frac{dP}{dt} \tag{13a}$$

$$Q_B = Q_{OB} - \chi_B V_B \frac{dP}{dt} \tag{13b}$$

where

Q_A, Q_B = fluid flow-rates

The total flow-rate at any time t is then

$$Q(t) = Q_A + Q_B = Q_O - (\chi_A V_A + \chi_B V_B) \frac{dP}{dt} \tag{14}$$

The mobile phase composition C_i^M delivered to the mixer at time t is then

$$C_i^M(t) = \frac{Q_B}{Q} = \frac{Q_{OB} - \chi_B V_B \frac{dP}{dt}}{Q_O - (\chi_A V_A + \chi_B V_B) \frac{dP}{dt}} \tag{15}$$

Eqns. 14 and 15 predict that the effect of fluid compressibility on gradient parameters will be greatest for fast gradient rates in systems operating at relatively high pressure (low column permeability, high flow-rates, viscous solvents) in which the mobile phase viscosity varies widely with composition.

Reversed-phase HPLC utilizes relatively viscous aqueous–organic mobile phases which are "associating" mixtures, leading to a wide range in η with composi-

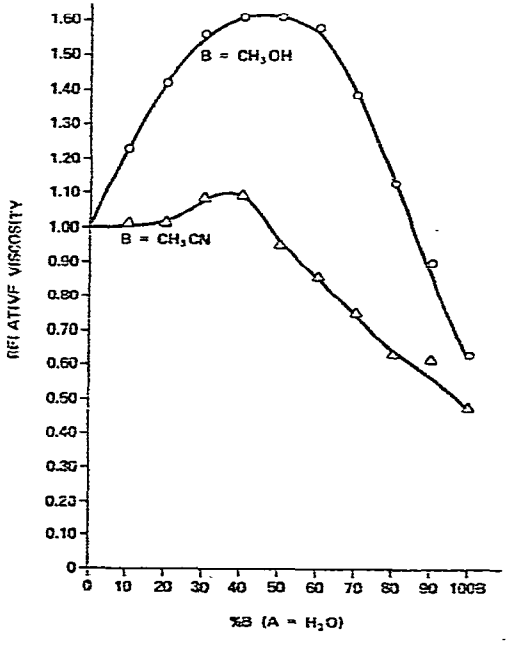


Fig. 5. Mobile phase relative viscosity as a function of composition for water–methanol and water–acetonitrile mixtures at a temperature of 25°. η (water) = 0.89 cP at 25°.

tion[4], as shown in Fig. 5. Thus, eqns. 14 and 15 predict greater compressibility effects in reversed-phase relative to normal-phase chromatography, in agreement with the data of Tables I and II.

Compressibility-generated flow deviations will diminish with successive chromatographic runs, as the pump volumes decrease. To evaluate the resultant deviations in solute retention for successive runs, one must calculate $Q(t)$ and $C_t^M(t)$ as functions of V_A and V_B and then substitute these terms into a form of the equation for solute migration (eqn. 9) derived in Section I. Since the flow velocity, u, is no longer a constant, the equation for solute migration becomes

$$F_t = \frac{0.01}{RL} \sum_0^t \frac{u_t}{1 + Ac^{m[C_t^M - R(V_{MC}/Q_t + F_{t-1}L/u_t)]}} \tag{16}$$

Eqns. 14 and 15 express $Q(t)$ and $C_t^M(t)$ in terms of the program settings $Q_O(t)$ and $Q_{OB}(t)$; pump volumes V_A, V_B; fluid compressibilities χ_A and χ_B; and the pressure differential in time dP/dt. dP/dt can be measured experimentally through use of a strain gauge pressure transducer. Thus, eqn. 16 can be solved to predict the effect of compressibility on gradient retention times.

IV. Effect of fluid compressibility on gradient retention times

Pressure was monitored during the gradient runs beginning with 218 ml and with 38 ml in each syringe pump (runs 1 and 9 of Table II). A plot of dP/dt during each gradient is presented in Fig. 6. The shape of the dP/dt curve follows the general

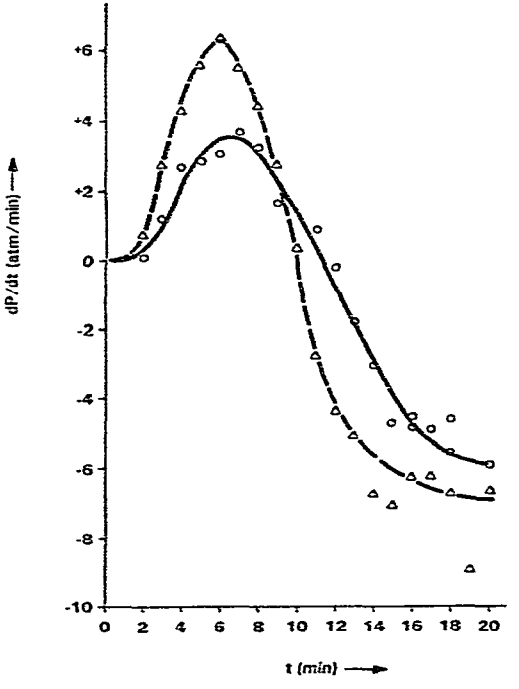
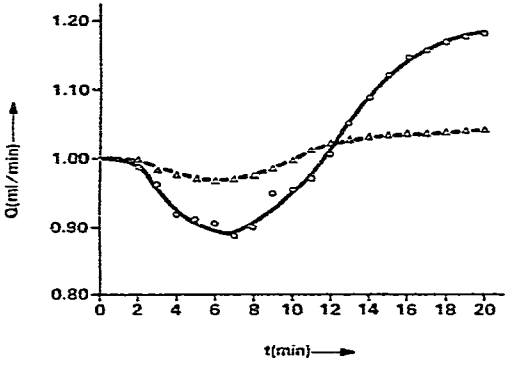


Fig. 6. Experimental values of dP/dt during reversed-phase gradient of Fig. 4, for syringe pumps initially at 218 ml (near full) (———) and at 38 ml (near empty) (— — —).

shape of the mobile phase viscosity curve of Fig. 5. The flow-rate and mobile phase composition delivered to the mixer were evaluated through use of eqns. 14 and 15. Fluid compressibilities for water and acetonitrile of 4.5×10^{-5} atm^{-1} and 9.6×10^{-5} atm^{-1}, respectively, were used[3]. Although fluid compressibility is a function of pressure, it is relatively constant in the pressure range of the gradient studied. $Q(t)$ and $C_i^M(t)$ are plotted in Figs. 7 and 8.



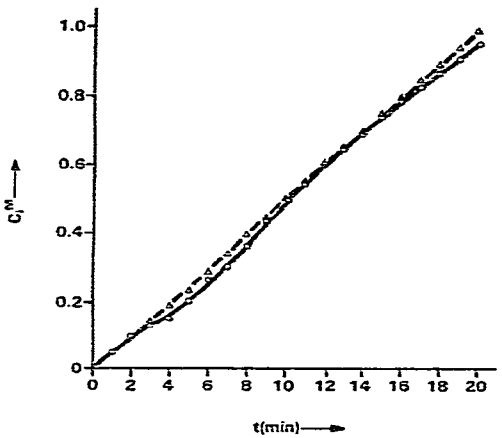


Fig. 7. Flow-rate Q, during reversed-phase gradient of Fig. 4, for syringe pumps initially at 218 ml (near full) (———) and at 38 ml (near empty) (— — —).

Fig. 8. Mobile phase composition, C_i^M, during reversed-phase gradient of Fig. 4, for syringe pumps at 218 ml (nearly full) (———) and at 38 ml (nearly empty) (— — —).

Inspection of Figs. 7 and 8 provides an intuitive grasp of the effects of compressibility on gradient retention time. Fig. 8 predicts the maximum compressibility-related variance in mobile phase composition to occur in the 20–50% and 80–100% B regions. The B concentration will be low for the 218-ml relative to the 38-ml case in both regions. However, whereas the compressibility-related flow deviation compounds the composition variance in the 20–50% B region, it counteracts it in the 80–100% B region (Fig. 7). Thus one would expect the compressibility-related retention variance to be greatest for phenol and benzene and significantly smaller for anthracene, benz[*a*]anthracene and benzo[*a*]pyrene (see Fig. 2).

The $Q(t)$ and $C_i^M(t)$ data were substituted into eqn. 16 along with experimentally determined values of A and m of several polynuclear aromatic hydrocarbons. Predicted values of retention time in the presence and absence of compressibility effects are compared to experimental data in Table III. An $F(t)$ plot for benzene in the presence and absence of compressibility effects is presented in Fig. 9.

The theoretical prediction of a systematic decrease in benzene retention time was confirmed by the experimental data. The observed range of 31.8 sec between the pumps near full at 218 ml and near empty at 38 ml agrees with the calculated value of 31.0 sec.

The theoretical prediction of only a slight effect of compressibility on the retention time of anthracene, benz[*a*]anthracene, and benzo[*a*]pyrene was also confirmed

TABLE III

COMPARISON OF THEORETICAL AND EXPERIMENTAL GRADIENT RETENTION TIMES OF POLYNUCLEAR AROMATIC HYDROCARBONS

Solute	*Peak retention time (sec)*							
	No χ effects		*χ effects operant*					
	*Theor.**	*Exp.***	*Theoretical*			*Experimental*		
			$V_A = V_B =$ 218 ml	$V_A = V_B =$ 38 ml	*Range*	$V_A = V_B =$ 218 ml	$V_A = V_B =$ 38 ml	*Range*
Phenol	273.7	266.7	314.8	294.2	20.6	319.8	295.8	24.0
Benzene	566.2	578.7	608.7	577.7	31.0	642.6	610.8	31.8
Anthracene	873.7	866.7	876.7	871.6	5.1	886.8	873.0	13.8
Benz[*a*]anthracene	1028.2	1021.2	1021.5	1023.7	−2.2	1015.8	1014 0	1.8
Benzo[*a*]pyrene	1141.3	1134.3	1139.7	1138.6	1.1	1124.4	1124.4	0

* Eqn. 16.

** Data obtained through the use of a constant-pressure valve in a dual syringe pump system. The observed retention time is corrected for a 7-sec delay due to transit through the valve volume

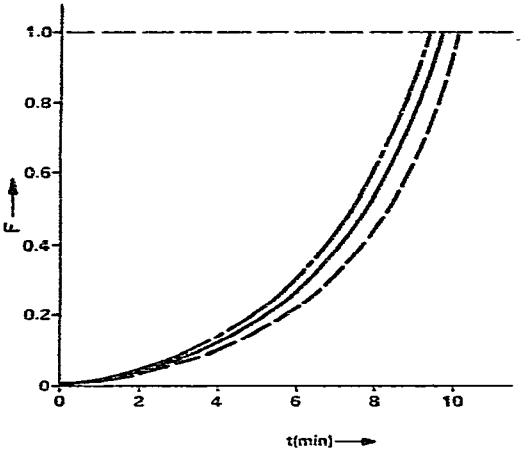


Fig. 9. Solute migration, $F(t)$, of benzene in the absence and presence of compressibility effects for reversed-phase gradient conditions of Fig. 4. – – –, No χ effects; ———, χ effects operant, $V_A = V_B = 38$ ml; – – –, χ operative, $V_A = V_B = 218$ ml.

by the experimental data. A systematic trend was not observed for benz[*a*]anthracene and benzo[*a*]pyrene. This observation is due to the fact that non-compressibility-related retention variances are similar to or exceed the slight (1–2 sec) variations predicted due to compressibility for these late eluting peaks.

The theoretical prediction of a 25-sec range in phenol retention time was also confirmed by the experimental data. However, intermediate runs did not show a clear trend in retention time. This observation suggests a confounding effect on retention. Phenol elutes early in the gradient (see Fig. 2), and thus the retention time may also be more sensitive to variations in column equilibration. This hypothesis is consistent with observations described in a later section of this paper.

V. Elimination of compressibility effects through use of a constant backpressure valve

The compressibility-generated variance in retention time can be eliminated through use of a "constant backpressure valve" (CPV) in the dual syringe pump chromatograph. An automatic valve installed at the outlet of the mixer maintains the system at a constant pressure set in excess of that developed by the column during the gradient. The valve operates so as to maintain $dP/dt = 0$. Compressibility effects on the gradient parameters are thus eliminated.

Retention time data obtained with such a system are presented in Table IV. The systematic trends previously observed for the retention times of benzene and anthracene were eliminated, confirming the hypothesis that these trends (Table II) had been compressibility-generated. The phenol (peak 1) retention time shows a variance similar to that observed for the system operated without a CPV. This may be due to still uncontrolled variations in column equilibration which should be more important for the early eluting phenol peak.

The confidence intervals for polynuclear aromatic hydrocarbons (PNA) gradient retention times were all within the goal of half a peak width with the CPV system. Confidence intervals for the latter system are compared to those for the system without the valve in Table V. Note the marked improvement in benzene and anthracene reproducibility

The observed retention times in the CPV system agree within about 3% or better with the times predicted by eqn. 9 when the experimentally determined A and m values of each PNA compound are used.

TABLE IV

RETENTION TIME REPRODUCIBILITY IN REVERSED-PHASE GRADIENT ELUTION WITH COMPRESSIBILITY EFFECTS AVOIDED THROUGH THE USE OF A CONSTANT-PRESSURE VALVE

Conditions: Column, 25 cm × 0.21 cm I.D. MicroPak CH-10; 60 ml/h; 35°; 0 → 100% B in 20 min at +5% B/min; A = water; B = acetonitrile. Pressure constant at 5000 p.s.i. during run due to the use of a constant pressure valve in the system.

Run	*Pump volume (ml)*		*Peak retention time (sec)*				
	V_A	V_B	*1*	*2*	*3*	*4*	*5*
1	218	218	281.4	583.8	889.2	1033.2	1140.6
2	195	195	276.0	588.6	889.2	1033.2	1138.8
3	173	173	274.2	588.0	888.6	1032.6	1139.4
4	150	150	280.2	585.6	890.4	1033.8	1139.4
5	128	128	285.0	588.0	892.8	1034.4	1138.8
6	105	105	264.6	582.6	890.4	1034.4	1140.0
7	83	83	273.6	585.0	891.6	1035.6	1141.8
8	60	60	264.0	585.6	891.6	1036.8	1143.0
9	38	38	264.0	583.8	892.2	1036.2	1143.6
$\bar{t}_R$, sec			273.7	585.7	890.7	1034.5	1140.6
Range, sec			17.4	6.0	3.6	4.2	4.8
σ			8.0	2.1	1.5	1.3	1.8
σ_{rel}, %			2.92	0.36	0.17	0.13	0.16
95% confidence interval, sec			13.4	3.6	2.6	2.2	3.0

TABLE V

EFFECT OF COMPRESSIBILITY AND TEMPERATURE ON GRADIENT RETENTION TIMES

Solute	*Retention time confidence interval (sec)*		
	Compressibility effects avoided; T constant at 35°	*Compressibility operative; T constant at 35°*	*Compressibility effects avoided; ambient T varies ~25 → 31° during experiment*
Phenol	13.4	13.1	36.6 (R)
Benzene	3.6	31.8 (R)*	9.0 (R)
Anthracene	2.6	13.8 (R)	11.4 (R)
Benz[*a*]anthracene	2.2	2.7	21.0 (R)
Benzo[*a*]pyrene	3.0	1.8	25.2 (R)

* (R) = Confidence interval taken as the range since the variation in retention time was systematic. All other data shown are 95% confidence intervals based on the retention time variance.

TABLE VI

REPRODUCIBILITY DATA —C_{18} REVERSED-PHASE GRADIENT ELUTION OF POLYNUCLEAR AROMATIC HYDROCARBONS

Compressibility effects are avoided by the use of constant-pressure valve. Ambient temperature increased from ~25–31° during the course of the experiment. Further conditions, same as in Table IV.

Run	*Pump volume (ml)*		*Peak retention time (sec)*				
	V_A	V_B	*1*	*2*	*3*	*4*	*5*
1	218	218	309.0	593.4	907.2	1065.0	1185.6
2	195	195	293.4	591.0	908.4	1068.0	1185.6
3	173	173	297.6	594.0	907.2	1062.0	1180.2
4	150	150	299.4	592.2	904.8	1060.2	1178.4
5	128	128	295.8	589.2	903.0	1057.2	1173.6
6	105	105	288.0	589.2	899.4	1051.2	1166.4
7	83	83	288.0	586.8	897.6	1048.8	1161.6
8	60	60	272.4	585.0	897.6	1048.2	1161.0
9	38	38	277.2	589.2	897.0	1047.0	1160.4
$\overline{t_R}$, sec			291.2	590.0	902.5	1056.4	1172.5
Range, sec			36.6	9.0	11.4	21.0	25.2

VI. Effect of temperature on gradient retention time

The previous reversed-phase gradient data had been obtained under controlled column temperature conditions* so as to allow the study of compressibility effects. Through the use of the constant pressure valve one can now eliminate compressibility effects, allowing the study of thermal effects on retention time variation.

A series of gradient runs was executed at ambient temperature throughout the course of an extremely hot day. The ambient temperature rose from 25° to 31° over

* Although the column temperature was controlled at 35°, the temperatures of the pump reservoir and the constant pressure valve were not controlled. However, thermal effects on retention time due to ambient temperature variation (25–31°) of these components should be significantly less than those due to column temperature variation.

the course of the runs. A systematic decrease in retention time on successive runs was observed for all five PNA peaks, as seen in Table VI. The temperature effect can be as significant as the compressibility effect on retention variance, as seen in Table V.

CONCLUSION

A dual syringe pump HPLC system incorporating a constant backpressure valve and column temperature control provide gradient retention time reproducibility to ≤ 6 sec out of retention times of up to 1200 sec. This reproducibility is comparable to that achievable in well-controlled isocratic elution[3] and is adequate for use of retention time for peak assignment in automated HPLC systems.

APPENDIX

In an exponential solvent gradient, the gradient rate can be described by

$$R = \frac{dC_i}{dt} = R_0 e^{at} \tag{A-1}$$

where a is a constant and R_0 is the initial gradient rate. The time τ spent at each 1 % B step in mobile phase composition is then

$$\tau = \frac{0.01}{R_0 e^{at}} \tag{A-2}$$

The mobile phase composition delivered to the mixer at time t is then

$$C_i^M(t) = C_i^0 + \int_0^t dC_i = C_i^0 + \frac{R_0}{a}(e^{at} - 1) \tag{A-3}$$

Substitution of eqn. A-3 into eqn. 9 yields the expression for solute migration in exponential gradient elution

$$F = \frac{0.01u}{L} \sum_0^t \frac{1}{R_0 e^{at}\left(1 + Ae^{n[C_i + R_0/a\,(e^{at}-1) - R_0 e^{at}(\tau_{MC} + F_i - \tau_{CO})]}\right)} \tag{A-4}$$

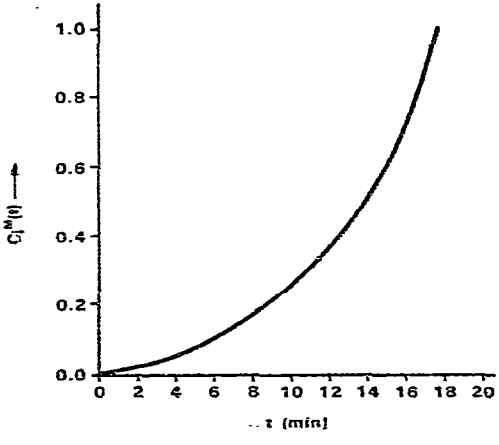


Fig. 10. $C_i^M(t)$ for typical exponential gradient: $R = R_0 e^{at}$, $a = 0.00268$.

A plot of $C_t^M(t)$ for a typical exponential gradient is presented in Fig. 10. A plot of $F(t)$ for polynuclear aromatic hydrocarbons, based on eqn. A-4 for the exponential gradient of Fig. 10, is presented in Fig. 11.

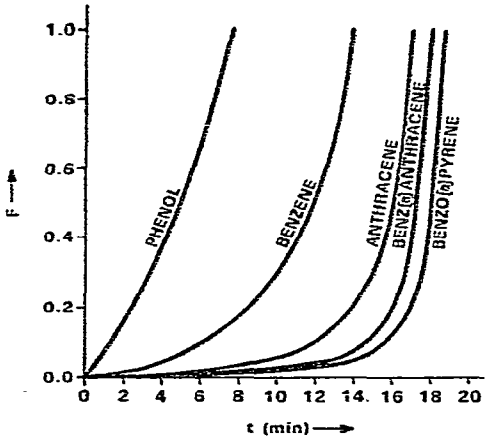


Fig. 11. Theoretical $F(t)$ migration of polynuclear aromatic hydrocarbons solutes in reversed-phase gradient elution. Column, mobile phase, and flow-rate, same as in Fig. 2. Exponential gradient profile of Fig. 10.

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