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LIQUID CHROMATOGRAPHY SYSTEM FOR FAST, ACCURATE ANALYSIS

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SUMMARY

The design characteristics of a liquid chromatograph that are essential for very fast separations are examined theoretically. Equations are derived that permit the calculation of the column length, column diameter, maximum instrument time constant and flow-rate to effect a given separation in the minimum time. A gradient elution system, that can provide gradients covering a wide range of solvent concentrations and gradient profiles that can be delivered in a few seconds, is disclosed. Examples are given of the rapid separation of multi-component mixtures in less than 30 sec by both isocratic and gradient elution. The separation times obtained experimentally are compared with those predicted theoretically. The quantitative precision that was obtained from fast liquid chromatography separations is also reported.

INTRODUCTION

The economic use of both instrumentation and manpower in a liquid chromatography laboratory is directly related to the speed of separation. The faster the separations can be achieved, the greater the number of analyses that can be obtained from a given number of instruments, by a given number of operators in a given time. However, the economy of a liquid chromatography system has only become a subject of concern over the past few years. Prior to about 1978, it was difficult enough to obtain the required separation at all and thus this aspect of the analysis provided more than sufficient concern, without being heedful of the time it took to achieve it. Today the separation process is far better understood and the conditions necessary to achieve even difficult separations are more readily arrived at; *eo ipso* the analysis time can now be given its meet attention.

One of the pioneers in high-speed liquid chromatography (LC) was Halász[1] who, in 1975, showed that the minimum analysis time that could be achieved for a given separation was limited by the available inlet pressure. It was also shown that the fastest separations would be obtained by employing the smallest particles, *e.g.*, particles having diameters of about 1 μm. These workers also pointed out that small particles inherently produced very low column permeability and thus caused considerable heat to be generated in the column, which would result in a serious deterio-

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ration in column performance. However, at this time the advantages of microbore columns had not been demonstrated[2] which, due to their small dimensions, could be made to dissipate the heat rapidly and thus maintain column performance. Halász *et al.*[3] extended their work in 1976 but, due to the difficulty experienced at that time in packing columns with particles of 5 μm in diameter or smaller, the analysis times were not reduced much below 5 min. Furthermore, due to the fact that fast data processing by computer was only in its infancy, the minimum analysis time for a chromatographic analysis that was considered practical was about 5 min. Guiochon[4] compared the relative analysis times that could be obtained from gas chromatographic and LC analysis when separating mixtures of comparable complexity. Guiochon concluded that both techniques were likely to provide similar analysis times, provided both systems were optimized to do so.

Fast LC separations, that is, separations that could be completed in less than 1 min and that could be used for accurate quantitative analysis, was first reported by Scott *et al.*[5] in 1979. These authors achieved their separations using microbore columns and demonstrated the necessity for employing low viscosity solvents and high linear mobile phase velocities in conjunction with a chromatographic system that had very fast detector sensor and amplifier response. The columns employed were about 25 cm long and were packed with silica gel having a particle diameter of 20 μm.

In 1981 DiCesare and co-workers[6–8] approached high-speed LC by a different route, employing short, wider columns packed with very small particles. Due to limitations in detector sensor response, an excessive electronic time constant and, to some extent, extra-column dispersion, DiCesare and his group only reduced analysis time to 1 or 2 min for a five- or six-component mixture. About the same time, Bannister *et al.*[9] developed some fast separations of the common tricyclic antidepressant drugs, and Burns[10] employed fast chromatography for automated pesticide analysis. Other contributions to high-speed LC over the last few years have been those of Dolan *et al.*[11], Noda *et al.*[12] and Akada *et al.*[13]. However, all those workers only managed to reduce analysis time to a few minutes. None achieved a separation of their mixtures of interest in less than 60 sec.

In this paper further developments in high-speed LC are reported based on the extension of the work of DiCesare and his co-workers. Short columns packed with particles 3 μm in diameter are employed but carefully designed to match the chromatography equipment with which they are used. The dimensions of the matched columns are arrived at by a rational approach employing accepted chromatographic theory. The problems associated with fast gradient elution development are also considered and the efficacy of the equipment used for fast gradient elution development demonstrated.

THEORETICAL

Today the speed of a chromatographic separation is not limited by the column performance that is attainable, but by instrument design. The properties of a chromatograph that control the speed of analyses are the maximum available inlet pressure, the maximum volume flow-rate, the speed of response of the overall detecting system, and the instrumental extra-column dispersion.

An optimized LC separation is achieved by designing a column with the req-

uisite number of theoretical plates and then operating the column under conditions that ensure that the separation takes place in the minimum time. Furthermore, the chromatographic system should provide the maximum solvent economy and mass sensitivity.

Consider a sample mixture that will be separated on a given phase system such that the last peak will elute at a capacity factor of k'_2. Moreover, let the closest eluted pair have a separation ratio of α and the *first* of the pair be eluted at a capacity factor of k'_1. Now the number of theoretical plates, N, necessary to effect the separation is given by the following equation[14]

$$N = S^2 (1 + k'_1)^2/(k'_1)^2 (\alpha - 1)^2 \quad (1)$$

where S is the distance between the peak maxima of the closest eluted pair of solutes measured in units of the standard deviation of the first peak. In many cases S is considered equal to four standard deviations for minimum acceptable resolution; that is, the distance between the peak maxima is equal to the base width of the first peak[14].

The analysis time, t, required to effect the separation is given by

$$t = (1 + k'_2)\, l/u \quad (2)$$

where l is the length of the column and u is the linear velocity of the mobile phase. Now

$$l = Nh \quad (3)$$

where h is the height of the theoretical plate. The relationship between h and u for a column packed with porous particles has been shown to be described by the Van Deemter equation[1,15]. Thus

$$h = A + B/u + Cu$$

where A, B and C are constants.

In high-speed separations, u is relatively large and therefore the longitudinal diffusion term, (B/u), becomes negligible and the Van Deemter equation reduces to:

$$h = A + Cu$$

Thus:

$$l = N(A + Cu) \quad (4)$$

However, the column length, l, is also related to the applied pressure, P, the diameter of the particles of the packing, d_p, the viscosity of the mobile phase, η, and the mobile phase velocity, u, as given by the following equation

$$u = \Psi P d_p^2/\eta l$$

where Ψ is a constant the numerical value of which is defined by units employed. Thus:

$$l = \Psi P d_p^2/\eta u \tag{5}$$

Equating eqns. 4 and 5, rearranging and solving for u:

$$u = A[(1 + 4\Psi PCd_p^2/\eta NA^2)^{1/2} - 1]/2C \tag{6}$$

Values for the constants in eqn. 6 that are compatible with general LC operating conditions are as follows: $P = 6000$ p.s.i.; $A = d_p$ cm; $N = 6000$; $\eta = 0.3$ cP; $\Psi = 35$ and $C = 10^{-3}$ sec.

Taking such values it can be seen that:

$$4\Psi PCd_p^2/\eta NA^2 \gg 1$$

Thus:

$$u = \frac{A}{2C}\left(\frac{4\Psi PCd_p^2}{\eta A^2 N}\right)^{1/2} = \left(\frac{\Psi P d_p^2}{\eta CN}\right)^{1/2}$$

Now from the Van Deemter equation[16]

$$C = \mathrm{f}(k')\, d_p^2/D$$

where D is the diffusivity of the solute in the mobile phase. Thus:

$$u = \left[\frac{\Psi PD}{N\eta \mathrm{f}(k')}\right]^{1/2} = \frac{k_1'(\alpha - 1)}{S(1 + k_1')}\cdot\left[\frac{\Psi PD}{\eta \mathrm{f}(k')}\right]^{1/2} \tag{7}$$

Substituting for l in eqn. 2 from eqn. 4 and substituting for u from eqn. 7 and noting that from the Van Deemter equation

$$A = 2\lambda d_p$$

where λ is a constant, and finally simplifying:

$$t = (1 + k_2')\, N\left\{\left[\frac{4N\eta \mathrm{f}(k')\, \lambda^2 d_p^2}{\Psi PD}\right]^{1/2} + \frac{\mathrm{f}(k')\, d_p^2}{D}\right\} \tag{8}$$

Hence, substituting for N from eqn. 1 in eqn. 8:

$$t = \frac{S^2(1 + k_1')^2\,(1 + k_2')}{(k_1')^2\,(\alpha - 1)^2}\cdot\left\{\frac{2S(1 + k_1')\,\lambda d_p}{k_1'(\alpha - 1)}\cdot\left[\frac{\eta \mathrm{f}(k')}{\Psi PD}\right]^{1/2} + \frac{\mathrm{f}(k')\, d_p^2}{D}\right\} \tag{9}$$

Eqn. 9 shows that one important factor controlling the minimum analysis time attainable for a separation is the maximum available pump pressure.

The minimum analysis time is achievable only at infinite pressure and is given by:

$$t = \frac{S^2(1 + k_1')^2 (1 + k_2') f(k') d_p^2}{(k_1')^2 (\alpha - 1)^2 D} \tag{10}$$

It is seen that the minimum analysis time that can be achieved for any given separation depends upon the C term of the Van Deemter equation and thus depends upon the square of the particle diameter and the reciprocal of the diffusivity of the solute in the mobile phase.

It is also seen from eqn. 9 that the pressure necessary to reduce the analysis time to within 10% of the absolute minimum is given by

$$\left[\frac{4N\eta f(k') \lambda^2 d_p^2}{\Psi P D}\right]^{1/2} = \frac{f(k') d_p^2}{10D} \tag{11}$$

or

$$P = \frac{400N\eta\lambda^2 D}{\Psi f(k') d_p^2} = \frac{S^2(1 + k_1')^2}{(k_1')^2 (\alpha - 1)^2} \cdot \frac{400\eta\lambda^2 D}{\Psi f(k') d_p^2} \tag{12}$$

By substituting for u in eqn. 5 from eqn. 7 a value for the column length can be obtained:

$$\begin{aligned} l &= \Psi P d_p^2/\eta \left[\frac{\Psi P D}{N\eta f(k')}\right]^{1/2} \\ &= N^{1/2} d_p^2 \left[\frac{\Psi P f(k')}{\eta D}\right]^{1/2} \\ &= \frac{S(1 + k_1') d_p^2}{k_1'(\alpha - 1)} \cdot \left[\frac{\Psi P f(k')}{\eta D}\right]^{1/2} \end{aligned} \tag{13}$$

Finally, the radius of the column needs to be determined together with the maximum permissible detector system time constant that can be tolerated. It has been shown[17] that

$$r^2 = (10N)^{1/2} \sigma_A/\theta\pi l \tag{14}$$

where r is the radius of the column, θ is the fraction of the cross-section of the column that is available for solvent flow, and σ_A is the volume standard deviation contributed by the apparatus. Substituting for l from eqn. 13 and rearranging:

$$r^2 = \left[\frac{\sigma_A}{\pi\theta} \cdot \frac{10\eta D}{\Psi P f(k') d_p^4}\right]^{1/2} \tag{15}$$

If the chromatographic system is operated to within 10% of the minimum analysis time, then the value for P given by eqn. 12 can be inserted in eqn. 5:

$$r = \left[\frac{\sigma_A k_1'(\alpha - 1)}{20 d_p \lambda \theta S(1 + k_1')}\right]^{1/2} \tag{16}$$

An expression for the maximum time constant that can be tolerated for the detector and its electronics is given by[18]

$$\tau = \sigma_t/(10)^{1/2} \tag{17}$$

where τ is the equivalent time constant of the detecting system, and σ_t is the standard deviation of the eluted peak at the dead time of the column. Thus from eqn. 2:

$$\sigma_t = l/uN^{1/2} \tag{18}$$

Substituting for l and u in eqn. 18 from eqns. 13 and 7 respectively, and substituting for σ_t in eqn. 17:

$$\tau = \frac{S(1 - k_1')\, f(k')\, d_p^2}{3.2 k_1'(\alpha - 1)\, D} \tag{19}$$

RESULTS

Eqns. 7 and 9 permit both the linear velocity and the analysis time to be calculated to effect a given separation. Examination of eqn. 9 indicates that the analysis time increases with the difficulty of the separation (lower values of α), the complexity of the separation (large values of k_2' to provide sufficient peak capacity) and with increased particle size, d_p (as expected from the Van Deemter equation). It is also seen that the analysis time is proportional to the inverse of the square root of the applied pressure and not inversely as the pressure which, at first sight, is not so obvious. It is also seen that at infinite pressure there will still be a finite time required for the separation, the magnitude of which is determined by the C term of the Van Deemter equation. The diffusivity of a solute in the mobile phase is inversely proportional to the viscosity of the mobile phase and thus eqn. 9 also confirms that the analysis time is proportional to the solvent viscosity.

Employing eqn. 9 the time required to effect a separation, where $\alpha = 1.08$, is shown plotted against the inlet pressure in Fig. 1 for columns packed with particles having diameters 3, 5, and 10 μm. The values for the constants used in eqn. 9 for these calculations were as follows: $S = 4$; $k_1' = 2.5$; $k_2' = 5$; $\lambda = 0.5$; $\eta = 3 \cdot 10^{-3}$ P, $D = 3 \cdot 10^{-5}$ cm^2/sec; $\Psi = 35$ and $f(k') = 0.26$.

It is seen that the larger the diameter of the particles, the less the pressure is required to effect the separation in the minimum time. For 10-μm particles an increase in pressure from 2000 to 6000 p.s.i. only reduces the analysis time by about 6%. However, reducing particle diameter to 3 μm and operating at the same inlet pressure of 6000 p.s.i. decreases the analysis time by over an order of magnitude. In Fig. 2 curves are shown relating analysis time and pressure for columns packed only with 3-

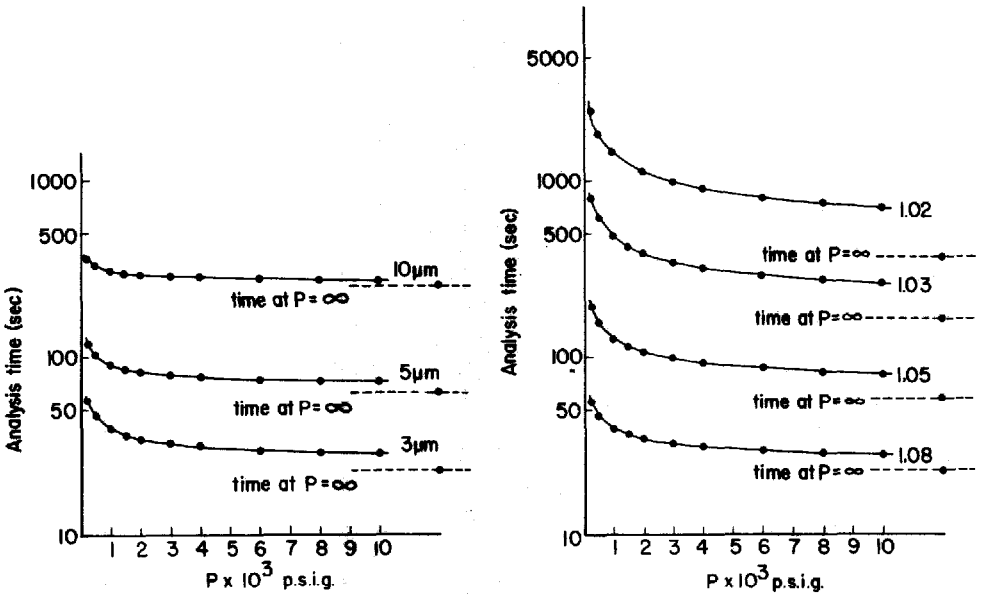

Fig. 1. Curves relating analysis time and pressure for columns packed with particles having diameters 3, 5 and 10 μm when separation ratio, α, is equal to 1.08.

Fig. 2. Curves relating analysis time and pressure for a column packed with 3-μm particles when separation ratios are equal to 1.02, 1.03, 1.05 and 1.08.

μm particles when separating mixtures of different intractability but otherwise eluted under the same conditions. It is seen that pressure is an extremely important factor controlling the speed of analysis when difficult samples are being analyzed (*i.e.*, $\alpha < 1.05$). Although 6000 p.s.i. is sufficient to reduce the analysis time to within 30% of the minimum for $\alpha = 1.08$; however, for $\alpha = 1.02$, 6000 p.s.i. will only reduce the analysis time to a value slightly greater than twice the minimum.

Eqn. 13 permits the length of the column, that is required to provide the minimum analysis time, to be calculated. It is seen that particles of large diameter or the use of high pressures will dictate the use of longer columns. It is interesting to note that as solvent viscosity is inversely related to solute diffusivity, the solvent viscosity does not affect the optimum column length. The equation that permits the calculation of the column radius eqn. 16 shows that the radius is directly related to the square root of the extra-column dispersion, the separation ratio and the reciprocal of the particle diameter. The overall instrument time constant is given by eqn. 19, and it is proportional to the square of the particle diameter and inversely proportional to the separation ratio and solute diffusivity.

Examination of a high-speed liquid chromatographic column

The apparatus consisted of a Perkin-Elmer Series 3B liquid chromatograph, a Model LC-85 UV detector, a Valco valve (UHP series) with a 0.2-μl injection loop and a Bascom-Turner Model 8120 recorder. The LC-85 detector had an experimental 1.4-μl flow cell and the response time of the detecting system was 73 msec (one

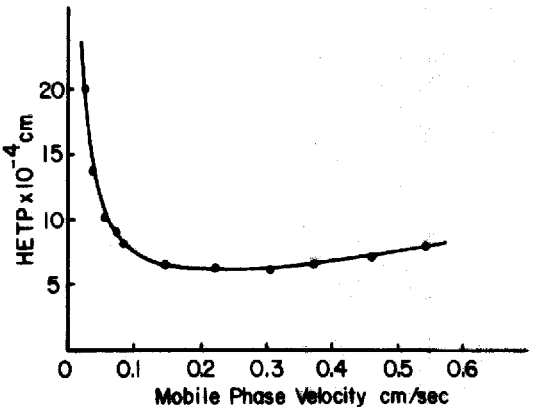

Fig. 3. Graph of HETP against mobile phase linear velocity for a short column. Packing: Spherisorb, 3 μm. Column: 4 × 0.4 cm I.D. Mobile phase: 4.3% (w/w) ethyl acetate in *n*-hexane. Solute: benzyl acetate.

standard deviation) as opposed to the new LC-85B which has a minimum response time of 5 msec. The column, 4 × 0.4 cm I.D., was packed with 3-μm Spherisorb silica gel having a mean particle diameter of 3.1 μm. The mobile phase was a 4.3% (w/w) solution of ethyl acetate in *n*-hexane. The test mixture consisted of two solutes, *p*-xylene and benzyl acetate, the former was eluted at a retention volume of 0.44 ml (which was taken as the dead volume of the column) and the latter at a retention volume of 1.08 ml ($k' = 1.5$).

Heights equivalent to a theoretical plate (HETP) for benzyl acetate were measured over a mobile phase linear velocity range of 0.02–0.6 cm/sec. The mobile phase linear velocity was determined as the ratio of the column length to the dead time.

Each HETP value was the average result of three or four determinations (each determination being within 5% of the relative standard deviation). At the minimum HETP curve the variance of the solute peak eluted at $k' = 1.5$ was determined to be 184 μl^2. The variance of the dispersion due to the chromatographic apparatus described above was measured to be 6.3 μl^2. Hence, the contribution of the instrumental dispersion to the column dispersion was about 3.5%. Therefore, the experimental HETP values were not distorted by extra-column effects and thus represented the true performance of the column over the linear velocity examined[17].

The HETP data were fitted to the Van Deemter equation[16], $h = A + B/u +$

TABLE I

RESULTS OF CURVE FIT OF VAN DEEMTER EQUATION, $h = A + B/u + Cu$, TO HETP DATA

$A = 1.75778 \cdot 10^{-4}$ cm
$B = 4.77211 \cdot 10^{-5}$ cm^2/sec
$C = 9.54624 \cdot 10^{-4}$ sec

Index of determination: 0.999182
Standard error: $6.04646 \cdot 10^{-6}$

Cu, and the result of the curve fit depicted by the solid line is shown in Fig. 3. Values for the coefficients *A*, *B*, and *C* from the curve fit are given in Table I. It is seen that the resistance to mass transfer effect (the *C* term) is very low for the 3-μm column which would be expected from the Van Deemter equation.

The performance of the total chromatographic system was studied over the linear velocity range of 0.02–0.6 cm/sec. However, since the column was to be used for high-speed analysis, it was necessary to determine the magnitude of dispersion effects resulting from the response time of the detecting system. The HETP values, therefore, were expressed as the time variance of the solute band and plotted in a similar way against the linear velocity of the mobile phase. This graph is shown in Fig. 4. At the optimum linear velocity (0.3 cm/sec) the solute peak had a variance of 0.176 sec^2 which equivalent to a time standard deviation, σ_t, of 0.420 sec. It has been shown[18] that the time standard deviation arising from the response time of the detecting system can be tolerated if it does not exceed 32% of that of the peak eluted from the column. Accordingly the maximum permissible response times, τ (sec), that are acceptable with no significant broadening of the peak could be calculated. For the peak having σ_t of 0.420 sec, the maximum permissible response time was determined to be 0.140 sec. For the peak having σ_t of 0.230 sec (at u = 0.55 cm/sec), the maximum permissible response time was 0.073 sec. It should be remembered that the detector response time was 0.073 sec. It followed that the operation of the column at higher linear velocities would result in significant loss in the column performance due to the slow response time of the detecting system. This necessitated the modification of the detector response time.

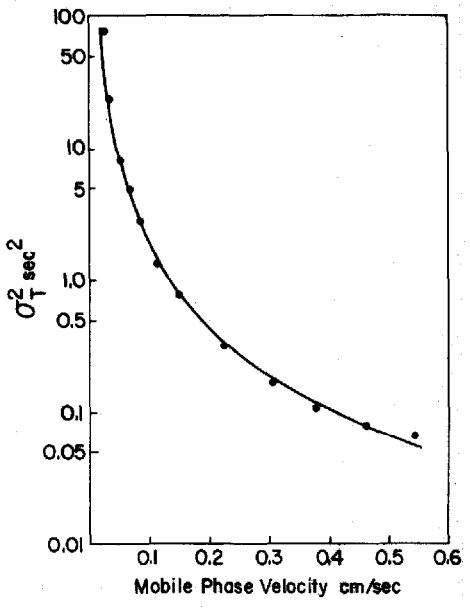

Fig. 4. Graph of peak variance (sec^2) against linear velocity (cm/sec). Column dimensions and conditions as in Fig. 3.

Performance of the chromatographic system in high-speed isocratic separations

Based on the results of the examination of the high-speed column, and the value of the effective time constant given by eqn. 19, the response time of the detecting system was reduced to about 14 msec (one standard deviation). The dispersion from the chromatographic apparatus with the modified response time constant in terms of volume flow of mobile phase was calculated to be less than 3 μl^2.

An example of a high-speed isocratic separation of an eight-component synthetic mixture on the 4 cm long column packed with 3-μm particles is given in Fig. 5. The concentration of the solutes in the mixture injected onto the column ranged from 0.5 to 2.5% (w/v). The linear velocity was 1.7 cm/sec which corresponded to a flow-rate of 12 ml/min. The inlet pressure at this flow ratio was 5600 p.s.i. (38 MPa). The chromatographic data were acquired at a rate of about 33 data points per sec utilizing the Bascom-Turner recorder.

In the separation of the mixture (Fig. 5) the last solute was eluted at $k' = 5.3$ and the analysis was completed in less than 16 sec. No significant loss in the column performance was observed even when operated at a linear velocity of 1.7 cm/sec. The observed efficiencies, the theoretical plates generated per second, the time standard deviation and the maximum permissible response time for selected solutes eluted at different k' values are given in Table II. It is seen from the values for the time standard deviation of the peak that there was no significant contribution to dispersion from the response time of the detecting system. It is also seen that the number of theoretical plates generated per second was greater than 2000 for the unretained solute and the column efficiency degrades as k' increases. This relationship which can be theoretically predicted, is only observed experimentally if the extra-column band broadening effects are rendered insignificant.

It should be noted that, the operating pressure was relatively high being equal to 5600 p.s.i. (38 MPa). However, even if pressure was not a limitation and the linear velocity was continuously increased by increasing the pressure, ultimately, the maxi-

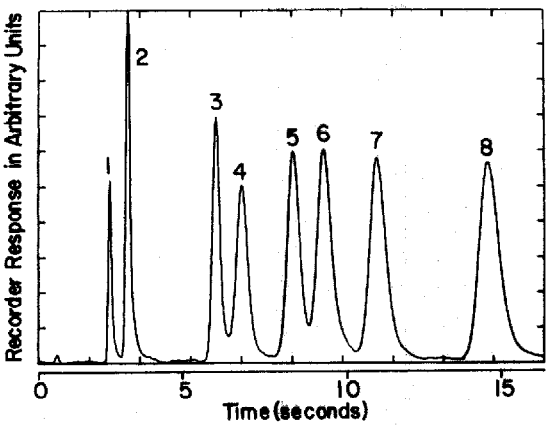

Fig. 5. High-speed isocratic separation of an eight-component synthetic mixture. Packing and column dimensions as in Fig. 3. Mobile phase: 4.1% (w/w) ethyl acetate in *n*-hexane. Linear velocity: 1.7 cm/sec. Peaks: 1 = *p*-xylene; 2 = anisole; 3 = benzyl acetate; 4 = dioctyl phthalate; 5 = dipentyl phthalate; 6 = dibutyl phthalate; 7 = dipropyl phthalate; 8 = diethyl phthalate.

TABLE II

COLUMN PERFORMANCE FOR AN EIGHT-COMPONENT SYNTHETIC MIXTURE SEPARATED ISOCRATICALLY

Packing: Spherisorb, 3 μm. Column: 4 × 0.4 cm I.D. Linear velocity: 1.7 cm/sec. σ_t = Standard deviation of peak; τ = maximum permissible time constant.

Compound	*k'*	*N*	*N/sec*	σ_t*(sec)*	τ*(sec)*
1 *p*-Xylene	0	5200	2200	0.032	0.010
2 Anisole	0.35	3800	1200	0.050	0.016
3 Benzyl acetate	1.47	3100	530	0.100	0.033
8 Diethyl phthalate	5.30	2300	150	0.310	0.100

mum flow-rate of the pump would be reached. *Ipso facto*, the flow-rate limit of the pump is another constraint in the design of the chromatographic system. Therefore, to increase the maximum possible linear velocity, and to remain within the pump flow-rate specifications, and also to provide some operational flexibility, the cross-sectional area of the column was reduced together with the column length.

To this effect, the internal diameter and the length of the column were reduced so that when the column was operated at the same volumetric flow, generating approximately the same inlet pressure, a significantly higher mobile phase linear velocity would be realized.

A column of 2.5 × 0.26 cm I.D. was packed with a 3-μm Hypersil silica gel material having an actual mean particle diameter of 3.4 μm. The efficient fast operation of this particular column required the speed of the response of the detecting system to be reduced to less than 6 msec (one standard deviation) to be commensurate with the column dispersion. In addition, since it was previously shown (eqn. 9) that the analysis time was proportional to the solvent viscosity, the least viscous solvent was chosen as the mobile phase.

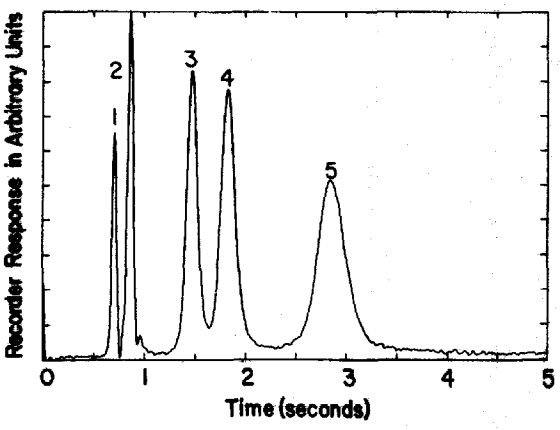

Fig. 6. High-speed isocratic separation of a five-component synthetic mixture. Packing: Hypersil, 3 μm. Column: 2.5 × 0.26 cm I.D. Mobile phase: 2.2% (w/w) methyl acetate in *n*-pentane. Linear velocity: 3.3 cm/sec. Peaks: 1 = *p*-xylene; 2 = anisole; 3 = nitrobenzene; 4 = acetophenone; 5 = dipropyl phthalate.

TABLE III

COLUMN PERFORMANCE FOR A FIVE-COMPONENT SYNTHETIC MIXTURE SEPARATED ISOCRATICALLY

Packing: Hypersil, 3 μm. Column: 2.5 × 0.26 cm I.D. Linear velocity: 3.3 cm/sec. Definitions as in Table II.

Compound	*k'*	*N*	*N/sec*	σ_t*(sec)*	τ*(sec)*
1 *p*-Xylene	0	1100	1450	0.023	0.007
2 Anisole	0.2	1080	1200	0.027	0.009
3 Nitrobenzene	1.0	840	560	0.052	0.016
4 Acetophenone	1.5	800	430	0.066	0.021
5 Dipropyl phthalate	2.9	450	160	0.140	0.043

An example of a high-speed separation of a five-component synthetic mixture on the 2.5 cm long column is shown in Fig. 6. The mobile phase was a 2.2% (w/w) solution of methyl acetate in *n*-pentane. The linear velocity was 3.3 cm/sec calculated from the retention time of *p*-xylene which corresponded to a flow-rate of 13 ml/min. The inlet pressure at this flow-rate was 5300 p.s.i. (36 MPa) and the chromatographic data was collected at an acquisition rate of 100 data points per sec. The reduction of the column internal diameter and the column length permitted the column to be operated at twice the mobile phase linear velocity. Consequently, the separation of the solutes eluted from the column up to a capacity factor of 3.0 was completed in about 3.5 sec. Table III summarizes the performance of this column operated at a linear velocity of 3.3 cm/sec.

There was no peak broadening due to detector response time, as was the case for the 4 cm long column. However, values for the number of theoretical plates for *p*-xylene and anisole were almost identical but this could be attributed to the contribution of the volume of the detector flow cell to the peak variance for such a small column.

Quantitative analysis in high-speed isocratic separations. Fast LC separations such as those shown in Figs. 5 and 6 can be employed in practice only if they can

TABLE IV

REPRODUCIBILITY OF QUANTITATIVE ANALYSIS BY PEAK AREA NORMALIZATION OF A FIVE-COMPONENT SYNTHETIC MIXTURE (ISOCRATIC HIGH-SPEED SEPARATION)

Chromatographic conditions as in Table III.

Compound	*Normalized peak area*						*Mean*	*σ (S.D.)*	*(Relative S.D.) (%)*
p-Xylene	9.8	9.8	9.6	9.8	9.8	9.6	9.73	0.10	1.1
Anisole	11.9	11.9	12.2	12.2	11.8	12.0	12.00	0.17	1.4
Nitrobenzene	24.2	23.9	24.7	24.2	24.0	24.3	24.22	0.28	1.2
Acetophenone	23.7	23.9	23.5	23.4	23.3	23.7	23.58	0.22	1.0
Dipropyl phthalate	30.3	30.5	30.0	30.4	31.1	30.4	30.45	0.36	1.2

TABLE V

RETENTION TIME REPRODUCIBILITY OF HIGH-SPEED SEPARATION OF A FIVE-COMPONENT SYNTHETIC MIXTURE (ISOCRATIC DEVELOPMENT)

Chromatographic conditions as in Table III.

Compound	*Retention time (sec)*						*Mean*	*σ (S.D.) (msec)*	*(Relative S.D.) (%)*
p-Xylene	0.74	0.73	0.74	0.73	0.73	0.75	0.74	6	0.8
Anisole	0.90	0.89	0.89	0.89	0.90	0.90	0.90	6	0.6
Nitrobenzene	1.50	1.50	1.49	1.49	1.49	1.51	1.50	8	0.5
Acetophenone	1.83	1.81	1.81	1.81	1.80	1.82	1.81	10	0.5
Dipropyl phthalate	2.78	2.79	2.76	2.76	2.76	2.78	2.77	14	0.5

provide accurate and reproducible results. The reproducibility of the chromatography system was determined using the 2.5 cm long column, operated at a linear velocity of 3.3 cm/sec. Six replicate samples of the five component mixture were injected. The normalized peak areas, their mean values, together with those of the standard deviation and the relative standard deviation for each solute are given in Table IV. It is seen that the quantitative reproducibility is very satisfactory, the values of the relative standard deviation for each solute being well within 1.5%. In Table V the retention times of the five solutes together with their mean values, the standard deviation and the relative standard deviation with respect to the mean values are given. It is seen that the standard deviation for each solute is within 1% of the mean value. The reproducibility of solute retention and analysis by normalized peak areas demonstrates that the precision obtained from fast LC isocratic separations is similar to that obtained from conventional chromatographic systems[19].

A pre-formed gradient system for high-speed liquid chromatography separations

The fast LC separations demonstrated earlier, which are believed to be the fastest achieved in LC so far, were performed utilizing isocratic development. However, isocratic elution has the disadvantage that it is unable to handle samples which contain many components covering a wide polarity range. In the analysis of complex samples, gradient elution is the preferred technique.

Obviously, columns of small dimension with low dead volumes are well suited for fast gradient elution analysis. However, the inherent large gradient delay volumes of conventional gradient mixing devices make them inappropriate for use in fast LC systems. A pre-formed gradient system, which was first proposed by Snyder and Saunders[20], was therefore employed in the design of a fast gradient elution system. Preliminary results of its utilization for rapid separations are reported here.

A schematic diagram of a pre-formed gradient system is shown in Fig. 7. The required gradient was formed in a column of 25 × 0.46 cm I.D. packed with glass beads having particle size of 40 μm. The gradient storage column had a dead volume of about 2 ml, and this constituted the total volume of the solvent program. The gradient column was filled with a given solvent program which was then displaced through the analytical column by the final solvent mixture.

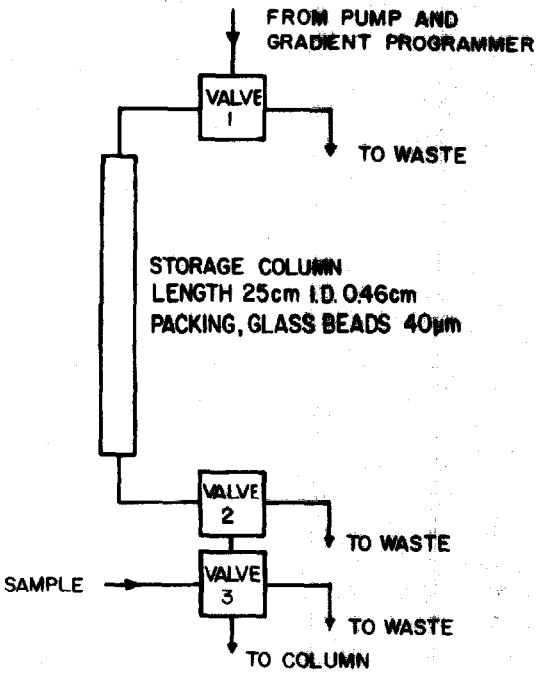

Fig. 7. Diagram of the pre-formed gradient system utilized for fast gradient elution separations.

The operational procedure of the pre-formed gradient system was as follows: a mobile phase mixture programmed to provide a given solvent concentration profile was formed over a period of time and pumped through the valves into the gradient storage column and the solvent previously contained in the gradient column passed to waste. When the complete solvent program was contained in the storage column, the flow was stopped. The sample was then charged into the injection loop of valve 3, and then valve 1 and valve 2 were changed. The flow was restarted and the final solvent allowed to displace the gradient from the storage column through the chromatographic column at a high flow-rate.

The chromatographic apparatus in which the gradient column was incorporated was the same as that used for high-speed isocratic separations. The column of 2.5 × 0.26 cm I.D., packed with a 3-μm Hypersil-C_{18} material (with an actual mean particle diameter of 3.1 μm) was employed as the analytical column.

A chromatogram of the separation of a thirteen-component synthetic mixture utilizing the pre-formed gradient system is given in Fig. 8. The mixture, made up in the mobile phase, contained components covering a wide polarity range at levels which varied from 0.01 to 1 % (w/w). To separate this mixture, the mobile phase was programmed to change linearly from 25 % acetonitrile in water to 100 % acetonitrile over 1 min at a flow-rate of 2 ml/min. This was the maximum speed at which the gradient system would provide a program with an automatic concentration profile. The final solvent used to develop the chromatogram in Fig. 8 was 100 % acetonitrile at a flow-rate of 5 ml/min.

It is seen that the separation of the thirteen-component mixture was completed

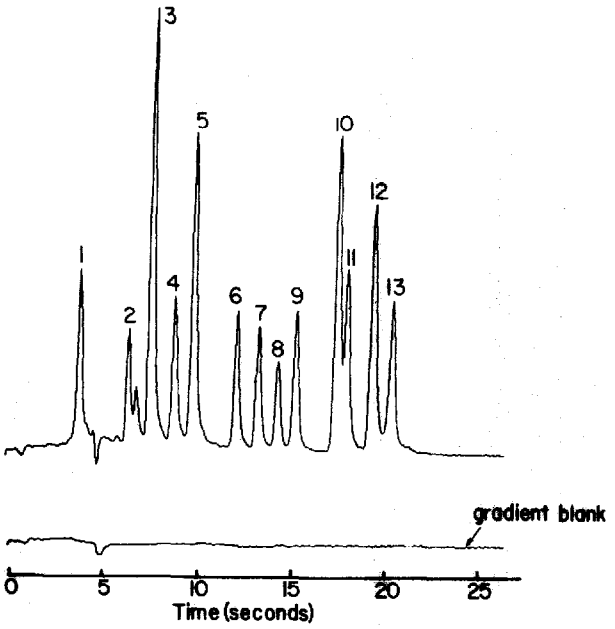

Fig. 8. Fast separation of a wide polarity range mixture by pre-formed gradient development. Column: 2.5 × 0.26 cm I.D. Packing: Hypersil-C_{18}, 3 μm. Solvents: I, 25% acetonitrile in water; II, 100% acetonitrile. Flow-rate: 5 ml/min. Peaks: 1 = 2,4-dinitrophenol; 2 = *p*-aminophenol; 3 = *o*-dinitrobenzene; 4 = benzyl chloride; 5 = benzene; 6 = *p*-xylene; 7 = isopropylbenzene; 8 = *tert.*-butylbenzene; 9 = dibutyl phthalate; 10 = benz[*a*]anthracene; 11 = dipentyl phthalate; 12 = benzo[*a*]pyrene; 13 = dihexyl phthalate.

in about 22 sec. However, the gradient cycle, including preparation, development and regeneration required 5–6 min. Thus to fully utilize the capabilities of this system, each chromatographic column would require a number of gradient columns that could be operated in parallel, if the gradient analysis was to be repeated continuously.

Reproducible results are required for solute identification and quantitative accuracy, but in general, it is more difficult to control experimental conditions in gradient elution analysis than in isocratic elution analysis. The reproducibility of the chromatographic system for fast isocratic separations was demonstrated to be very satisfactory; however, it was also important to evaluate the precision and accuracy of the fast gradient elution chromatographic system. To determine reproducibility of retention time and quantitative analysis that can be obtained from this particular system, eight replicate samples of the thirteen-component mixture were chromatographed. The results of the repeatability study are given in Tables VI and VII. The normalized peak area (obtained by cutting the peak out and weighing), their mean value, the standard deviation, and the relative standard deviation for each solute are given in Table VI. It is seen that the relative standard deviation lies within 3–4% of the mean value with the exception for 2,4-dinitrophenol and dipentyl phthalate, the latter being not completely resolved from the benz[*a*]anthracene peak. The deviation for the first peak (5.5% relative S.D.) can be probably explained by the fact that the pressure does not rise instantaneously when the system is turned on to develop the

TABLE VI

REPRODUCIBILITY OF QUANTITATIVE ANALYSIS OF PEAK AREA NORMALIZATION OF A THIRTEEN-COMPONENT SYNTHETIC MIXTURE (PRE-FORMED GRADIENT DEVELOPMENT)

Packing: Hypersil-C_{18}, 3 μm. Column: 2.5 × 0.26 cm I.D. Flow-rate: 5 ml/min.

Compound	*Peak area (by weighing, mg)*								*Mean*	*σ (S.D.)*	*(Relative S.D.) (%)*
1 2,4-Dinitrophenol	16.67	16.14	15.60	15.06	17.37	15.34	15.68	17.34	16.15	0.89	5.5
2 *p*-Aminophenol	8.67	7.75	8.51	8.13	8.48	8.59	8.32	8.41	8.36	0.30	3.5
3 *o*-Dinitrobenzene	38.90	37.80	38.80	38.80	37.90	38.30	38.00	40.10	38.57	0.76	2.0
4 Benzyl chloride	15.10	14.00	14.30	14.10	14.30	14.80	14.80	14.90	14.54	0.41	2.8
5 Benzene	31.80	29.80	31.00	31.70	31.00	31.10	31.60	32.10	31.26	0.72	2.3
6 *p*-Xylene	13.10	12.60	13.40	13.40	13.10	12.60	12.90	13.20	13.04	0.32	2.4
7 Isopropylbenzene	12.52	11.34	12.05	12.00	12.00	12.00	12.00	12.30	12.03	0.34	2.8
8 *tert.* Butyl-benzene	8.72	8.54	8.67	8.40	8.52	8.38	8.78	8.69	8.59	0.15	1.7
9 Dibutylphthalate	15.06	14.22	15.32	14.57	14.91	14.57	14.28	14.39	14.67	0.39	2.7
10 Benz[*a*]anthracene	31.10	28.80	31.30	29.40	31.20	30.80	29.70	31.40	30.46	1.0	3.3
11 Dipentyl phthalate	20.10	18.30	19.60	17.90	19.80	20.40	19.20	21.40	19.59	1.13	5.8
12 Benzo[*a*]pyrene	27.00	26.80	28.20	26.20	27.30	26.50	27.60	28.20	27.23	0.74	2.7
13 Dihexyl phthalate	17.07	15.86	17.02	15.85	17.17	16.25	17.09	17.33	16.71	0.63	3.8

TABLE VII

RETENTION TIME REPRODUCIBILITY OF HIGH-SPEED SEPARATION OF A THIRTEEN-COMPONENT SYNTHETIC MIXTURE (PRE-FORMED GRADIENT DEVELOPMENT)

Chromatographic conditions as in Table VI.

Compound	*Retention time (sec)*								*Mean*	*σ (S.D.)*	*(Relative S.D.) (%)*
1 2,4-Dinitrophenol	3.76	3.84	3.86	3.89	4.17	4.11	4.07	4.08	3.97	0.15	3.8
2 *p*-Aminophenol	6.45	6.46	6.46	6.48	6.74	6.71	6.60	6.53	6.55	0.12	1.8
3 *o*-Dinitrobenzene	7.69	7.65	7.61	7.58	7.89	7.85	7.69	7.61	7.70	0.11	1.5
4 Benzyl chloride	8.96	8.86	8.83	8.82	9.15	9.22	8.89	8.85	8.95	0.15	1.7
5 Benzene	9.94	9.89	9.85	9.82	10.13	10.19	9.81	9.80	9.92	0.13	1.3
6 *p*-Xylene	12.24	12.15	12.13	12.14	12.40	12.43	12.11	12.06	12.21	0.14	1.1
7 Isopropylbenzene	13.49	13.34	13.30	13.26	13.54	13.61	13.24	13.23	13.38	0.15	1.1
8 *tert.*-Butylbenzene	14.54	14.40	14.33	14.33	14.59	14.62	14.33	14.33	14.43	0.13	0.9
9 Dibutyl phthalate	15.49	15.31	15.28	15.23	15.56	15.60	15.23	15.28	15.37	0.15	1.0
10 Benz[*a*]anthracene	17.65	17.54	17.47	17.43	17.68	17.79	17.38	17.51	17.56	0.15	1.0
11 Dipentyl phthalate	18.19	18.02	17.97	17.91	18.20	18.30	17.91	18.05	18.07	0.15	0.8
12 Benzo[*a*]pyrene	19.57	19.43	19.39	19.32	19.64	19.69	19.29	19.54	19.48	0.15	0.8
13 Dihexyl phthalate	20.60	20.43	20.38	20.37	20.67	20.71	20.37	20.56	20.51	0.14	0.7

chromatogram. Retention times for the thirteen solutes, their mean values, together with the standard deviation and the relative standard deviation are listed in Table VII. It is seen that the reproducibility is satisfactory for each solute with the exception for the first peak, which again can be explained by the reason mentioned above. It is apparent also from Tables IV–VII that identification by retention time measurements and quantitative analysis by peak area would be significantly better with the fast isocratic separations than with fast gradient separations, but in both cases the level of repeatability would be adequate for most LC analyses.

In addition to the determination of the reproducibility of the fast gradient system preliminary results on the accuracy of the gradient program were obtained. It was found that the actual gradient profile was somewhat different to that programmed, and this was apparently due to some intermixing of the solvents in the storage column, and appeared to be caused by the difference in the viscosity of the two solvents.

DISCUSSION

It was interesting to compare values of the separation times obtained experimentally (Figs. 5 and 6), together with the column length, column radius, flow-rate and maximum instrument time constant that were actually employed to the values that were predicted by equations previously derived. Eqns. 7, 9, 13, 16 and 19 were used to calculate the theoretical values of flow-rate, analysis time, column length, column radius and maximum instrument time constant, respectively. The experimental and theoretical values obtained are summarized in Table VIII, and experimental values used for the calculations are also included.

p-Xylene, which was used as the unretained solute for purposes of calculating linear velocity, was in fact slightly retained. Therefore to improve precision, an estimation of the column dead volume was made for both 4 cm and 2.5 cm long columns on the basis of their geometry. A value of 0.75 was taken as the column fraction available for the unretained solute to permeate through a packed bed. The column dead volume so estimated was used for the calculation of all k' values as well as for values of the linear velocity, u_A, and these data are listed in Table VIII. Values for S

TABLE VIII

EXPERIMENTAL AND THEORETICAL VALUES FOR THE FLOW-RATE, ANALYSIS TIME, COLUMN LENGTH, COLUMN RADIUS AND MAXIMUM INSTRUMENT TIME CONSTANT

Column dimensions (cm)	u_A *(cm/sec)*	u_T *(cm/sec)*	t_A *(sec)*	t_T *(sec)*	l_A *(cm)*	l_T *(cm)*	r_A *(cm)*	r_T *(cm)*	τ_A *(sec)*	τ_T *(sec)*
4 × 0.4	2.1	1.7	14.8	19.5	4.0	3.5	0.2	0.18	0.014	0.012
2.5 × 0.26	5.4	4.2	2.9	3.4	2.5	2.1	0.13	0.13	0.006	0.005
	$\Psi = 35$, f(k') = 0.26, $\theta = 0.4$, $\lambda = 0.5$, $d_p = 3 \cdot 10^{-4}$ cm									
4 × 0.4	$S = 8.25$, $k'_1 = 2.0$, $k'_2 = 6.8$, $\alpha = 1.24$, $\eta = 3 \cdot 10^{-3}$ P, $D = 3.07 \cdot 10^{-5}$ cm^2/sec, $\sigma_{A(calc.)} = 2.1$ µl, $P = 5600$ p.s.i.									
2.5 × 0.26	$S = 6.8$, $k'_1 = 2.28$, $k'_2 = 5.3$, $\alpha = 1.34$, $\eta = 2 \cdot 10^{-3}$ P, $D = 4.07 \cdot 10^{-5}$ cm^2/sec, $\sigma_{A(calc.)} = 0.6$ µl, $P = 5300$ p.s.i.									

and α were calculated for benzyl acetate and dipentyl phthalate (Fig. 5), and for nitrobenzene and acetophenone (Fig. 6) which were taken as the pairs of closest eluting compounds defined in eqn. 8. To calculate the column radius, r, the volume standard deviation, σ_A, contributed by the apparatus was determined according to the relationship suggested by Klinkenberg[21]

$$\sigma_A = \sigma_c/(10)^{1/2}$$

where σ_c is the volume standard deviation of the unretained peak eluted from the column. Values for σ_c were calculated assuming a value of 2.5 for the reduced plate height for both columns. Furthermore, a value of 0.4 was assumed for θ, which was the fraction of the cross-section of the column available for the solvent flow in a bed packed with porous particles. Values for the mobile phase viscosity, η, were taken from published data[15], together with values for f(k'), λ, and D (ref. 22).

It is seen in Table VIII that an excellent agreement between the experimental and theoretical values is found. It should be pointed out that in the derivation of the equations, the Van Deemter function describing the relationship between h and u was employed and its successful utilization confirms its applicability to LC columns.

It is seen that the equations permit the calculation of those column parameters that allow the column to be matched to a particular chromatographic apparatus. According to eqn. 16 the column radius, r, is proportional to the square root of the instrument dispersion, σ_A, and hence, it follows that the quality of the instrument design controls the minimum radius that can be used and consequently, the solvent economy. It should be pointed out that the column of 2.5 × 0.26 cm I.D. could only be utilized successfully in fast LC separations where the instrument dispersion was not significant compared with that of the column. At lower linear velocities, where a higher column efficiency would be realized, the solute bands would be more narrow and this would dictate the use of an instrument with even less dispersion. According to eqn. 19 the maximum instrument time constant is proportional to the square root of the number of theoretical plates and to the C term in the Van Deemter equation, the magnitude of which is mainly determined by the particle diameter. Thus, at a given linear velocity, the smaller the particle diameter employed, the smaller the value τ must be for any instrument with which it would be used. It can be calculated, for example, that a value of 0.002 sec would be necessary for a column 2.5 cm long packed with 1-μm particles. Finally, according to eqn. 9, the ultimate factor controlling the analysis time is the pump pressure. It was shown that the chromatographic system with which the 2.5 cm long column was used was operated almost at the maximum pump pressure to effect a separation of two components having separation ratio, α, equal to 1.34. It is interesting to note that if an inlet pressure of 10,000 p.s.i. had been available, and the column length was adjusted to give the minimum analysis time, then the flow-rate could be increased by a factor of 1.4, but the analysis time however would be reduced only by about 3%. Thus the C term was the predominant factor and the pressure available was quite adequate for a column packed with 3-μm material to separate a particular mixture in the minimum time.

The results demonstrated that the separations were obtained in the minimum analysis time achievable and further reduction of the analysis time was restricted by the chromatographic apparatus that was currently available.

CONCLUSIONS

Equations based on accepted chromatographic theory can be derived that permit the calculation of the column length, column diameter, maximum instrument time constant and flow-rate to obtain a given separation in the minimum analysis time for a given chromatographic system. LC columns have been carefully designed on the basis of the equations derived to be commensurate with the dispersion of the instrument and other physical properties of the system. The agreement between the experimental values obtained, utilizing the chromatographic system for rapid separations, and those predicted theoretically has been demonstrated to be very good. Gradient elution systems can be designed that are suitable for fast liquid chromatography analysis. The rapid separation of multi-component mixtures in less than 30 sec has been achieved utilizing both isocratic and gradient elution development. Finally, it has been shown that the chromatographic systems designed for fast analysis by both isocratic and gradient elution development can provide accurate and reproducible results and therefore, can be used for solute identification and quantitative analysis.

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