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High speed gradient elution reversed-phase liquid chromatography

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Abstract

A major disadvantage of gradient elution in terms of speed results from the need to adequately re-equilibrate the column. This work distinguishes two states of re-equilibration: (1) run-to-run repeatability and (2) full equilibration. We find that excellent repeatability (±0.002 min in retention time) is achieved with at most 2 column volumes of re-equilibration whereas full equilibration can require considerably more than 20 column volumes. We have investigated the effects of adding ancillary solvents (e.g. *n*-propanol, *n*-butanol) to the eluent and changing the particle pore size, initial eluent composition and type, column temperature and flow rate on the speed of full equilibration. Full equilibration seems to be more thermodynamically limited than kinetically controlled. Also, we show that the main limitation to reducing the full equilibration time is related to instrument design issues; a novel approach to overcome these instrumental issues is described.

Keywords: Gradient elution; Speed; Equilibration; Flush-out volume

1. Introduction

The ultimate goal of chromatographic method development is to obtain acceptable resolution of all components within a reasonable analysis time. This goal has led many to investigate the limits of speed in chromatography [1–10]. In gradient elution HPLC, the analysis time is determined by the cycle time, which is the sum of the time required for the separation (i.e. gradient development time) and the time required to re-equilibrate the column to the initial eluent to prepare it for the next gradient run (i.e. re-equilibration time). For an optimized separation (in terms of resolution), the gradient development time should be considered fixed; therefore, the only way to minimize the cycle time (i.e. optimize the speed of gradient elution) is to minimize the re-equilibration time.

In 1990, Cole and Dorsey [11] devised a method to reduce the re-equilibration time by addition of 3% *n*-propanol to the initial eluent. After equilibrating the column with 100% acetonitrile, they flushed the column with the initial eluent (water, 3% *n*-propanol in water, etc.). The retention of acetone (injected every minute) indicated the degree of column re-equilibration; they claimed that equilibration occurs when the retention of acetone becomes constant. Cole and Dorsey demonstrated that re-equilibration is highly dependent on the eluent composition and bonding density of the stationary phase. In 1997, Warner and Dorsey claimed that the addition of 3% *n*-propanol to the eluent decreased the cycle time for dansyl-L-amino acids, phenols and polyaromatic hydrocarbons anywhere from 15 to 45% [12]. Also, the addition of 3% *n*-propanol had minimal effects on the retention order, retention time and resolution for all solutes studied. Unfortunately, this most recent study by Warner and Dorsey provides no data indicating the equilibration state of the column as a function of the re-equilibration conditions [12]. Instead, Warner and Dorsey assumed that a particular re-equilibration protocol was adequate based on prior work using different columns and the reproducibility of only acetone as the test solute [11] which makes their conclusions pertaining to the effect of *n*-propanol on column equilibration less than general.

Ultimately we are interested in optimizing the speed of gradient elution RPLC by reducing the re-equilibration time. We must distinguish between two very different definitions

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of "column equilibration" in gradient elution RPLC. First, most routine analytical work involving gradient elution is concerned only with achieving run-to-run repeatability of retention time for runs with fixed re-equilibration times and, of course, fixed gradient conditions. In this case, one obtains acceptable run-to-run repeatability (i.e. standard deviation <0.002 min) by reproducibly conditioning the column to the initial eluent before the next analysis. However, conditioning a column to provide acceptable run-to-run repeatability in retention time does not necessarily require full equilibration of the column to the initial eluent; solute retention times vary with respect to the re-equilibration time. In contradistinction, when a column provides retention times for all solutes which are independent of the re-equilibration time, the column has reached a state of full equilibration. Obviously one expects acceptable run-to-run repeatability when one achieves full equilibration of the column. In this paper, we investigate the minimum re-equilibration times required to provide acceptable run-to-run repeatability in retention time or full equilibration of the column for non-ionizable solutes using non-buffered eluents. Of all the relevant studies involving column equilibration [11,13–21], we believe this is the only study that provides a clear distinction between run-to-run repeatability and full equilibration.

The most widely accepted method of equilibration involves passing 10 column volumes of the initial eluent through the column before the next analysis [13]. We assume that this rule refers to equilibration as the state of full equilibration as defined above. Unfortunately, the literature provides very little data that substantiates this rule of thumb. Thus, the present work provides validated guidelines to properly investigate the state of column equilibration under gradient conditions.

Cole and Dorsey claimed that full equilibration could be obtained with less than three column volumes of 3% *n*-propanol in water (v/v) or just over 10 column volumes of eluent comprised of pure water [11]. However, they did not report the reproducibility of their retention data. Without this data we feel one cannot make an accurate estimate of the time required to achieve full equilibration (see Section 2 below). Regardless, the more than three-fold difference in the required equilibration time motivated us to investigate the effects of initial eluent strength (i.e. % acetonitrile, v/v), column temperature, flow rate and particle pore size on the required time to achieve acceptable run-to-run repeatability or full equilibration of the column. Following the key idea of Cole and Dorsey, we modified the initial eluent with judiciously selected ancillary solvents such as *n*-propanol, *n*-butanol and *n*-octanol to determine their effect on the column equilibration time and compare them to gradients done by common practice, that is, from a low percentage of acetonitrile (10/90, v/v acetonitrile/water) to pure acetonitrile.

As addition of ancillary solvents to the eluent can affect the viscosity of the eluent and retention times of the solutes, especially the early eluters, we outline a scheme to reduce re-equilibration time without significantly affecting either the viscosity of the eluent, or the retention times of the solutes. This study is not concerned with the details of the mechanism of how a specific change in conditions affects the required re-equilibration time; however, we did vary flow rate and temperature to investigate whether column equilibration is mainly a "thermodynamic" or a "kinetic" process.

Although changes in separation conditions are easy to implement, we are also concerned with the relationship between instrument design and the re-equilibration time. Specifically, at the end of a linear gradient the instrument must flush out the final eluent from the pumping system and connecting tubing before fresh initial eluent arrives at the column inlet. We believe the flush-out time of even well-designed popular instruments is a very significant contribution to the re-equilibration time and thus to the overall gradient cycle time. To test this hypothesis, we describe a novel instrument modification which virtually eliminates the entire flush-out volume and begins the immediate re-equilibration of the column at the end of the gradient. Through combination of a reduced flush-out volume and the appropriate eluent composition, we show that a dramatic reduction in the re-equilibration time is possible such that only one to two column volumes of eluent are required to achieve full equilibration.

The main advantage of full equilibration is that one obtains a value of retention time for each peak which is both accurate and precise from run-to-run. With an accurate value of retention time we expect that one can accurately predict separations using gradient elution training data [22–24]. A major limitation of using experimental runs to predict separations under other conditions is that the retention of the experimental runs might change from batch-to-batch of eluent and/or from day-to-day of data collection. Although these errors are correctable with the proper controls, we feel that proper eluent preparation, instrument maintenance and experimental conditions providing full equilibration will minimize these errors. We investigate both the accuracy and repeatability in retention time using different batches of eluent on the same day and using the same batch of eluent on different days. These results provide an indication of the reproducibility of the instrument and the gravimetric precision required in eluent preparation to obtain accurate and reproducible values of retention time.

2. Experimental

2.1. Instrumentation

All chromatographic experiments were conducted using three HP 1090 Series I chromatographic instruments controlled by version A. 10.01 Chemstation software (Agilent Technologies, Palo Alto, CA). Each instrument was equipped with a low pressure mixing chamber, autosampler, photodiode array UV detector and binary pump. The dwell volumes of instruments A–C, including all tubing required to

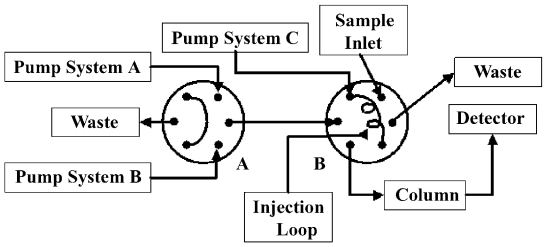

Fig. 1. Schematic diagram of the modified instrument design used to reduce the flush-out volume of the instrument. When valve A is in position 1, pump system A delivers eluent to valve B and eluent from pump system B is diverted to waste; position 2 reverses the destination of eluent from pump systems A and B. Valve B allows for 0.65 μl injections of sample delivered by pump system C. The "load" position of valve B directs eluent from valve A through the column and flushes the injection loop with sample from pump C. The "inject" position of valve B flushes the injection loop with eluent from valve A while diverting sample from pump C to waste.

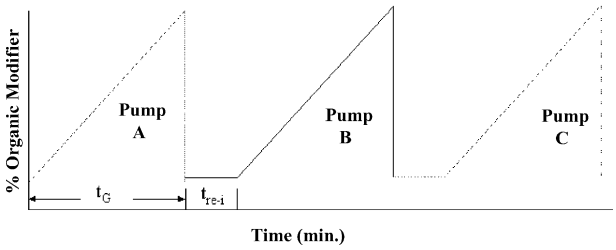

Fig. 2. Schematic representation of the gradient program used to measure the effect of a reduced flush-out volume on column equilibration as a function of the re-equilibration time ($t_{re,i}$ where 'i' is the time in min). The gradient time (t_G) is 1 min and $t_{re,i}$ is 2 min for the control runs and 0.15, 0.25, 0.50, 1.0 or 1.5 min for the experimental runs. Four control runs ($t_{re,2}$) were performed using only pump A before and after a series of seven experimental runs ($t_{re,i}$); four runs were performed with pump A and three runs were performed with pump B. The position of valve A in Fig. 1 determines which pump is delivering eluent.

connect the column, were determined to be 0.35, 0.38 and 0.42 ml, respectively, using the technique found in chapter 8 of ref. [25]. A prototype eluent pre-heater and column heating jacket obtained from Systec Inc. (New Brighton, MN) were used to pre-heat the mobile phase and maintain the column at 40.0 ± 0.1 °C unless stated otherwise; a thermocouple and Omega CN9000 display (Omega Engineering Inc., Stamford, CT) were used to monitor the eluent temperature at the column exit. The flow rate of each instrument was checked using a 10 ml volumetric flask and a stopwatch, and was determined to be consistently accurate to within 1% of the set-point.

Instrument A was used to investigate column equilibration with a conventional flush-out volume as a function of the eluent composition and re-equilibration time. The re-plumbed instrument used to reduce the instrument flush-out volume is shown in Fig. 1; instruments B and C are referred to as pump systems A and B, respectively. The two six-port dual position injection valves (Model 7000) were obtained from Rheodyne LLC (Rohnert Park, CA) and the HP 1040A diode array UV detector was obtained from Hewlett Packard S.A. (Wilmington, DE). An Altex Scientific Inc. (Berkeley, CA) model 110A pump (designated as pump system C in Fig. 1) was used to provide a continuous flow of sample to injection valve B at a 0.2 ml/min flow rate.

LabVIEW 6.0 software (National Instruments Inc., Austin, TX) and a 6024E data acquisition board were used to control the timing of the valves, detector and pump systems A–C in Fig. 1. We programmed valve A and pump systems A and B to deliver the gradient profile shown in Fig. 2. Using only pump system A and valve B (i.e. turning off pump B and keeping valve A static) allowed us to perform experiments using an instrument with a conventional flush-out volume of 0.80 ml using a 1.0 ml/min flow rate. Using both valves and all three pump systems allowed us to reduce the flush-out volume to 0.012 ml (i.e. the volume of the tubing between valve A and B) at the same flow rate. For clarification, Figs. 4–15 represent data obtained using an instrument with a conventional flush-out volume; Figs. 16–20 represent data obtained using a modified instrument with a reduced flush-out volume.

2.2. Reagents

All solutes were of reagent grade or better and were used as obtained from the manufacturer without further purification. Uracil, acetone, *N*-benzylformamide and alkylphenones (acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone and heptanophenone) were obtained from Aldrich (Milwaukee, WI). These solutes were diluted into one sample using a 10/90 (v/v) acetonitrile/water eluent; the concentration of uracil and acetone, *N*-benzylformamide and each alkylphenone was 1 mg/ml, 100 and 1 μg/ml, respectively. Uracil was used to measure the kinetic dead volume of the column.

The eluent reservoirs and filtration apparatus glassware were scrupulously cleaned, rinsed with water then acetone, and dried using nitrogen before use. The organic co-solvents in this study were used as obtained from the manufacturer; acetonitrile was obtained from Burdick and Jackson (Muskegon, MI), *n*-propanol, *n*-butanol and *n*-octanol were obtained from Fisher (FairLawn, NJ). HPLC grade water was obtained in-house from a Barnstead Nanopure Deionizing system (Dubuque, IA). This water was boiled to remove carbon dioxide and cooled to room temperature before use.

All eluents were prepared gravimetrically (±0.01 g) based on the density (17) at room temperature (25 °C) of acetonitrile, *n*-propanol, *n*-butanol, *n*-octanol and water where eluent composition is reported as the v/v ratio. Ternary solvents were made by first adding the alcohol to acetonitrile followed by dilution with water. The eluents were stirred magnetically until they reached room temperature. All eluents were passed through a 0.45 μm nylon filtration apparatus (Lida Manufacturing Inc., Kenosha, WI) immediately before use. These eluents were not degassed to any extent beyond the degassing that occurred during filtration.

2.3. Columns

Two 15 cm × 4.6 mm i.d. columns with 5 μm SB-C_{18} particles and pore sizes of 80 and 300 Å were gifts from Agilent Technologies. These columns were used to study the effect of the eluent composition and re-equilibration time on column equilibration. A 5 cm × 2.1 mm i.d. column with 5 μm highly-crosslinked C_{18} modified particles (HC-C_{18}) with 100 Å pores developed in our lab [26] was used for the reduction of instrument flush-out volume studies. The stainless steel column hardware was obtained from Isolation Technologies (Hopedale, MA). The HC-C_{18} particles were slurried in 2-propanol and sonicated (model PC3, L&R Manufacturing, Kearny, NJ) for 20 min before packing. The column was packed using the downward slurry method technique at a packing pressure of 48 MPa using pure 2-propanol as the driving solvent and a Haskel 16501 high-pressure pump (Haskel International Inc., Costa Mesa, CA).

2.4. Chromatographic conditions: conventional flush-out volume

All gradient elution conditions for the column equilibration study with a conventional flush-out volume were as follows unless stated otherwise. Detection was performed at 254 nm and 5 μl injections of sample were made. The instrument was programmed to form a gradient from 100% channel A to 100% channel B in 10.00 min at a flow rate of 1.00 ml/min followed by a step change back to 100% channel A. The instrument was flushed with 100% channel A for a desired re-equilibration time before ending the run (i.e. stopping data collection and beginning data analysis). The time between the end and beginning of two consecutive runs in the sequence (i.e. the instrument cycle time) was approximately 30 s; this time results in additional re-equilibration of the column which is not included in the reported re-equilibration times below.

The sequence shown in Fig. 3 was used in the column equilibration studies. First, six control runs were performed using a 15 min re-equilibration time between gradients; 15 min is roughly equivalent to 10 column volumes for a 15 cm × 4.6 mm i.d. column. Most chromatographers believe that at least 5–10 column volumes are required to fully re-equilibrate the column. The initial six control runs (only two are shown in Fig. 3) ensured that the column was properly heated and conditioned before experimental data were collected. Next, four experimental runs (runs 3–6 in Fig. 3) using the test re-equilibration time between gradients were done, followed by two control runs (runs 7–8 in Fig. 3). This pattern of four experimental and two control runs was repeated at all test re-equilibration times. Typically, we gathered data for test re-equilibration times of 2, 5, 7, 10, 20, 25, 30, 35, and 40 min. As the re-equilibration period occurred after the gradient run, the data for a specific re-equilibration time was contained in the following run.

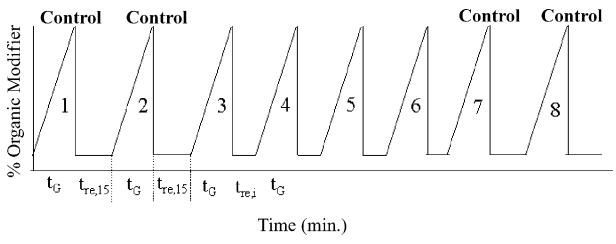

Fig. 3. Schematic representation of the gradient program used to measure differences in gradient retention time as a function of the re-equilibration time ($t_{re,i}$; see Fig. 2) using an instrument with a conventional flush-out volume. The gradient time (t_G) is 10 min and $t_{re,i}$ is 15 min for the control runs ($t_{re,15}$) and varied for a set of four experimental runs ($t_{re,2}$, $t_{re,5}$, $t_{re,10}$, etc.).

The average of four control runs (the two before and two after a set of four experimental runs, see Fig. 3) was used as the control retention time; the average of the four experimental runs is used as the experimental retention time. The "worst" solute, which is listed in Table 1 for all conditions studied using a conventional flush-out volume, displays the largest differences in the control and experimental retention time and indicates the degree of column equilibration at various equilibration times. All plots of the difference in control and experimental retention time versus re-equilibration time are generated using data from the "worst" solute unless otherwise indicated. After devising this sequence of runs we noticed that Patthy et al. [15] used a similar scheme to study the equilibration process for polar solutes and buffered eluents. However, we feel our scheme is better due to the use of controls between each set of experimental runs and the number of runs in each average.

2.5. Chromatographic conditions: reduced flush-out volume

All gradient elution conditions for the study of the reduction of the flush-out volume were as follows unless stated otherwise. Pump systems A and B were programmed to form a gradient from 100% channel A to 100% channel B in 1.00 min at a flow rate of 1.00 ml/min followed by a step change back to 100% channel A before the run was ended. Channel A contained 3/7/90 *n*-propanol/acetonitrile/water (v/v/v) and channel B contained pure acetonitrile; pump systems A and B used the same eluent reservoir (i.e. the same eluent) for each respective channel. The control method consisted of a 2.00 min re-equilibration time which is approximately 12 column volumes of eluent for the 5 cm × 2.1 mm i.d. column. Experimental re-equilibration times of 0.15, 0.25, 0.30, 0.50, 1.00 and 1.50 min were used.

2.6. Data analysis

The Chemstation software reports retention time data to the nearest 0.001 min by default. In some cases, especially for the 5 cm × 2.1 mm i.d. column, we obtained a 0.000 min standard deviation in run-to-run retention time. Fortunately,

Table 1
Summary of conditions used for the column equilibration study[a]

Condition	Column[b]	Initial eluent	Final eluent	Flow rate (ml/min)	Temperature (°C)	Worst solute
1	A	1/99 ACN/water	ACN	1.0	40.0	Acetone
2	A	10/90 ACN/water	ACN	1.0	40.0	Acetone
3	A	30/70 ACN/water	ACN	1.0	40.0	*N*-Benzylformamide
4	A	50/50 ACN/water	ACN	1.0	40.0	*N*-Benzylformamide
5	B	10/90 ACN/water	ACN	1.0	40.0	*N*-Benzylformamide
6	A	3/97 *n*-PrOH/water	ACN	1.0	40.0	Acetone
7	A	6/94 *n*-PrOH/water	ACN	1.0	40.0	Acetophenone
8	A	6/94 *n*-PrOH/water	94/6 ACN/*n*-PrOH	1.0	40.0	Acetophenone
9	A	10/90 ACN/water	ACN	3.0	40.0	Acetone
10	A	10/90 ACN/water	ACN	1.0	80.0	Acetone
11	A	10/3/87 ACN/*n*-PrOH/water	ACN	1.0	40.0	*N*-Benzylformamide
12	A	10/1/89 ACN/*n*-BuOH/water	99/1 ACN/*n*-BuOH	1.0	40.0	Acetone
13	A	4/1/95 ACN/*n*-BuOH/water	99/1 ACN/*n*-BuOH	1.0	40.0	Acetone
14	A	10/0.05/89.95 ACN/*n*-OcOH/water	99.95/0.05 ACN/*n*-OcOH	1.0	40.0	Butyrophenone

[a] Instrument A with a conventional flush-out volume (see Section 2.1) was used to perform these runs.
[b] Column A was a 15 cm × 4.6 mm column packed with 5 μm, 80 Å SB-C_{18} particles and column B was the same except the particles were 300 Å.

the Chemstation software records data to more than three decimal places. To obtain this data, we used a macro graciously provided by Agilent Technologies from the User Contributed Software Library to extract data (i.e. retention time, peak width, asymmetry, etc.) directly into Microsoft Excel to the specified number of decimal places. Overall, we obtained a more accurate estimate of run-to-run standard deviation in retention time (to at least five decimal places).

3. Results/discussion

3.1. Equilibration study using a conventional flush-out volume

The eluent composition has an important effect on the speed of equilibration in gradient elution. Thus, we first investigated the conditions required to obtain acceptable run-to-run repeatability in retention time and then searched for conditions that fully equilibrated the column using an instrument with a conventional flush-out volume as outlined in Figs. 3–15. After finding acceptable eluent compositions to reduce the time required for full equilibration, we further increased the speed of gradient elution by dramatically reducing the flush-out volume through appropriate modification of the instrumentation (see Figs. 1 and 2) to obtain the data in Figs. 16–20. We stress that the following results are specific to the conditions (i.e. stationary phases, solutes, eluents, instrumentation, etc.) investigated, but the methodology used to determine when one obtains repeatability and/or full equilibration is applicable to all other gradient elution RPLC conditions.

3.2. Run-to-run retention time repeatability

As most chromatographers are only concerned with obtaining reproducible data, we first investigated the re-equilibration time required to obtain acceptable run-to-run repeatability in retention time as good as one would expect from isocratic elution (<0.002 min). Fig. 4 shows a typical chromatogram of a mixture of uracil, acetone and seven alkylphenones (C_2–C_8). The repeatability of four replicate runs for all peaks was less than 0.002 min under all conditions used (see Table 1). As an example, Table 2 shows that run-to-run repeatability is excellent and independent of the composition of the initial acetonitrile/water eluent, and the re-equilibration time used. This is a surprising result. We had expected the retention repeatability to degrade at such short re-equilibration times. We chose not to use a re-equilibration time between 0 and 2 min under the assumption that at least one column volume of eluent must flush the column before the next run. Injecting samples before passing one column volume of initial eluent through the column for re-equilibration leads to larger

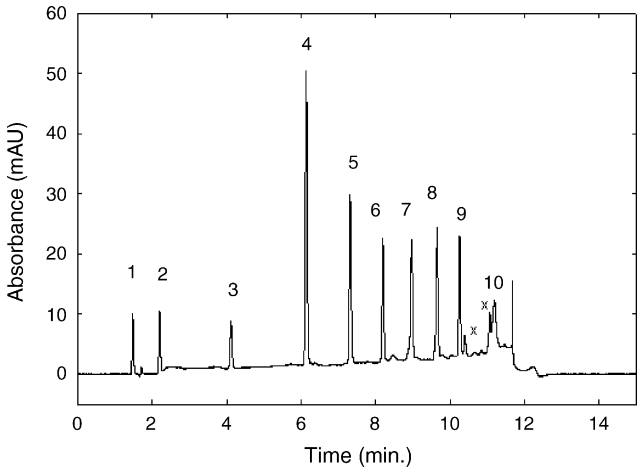

Fig. 4. Example of a gradient elution separation on column A (see Table 1) using an instrument with a conventional flush-out volume. Conditions: 10/90 to 100/0 acetonitrile/water in 10 min at a flow rate of 1 ml/min; 254 nm detection; 40 °C; solutes: uracil (1), acetone (2), *N*-benzylformamide (3), C_2–C_8 alkylphenones (4–10), impurity (×).

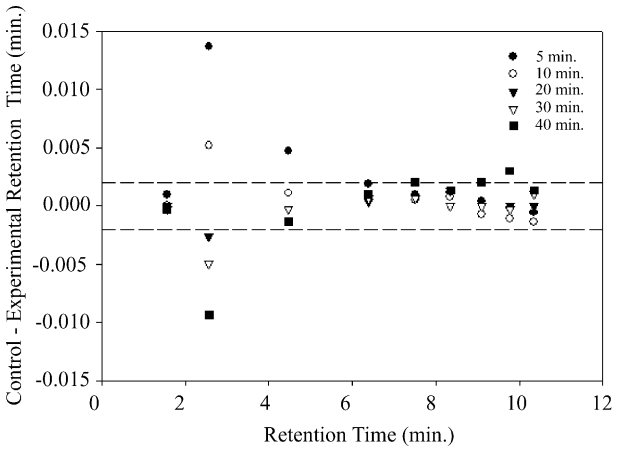

Fig. 5. Effect of re-equilibration time on the difference between the control and experimental gradient retention times (obtained as described in Fig. 3), which indicates the degree of column equilibration, as a function of the retention time for condition 2 (see Table 1). The dotted horizontal lines (– – –) represent the pooled standard deviation of the control and experimental runs averaged for each solute. All other conditions are the same as in Fig. 4. The experimental re-equilibration times used are 5 min (●), 10 min (◯), 20 min (▼), 30 min (▽) and 40 min (■), as indicated in the plot.

errors in run-to-run repeatability of retention time (data not shown). Regardless, our results show that excellent repeatability in gradient retention times is possible independent of the separation conditions (i.e. temperature, flow rate, eluent composition, etc.) when the column is flushed with only a single column volume of eluent (data not shown). We do not believe the superb repeatability in gradient retention time (<0.002 min) reported here resulted from the strict control of the column temperature (±0.1 °C); we are currently investigating the effect of temperature control on the repeatability of isocratic and gradient retention time.

When ionizable solutes and buffered eluents are used we expect the run-to-run repeatability to be much worse at least

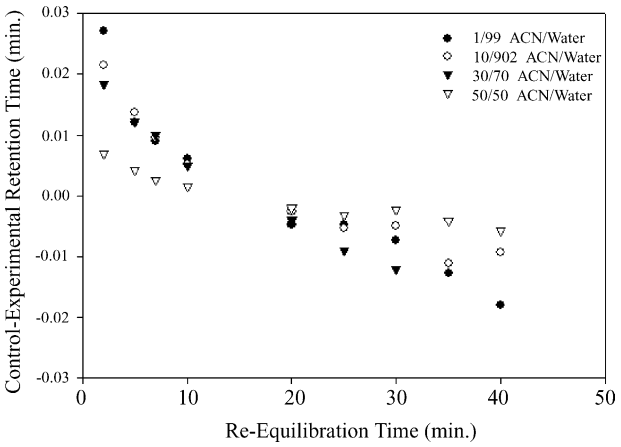

Fig. 6. Effect of the initial eluent composition on equilibration (see Fig. 5) as a function of the re-equilibration time. All other conditions are the same as in Fig. 4 except that the symbols represent conditions 1 (●), 2 (◯), 3 (▼) and 4 (▽) (see Table 1) of different initial eluent compositions as indicated in the plot.

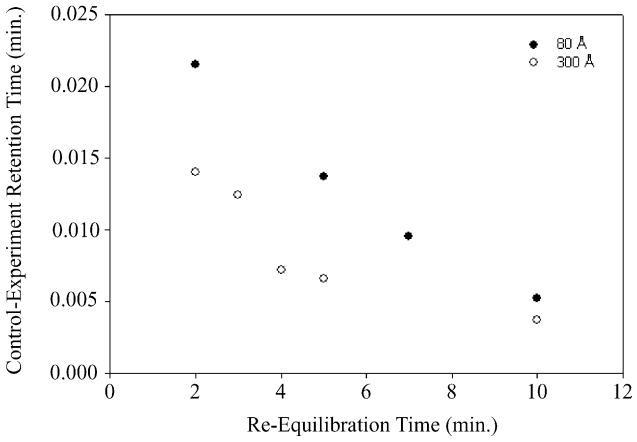

Fig. 7. Effect of the stationary phase pore size on equilibration (see Fig. 5 as a function of the re-equilibration time. All other conditions are the same as in Fig. 4 except that the symbols represent conditions 2 (●) and 5 (◯) (see Table 1) of different pore sizes as indicated in the plot.

on some specific column types based on the recent findings of Marchand et al. [14]. We expect that the factors controlling the repeatability for ionizable solutes are different and more complex than the factors affecting the repeatability of non-ionizable solutes in this study; this work is in progress.

3.3. *Effect of eluent strength, pore size, temperature and flow rate on full equilibration*

Although only a short re-equilibration time is required to achieve excellent retention time repeatability, such short re-equilibration times do not suffice to achieve full equilibration. Using the scheme shown in Fig. 3 and explained earlier, we measured the difference between the control and experimental retention times for each peak for various re-equilibration times; this retention time difference serves as an indication of the degree of full equilibration. Fig. 5 shows a plot of this dif-

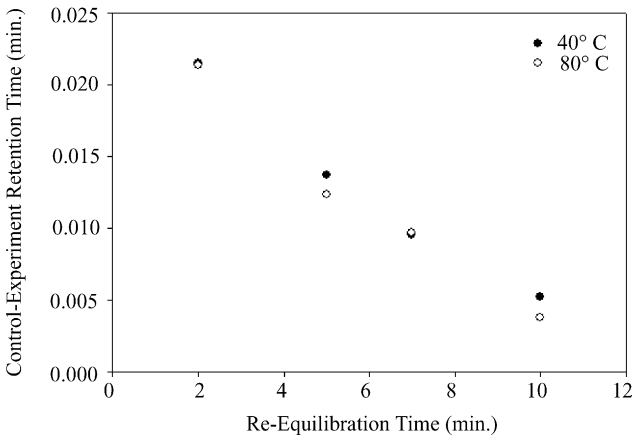

Fig. 8. Effect of the column temperature on equilibration (see Fig. 5) as a function of the re-equilibration time. All other conditions are the same as in Fig. 4 except that the symbols represent conditions 2 (●) and 10 (◯) (see Table 1) of different temperatures as indicated in the plot.

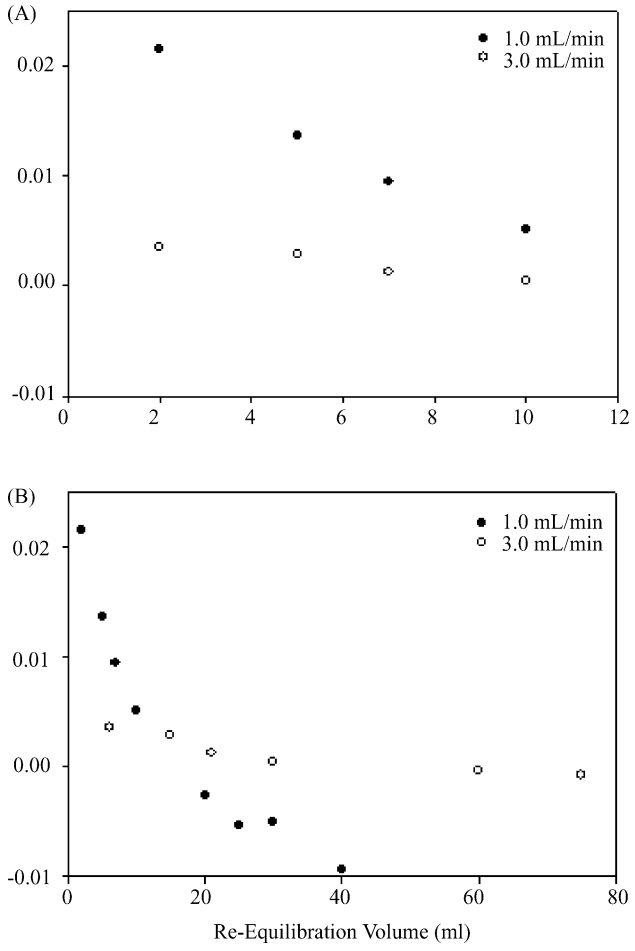

Fig. 9. Effect of the eluent flow rate on equilibration (see Fig. 5) as a function of the re-equilibration time (A) or the re-equilibration volume (B). All other conditions are the same as in Fig. 4 except that the symbols represent conditions 2 (●) and 9 (◯) (see Table 1) of different flow rates as indicated in the plot.

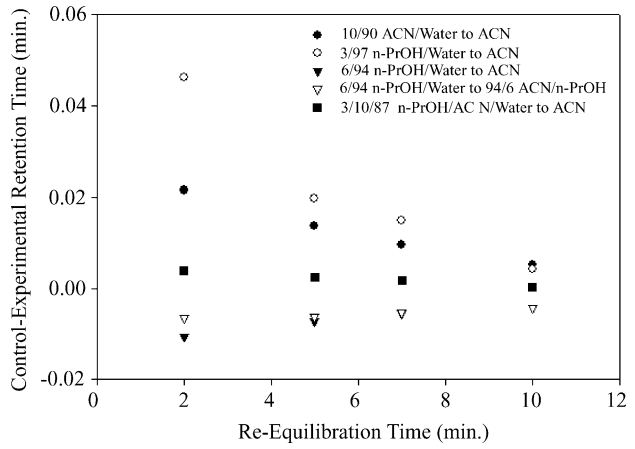

Fig. 10. Effect of the ternary composition of *n*-propanol/acetonitrile and water in the eluent on equilibration (see Fig. 5) as a function of the re-equilibration time. All other conditions are the same as in Fig. 4 except that the symbols represent conditions 2 (●), 6 (◯), 7 (▼), 8 (▽) and 11 (■) (see Table 1) of different eluent compositions as indicated in the plot.

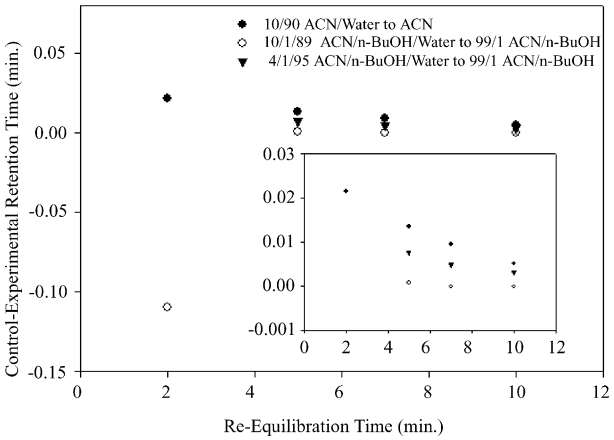

Fig. 11. Effect of the ternary composition of *n*-butanol/acetonitrile and water in the eluent on equilibration (see Fig. 5) as a function of the re-equilibration time. All other conditions are the same as in Fig. 4 except that the symbols represent conditions 2 (●), 12 (◯) and 13 (▼) (see Table 1) of different eluent compositions as indicated in the plot. The data point for condition 13 at ∼2 min is intentionally excluded for clarity.

ference in retention times as a function of the solute retention time.

The horizontal dashed lines in Fig. 5 show the pooled standard deviation of the control and experimental runs averaged for each solute, this value is no worse than 0.002 min for all conditions studies (see Table 1). When the dotted lines bracket a solute, the experimental and control retention times are statistically the same. The 90% confidence interval is roughly 0.0008 min using the pooled standard deviation from 18 sets of data. An interesting aspect of this graph is that the retention of only two solutes (*N*-benzylformamide and acetone) seems to depend strongly on the re-equilibration time. The later eluting solutes are almost insensitive to the column's pre-equilibration condition (i.e. the late eluters have no "memory" of the column's state before full equilibration was achieved). We also note that the retention time of the dead

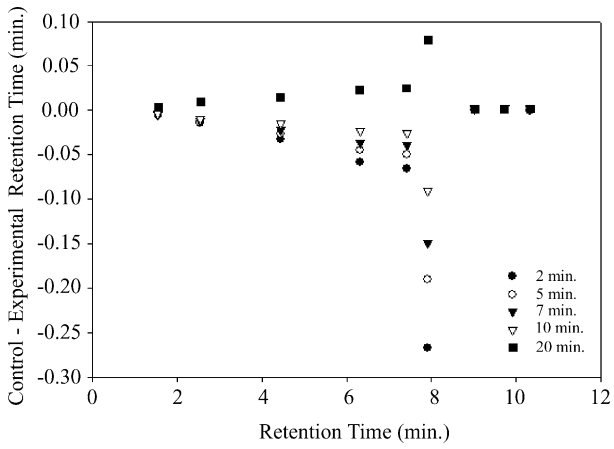

Fig. 12. Plot of column equilibration (see Fig. 5) vs. retention time using a ternary mixture of n-octanol/acetonitrile/water as the eluent (see condition 14 in Table 1). All other conditions are the same as in Fig. 4 using experimental re-equilibration times of 2 min (●), 5 min (◯), 7 min (▼), 10 min (▽) and 20 min (■).

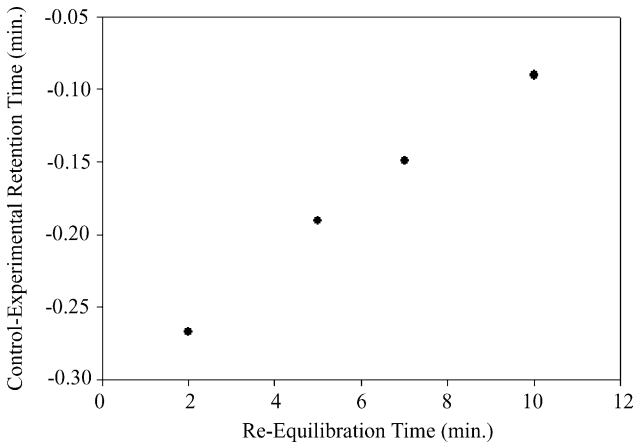

Fig. 13. Plot of column equilibration (see Fig. 5) vs. re-equilibration time using a ternary mixture of *n*-octanol/acetonitrile/water as eluent (see condition 14 in Table 1); all other conditions are the same as in Fig. 4.

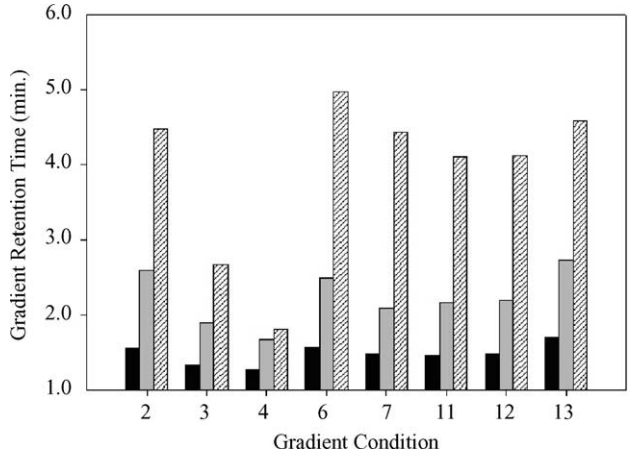

Fig. 14. Effect of the ternary composition of the eluent on gradient retention time for the gradient conditions described in Table 1. All other conditions are the same as in Fig. 4 and the solutes shown are uracil (▬), acetone (▭) and *N*-benzylformamide (▭).

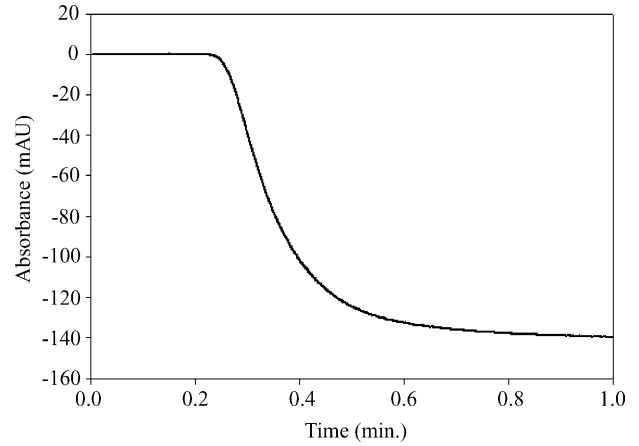

Fig. 15. Flush-out profile of an instrument with a conventional flush-out volume at 1.0 ml/min. using a step change from water with 0.1% acetone in channel B to 100% water in channel A and detection at 254 nm.

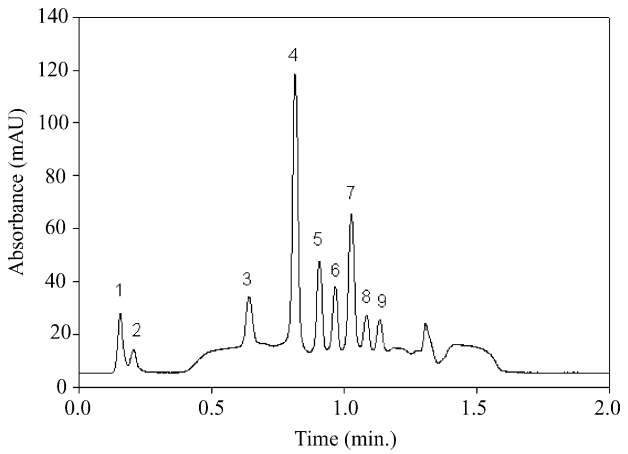

Fig. 16. Typical gradient elution separation on the 50 mm × 2.1 mm HC-C_{18} column using the instrument design in Fig. 1 with a conventional flush-out volume (valve A remained static and only pump A was used). All other conditions are the same as in Fig. 4 except that the gradient was from 3/7/90 to 3/97/0 *n*-propanol/acetonitrile/water in 1 min and octanophenone was not included in the solute mixture.

time marker (uracil) is essentially invariant and independent of the state of column equilibration. For the rest of this study, we use the "worst" solute (see chromatographic conditions and Table 1) to probe the state of column equilibration; full equilibration is deemed to occur when the retention of the "worst" solute becomes independent of the re-equilibration time.

To determine the minimum flushing time required to achieve full equilibration we plot the retention difference for the worst solute versus the re-equilibration time for the various separation conditions tested (see Table 1 and Figs. 6–11 and 13). Qualitatively, full equilibration occurs when the data (e.g. see Fig. 6) approach a horizontal asymptote. Statistically, full equilibration occurs when the measurement, i.e.

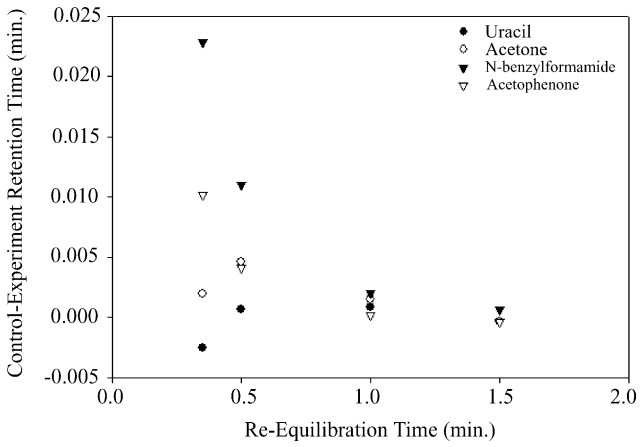

Fig. 17. Effect of solute retention on equilibration as a function of the re-equilibration time using an instrument with a reduced flush-out volume (see Fig. 1). The scheme for obtaining the difference in the control and experimental retention times (i.e. the degree of equilibration) is described in Fig. 2. All other conditions are the same as in Fig. 16 and the solutes shown are uracil (●), acetone (○), *N*-benzylformamide (▼) and acetophenone (▽).

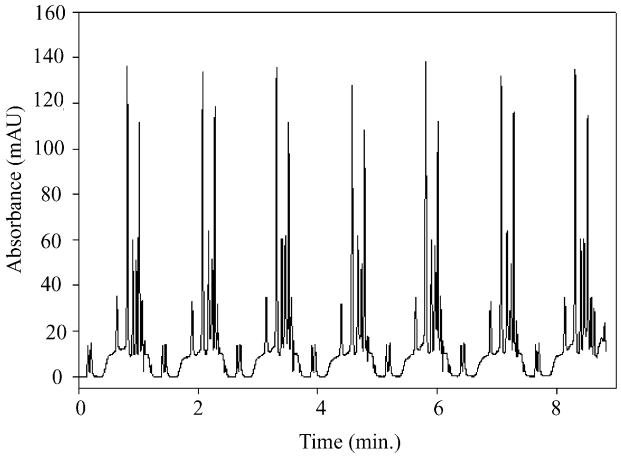

Fig. 18. Chromatogram of seven experimental runs obtained using an instrument with a reduced flush-out volume (see Figs. 1 and 2) for $t_{re,0.25}$; all other conditions are the same as in Fig. 16.

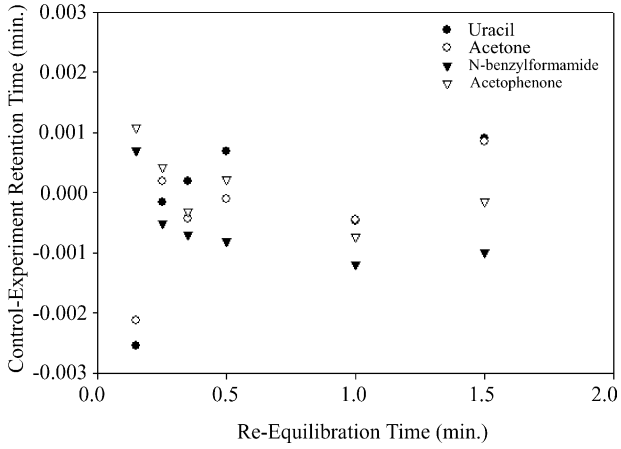

Fig. 19. Effect of solute retention on equilibration (see Fig. 17) as a function of the re-equilibration time using an instrument with a reduced flush-out volume (see Figs. 1 and 2). All other conditions are the same as in Fig. 16 and the solutes plotted are the same as those in Fig. 17.

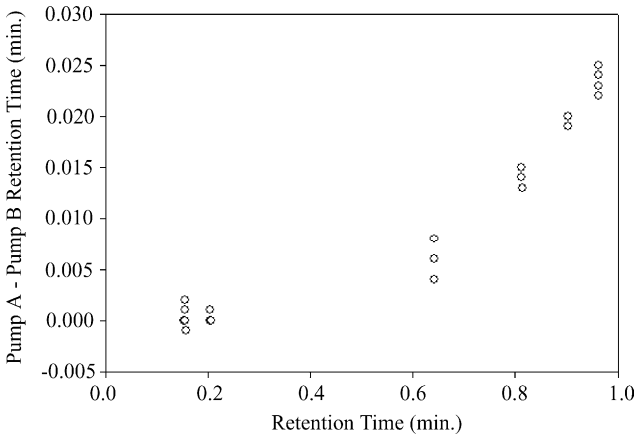

Fig. 20. Difference in retention time for pumps A and B (see Fig. 1 as a function of retention time using a system with a reduced flush-out volume for $t_{re,0.25}$ (see Fig. 2); all other conditions are the same as in Fig. 16.

the difference between the control and experimental retention time, no longer systematically varies by more than 0.002 min upon further increases in the re-equilibration period.

3.4. Effect of eluent strength on full equilibration

In Fig. 6, we compare the equilibration state of the column obtained using initial eluent strengths of 1/99, 10/90, 30/70 and 50/50 acetonitrile/water. The first important observation is that all conditions show that full equilibration is not complete even within 40 min (more than 25 column volumes). This is a most surprising result in that it corresponds to much more than ten column volumes of eluent. There is a similar trend in re-equilibration for all conditions (except 50/50 acetonitrile/water) for re-equilibration times shorter than 15 min. These data suggest that higher initial eluent strengths speed up full equilibration. Quantitative determination of the time required for full equilibration at each eluent composition is difficult because of the weak dependence of the retention difference on the re-equilibration time and the error in the retention data. However, it appears that full equilibration of the column becomes faster as the initial eluent strength is increased.

Table 2
Standard deviation of retention time for various gradient conditions and re-equilibration times

Peak number	Gradient condition[a]				Re-equilibration time (min)[b]			
	1	2	3	4	2	5	7	15
1	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001
2	0.002	0.001	0.000	0.000	0.000	0.001	0.002	0.001
3	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
4	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001
5	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.000
6	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001
7	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001
8	0.002	0.001	0.001	0.001	0.002	0.001	0.001	0.001
9	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.001

[a] Standard deviation of four control runs for various gradient conditions (see Table 1).
[b] Standard deviation of various re-equilibration times for condition 2 (see Table 1).

3.5. Effect of pore size on full equilibration

Fig. 7 shows the effect of the stationary phase pore size on column re-equilibration. The wider pore stationary phase (300 Å) re-equilibrates faster than the narrow pore stationary phase (80 Å).

3.6. Effect of temperature and flow rate on full equilibration

We were interested in determining whether equilibration is a thermodynamically or kinetically controlled process. To investigate the potential importance of any kinetic effects related to column equilibration we varied the temperature from 40 to 80 °C. As seen in Fig. 8, temperature has almost no effect on the speed of column equilibration. Thus, we believe that column equilibration is not a kinetically controlled process at room temperature and above.

To determine if equilibration is controlled by a thermodynamic process, that is by the extent to which the eluent is sorbed to the stationary phase, we used two flow rates of 1.0 and 3.0 ml/min and appropriately adjusted the gradient time to 3.33 min (at 3.0 ml/min) to maintain the gradient steepness (i.e. selectivity). At 1.0 ml/min there is no indication that full equilibration is obtained whether monitoring the equilibration state as a function of time (Fig. 9A) or volume (Fig. 9B). However, at 3.0 ml/min full equilibration of the column is evident in about 10 min (i.e. 30 ml) which suggests that use of the higher flow rate drastically reduced both the time and volume required to fully equilibrate the column. Based on these studies, we believe that column re-equilibration is mainly a thermodynamic process and the mass transfer kinetics of moving the solvent out of the pores is not a limiting factor.

3.7. Effect of ancillary solvents on full equilibration

In this section, we expand on the work of Dorsey and co-workers [11,12] by investigating the speed of full equilibration upon introduction of ancillary solvents such as *n*-propanol and *n*-butanol into the initial and/or final eluent. Fig. 10 shows the effect of adding *n*-propanol to acetonitrile/water eluents. Addition of 3% *n*-propanol to an initial eluent of water and keeping 100% acetonitrile as the final eluent (condition 6) decreased the rate of re-equilibration relative to the reference gradient (condition 2). Also, re-equilibration becomes much faster when a higher percentage of *n*-propanol (6%) is present in the initial mobile phase (condition 7). There are at least two explanations for this result. A higher *n*-propanol concentration might displace acetonitrile from the stationary phase more efficiently, or the amount of acetonitrile flushed through the column was not able to completely remove the higher concentration of *n*-propanol in the stationary phase [17]. In any case, it is clear that a higher concentration of *n*-propanol in only the initial mobile phase dramatically decreases the time required to achieve full equilibration. Further studies are required to understand the mechanistic issues at hand.

We also tested the rate of column equilibration with 6% *n*-propanol in both the initial (100% water) and final (100% acetonitrile) eluents (condition 8). Comparison of conditions 7 and 8 in Fig. 10 shows that the addition of 6% *n*-propanol to the final eluent has little or no effect on the speed of re-equilibration.

Last, we tested an initial eluent of 3/10/87 *n*-propanol/acetonitrile/water (condition 11) to determine the effect of the amount of acetonitrile in the initial eluent. Overall, the ternary initial eluent provided the fastest full equilibration compared to any other conditions described thus far. We believe that full equilibration occurs quickly when the initial eluent is most similar to the final eluent and contains an ancillary solvent that efficiently wets the stationary phase. Although we have not fully explored the effects of altering the composition of the initial and final eluents, we have found conditions that greatly speed-up re-equilibration of the column relative to the reference gradient (condition 2).

It is clear that *n*-propanol/water mixtures, which are known to effectively wet the stationary phase [17], are excellent alternatives to acetonitrile/water mixtures in terms of achieving full re-equilibration. Scott and Simpson [17] have investigated the stationary phase wetting effectiveness of various alcohols and found that the order (from worst to best) was methanol > ethanol > *n*-propanol > *n*-butanol. Thus, we added a small amount of *n*-butanol to the eluent; the results in Fig. 11 confirm our expectation that *n*-butanol would hasten equilibration. The addition of only 1% *n*-butanol to both the final and initial eluent and using 10% acetonitrile in the initial eluent provided the fastest full equilibration of the column compared to all other conditions; only 2–3 column volumes of eluent were required. Compared to the standard gradient profile (condition 2) which appears to never reach full equilibration, the addition of a small amount of *n*-butanol to the initial and final eluent greatly reduced the re-equilibration time. All results suggest that the ability of the initial eluent to wet the stationary phase has a strong influence on the time required to achieve full equilibration whereas the composition of the final eluent seems less important. Therefore, we strongly recommend the incorporation of *n*-butanol into the eluent when one demands full equilibration and a short cycle time in gradient elution.

3.8. Effect of n-octanol on full equilibration

The results of Scott and Simpson [17] along with the work described so far suggest that even longer alcohols might provide shorter re-equilibration times required for full equilibration. A major problem with using a longer chain alcohol such as *n*-octanol is its relatively low solubility in highly aqueous eluents. However, we decided to use a gradient from 0.05/10/89.95 to 0.05/99.95/0 *n*-octanol/acetonitrile/water (condition 14) to determine the effect of *n*-octanol on full equilibration. As seen in Fig. 12, the “worst” solute

(butyrophenone) is not near the beginning of the gradient as was the "worst" solute in all other cases. Also, Fig. 13 indicates that the column will not achieve full equilibration with *n*-octanol in the eluent. Although this result is puzzling, we recommend avoiding the use of alcohols with chains longer than *n*-butanol.

3.9. Effect of eluent composition on retention

An important consequence of changing the initial eluent composition in gradient elution is that the retention of early eluting solutes changes. Changes in the eluent viscosity and system back pressure also occur but we are less concerned with those differences because the effects are small. Fig. 14 shows the gradient retention time for the three earliest eluting solutes (i.e. those most effected by the initial eluent composition). As expected, the kinetic dead time of the column changed dramatically as the initial eluent composition was varied [27]. However, Fig. 14 does show that some eluent compositions both shorten full equilibration of the column and give retentions for most peaks similar to those under the original (binary solvent) condition (condition 2). These data support our prior conclusion that an acetonitrile/water mixture with a small amount (1%, v/v) of *n*-butanol is best because it both provides the fastest rate of full equilibration and has the least impact on retention. We strongly urge that one incorporate the alcohol in the eluent from the beginning of method development.

4. Equilibration study using a reduced flush-out volume

All of the work presented above is concerned with the reduction of the re-equilibration time through changes in the eluent composition, system temperature, or flow rate using an instrument with a conventional flush-out volume. A significant finding was that we achieved full equilibration in less than three column volumes, using a 15 cm × 4.6 mm i.d. column. Fig. 15 shows a profile of the instrument flush-out volume upon a step change from 100% channel B to 100% channel A, where B is 0.1% acetone in water, and A is water. The time required for the instrument to flush-out the strong solvent channel to begin delivering initial eluent to the head of the column is at least as large as the instrument dwell volume (∼0.35 ml), and in this case (1.0 ml/min), it is more than twice as large as the dwell volume (∼0.80 ml). We conservatively estimate the flush-out volume as the volume when the eluent at the column inlet contains 3% of the final eluent (i.e. the pumping system is 97% flushed). Obviously, the column cannot begin equilibrating with the initial eluent until the initial eluent actually starts flowing through the column. When a 15 cm × 4.6 mm i.d. column is used, this flush out represents a small fraction of the column volume itself, and therefore does not add appreciably to the time required for full equilibration. However, when the column volume is small compared to the flush-out volume, as in the case of short narrow-bore columns (i.e. 5 cm × 2.1 mm i.d., column volume is ∼0.15 ml), the flush-out volume adds significantly to the required re-equilibration time increasing it by a factor of five or more.

Our initial underlying motivation in this work was to better understand the limits of speed in gradient RPLC by minimizing the re-equilibration time. In this section, we present a novel instrument modification that dramatically reduces the system flush-out volume by making an immediate switch from the final eluent to the initial eluent at the column inlet. Thus, we used an apparatus consisting of two pumps, two switching valves and a narrow bore column, as shown in Fig. 1, to produce the gradient profile shown in Fig. 2. The chromatogram shown in Fig. 16 represents a typical separation of the solute mixture (without octanophenone) on the narrow-bore column using a "standard" system with a flush-out volume of approximately 0.80 ml (roughly twice the dwell volume of the instrument). The abnormal baseline (in Figs. 16 and 18) results from elution of built-up solvent impurities on the column under conditions of a steep gradient slope; this problem is not seen using longer gradient times. We expect that higher purity solvents will remedy this problem for quantitative purposes in fast gradient elution.

Fig. 17 is a plot indicating the degree of full equilibration of the narrow-bore column. We believe that full equilibration of the narrow-bore column is obtained in this case after a 1.0 min re-equilibration time, which is roughly ten column volumes. This result is consistent with the fact that the flush-out volume is relatively large (∼0.80 ml), and the fact that a primarily acetonitrile/water mixture with a small amount of *n*-propanol provided one of the fastest rates of full equilibration in the first part of the study.

To determine if a reduction in the flush-out volume of a "standard" system would reduce the time required for full equilibration, we used the instrument design and gradient profile in Figs. 1 and 2 to obtain the chromatogram shown in Fig. 18. The flush-out volume of the modified instrument is the volume of the tubing between valve A and the column inlet, which is 0.012 ml. Due to this small volume we were not able to accurately measure a flush-out volume profile. However, Fig. 19 provides an indication of when full equilibration occurs for the system with reduced flush-out volume. From the plot in Fig. 19, we believe full equilibration occurs before 0.25 min for all solutes. The fact that full equilibration was achieved with only two column volumes of eluent (compared to the ten column volumes using a larger flush-out volume) confirms our hypothesis that the system flush-out volume is an important factor in minimizing the cycle time in gradient elution. Although instrument manufacturers are beginning to realize the importance of the flush-out volume [28], we are not aware of any instrument design that reduces the flush-out volume in the fashion described here.

Alternative approaches employing two gradient pumping systems and two HPLC columns (i.e. parallel separations) where one column is used for separation while the other is

re-equilibrated have been reported [29]. However, we believe the approach presented here is an improvement in that it eliminates the possibility of differences in retention time data between two different HPLC columns (i.e. only one column is used for the separation), leaving only the variability introduced by differences in the two fluid pumping systems. Also, the reduced flush-out volume will always make the system faster, no matter how many columns are used. For example, a system using a reduced flush-out volume with one column might be as fast as parallel chromatography with two columns, but performing parallel chromatography using a system with a reduced flush-out and two columns would be even faster.

A limitation of our instrument design is that the two pumps are not identical in their flow characteristics, despite the fact that they are the same brand and model. Due to the slight differences in pump systems A and B in Fig. 1, the retention time of peaks eluted by each pump system was not the same. Fig. 20 shows the difference between the retention times obtained for each peak eluted with pump systems A and B, versus the peak retention time for the chromatogram obtained in Fig. 18. Although the difference in retention times obtained by each pump is significant (i.e. >0.001 min), the differences in retention time are reproducible and a function of retention time. Thus, one can calibrate (i.e. adjust) the retention time obtained using each pump. However, we chose to use data from one pump only for the plot in Fig. 20 to indicate when full equilibration was achieved. This work demonstrates that the main limitation to fast cycle times in gradient RPLC is not the HPLC column, but rather the design of the HPLC system itself. We hope the manufacturers of HPLC instrumentation solve this engineering challenge and thereby remove a major barrier to fast gradient RPLC cycle times.

5. Day to day precision in retention time

Although this study is mainly concerned with finding ways to reduce the time required to achieve full equilibration, we also investigated the precision of retention times obtained using different batches of eluent on the same day, and using the same batch of eluent on different days. The advantage of obtaining full equilibration is that one obtains the true or absolute value of retention instead of the less meaningful value of retention when conditioning the column to provide reproducible retention. For example, when using gradient retention times to predict an isocratic or gradient separation, the gradient retention times used must be accurate [30,31]. Thus, we were interested in determining the required batch-to-batch precision in eluent preparation to obtain retention times within 0.001 min on the same day. Using a balance with a precision of ±0.01 g, we prepared four batches of eluent (8.06 g/77.66 g/887.42 g *n*-butanol/acetonitrile/water) and calculated the average retention time of each peak using control runs (i.e. 15 min re-equilibration time) and column A (see Table 1).

As shown in Table 3, the standard deviation of the average retention time for each peak using four batches of eluent was quite acceptable at <0.003 min. This suggests that eluents must be prepared gravimetrically with a precision of at least ±0.01 g (out of a total of >750 g) to obtain retention times with a precision of 0.003 min or less. From Table 3, this correlates into a %R.S.D. of 0.05% or less for each peak, which we believe is an acceptable error in retention time to obtain reasonable predictions in method development.

Another cause of errors in predictions using gradient retention times is deviation in the performance of the instrument itself. We have already shown that on the same day our instrument provided run-to-run repeatability in retention time comparable to that expected in isocratic elution (<0.002 min) for all conditions and re-equilibration times (see Table 2). However, we were also interested in the day-to-day reproducibility of the instrument using the same batch of eluent. Therefore, we calculated the average and standard deviation of retention times for each peak chromatographed under identical conditions on three different days (see Table 4). This result indicates that retention times obtained on different days using the same batch of eluent are highly reproducible (<0.002 min) if

Table 3
Eluent batch-to-batch precision in retention time[a]

Peak number	Eluent batch[b]				Average[c]	S.D.[d]
	1	2	3	4		
1	1.492 ± 0.001	1.491	1.490	1.491	1.491	0.000
2	2.225 ± 0.001	2.224	2.223	2.223	2.223	0.000
3	4.199 ± 0.001	4.192	4.192	4.195	4.193	0.002
4	6.218 ± 0.001	6.211	6.211	6.216	6.212	0.003
5	7.390 ± 0.001	7.384	7.384	7.388	7.385	0.002
6	8.262 ± 0.001	8.256	8.257	8.260	8.257	0.002
7	9.021 ± 0.001	9.017	9.018	9.020	9.019	0.002
8	9.699 ± 0.001	9.696	9.696	9.698	9.696	0.001
9	10.297 ± 0.001	10.295	10.293	10.295	10.294	0.001

[a] Data was collected using condition 12 (see Table 1).
[b] The average retention time for four experimental runs ($t_{re,15}$) is reported and the standard deviation, as shown for eluent batch 1, was always less than ±0.001 min for all batches.
[c] Average retention time for the 16 runs from eluent batches 1–4.
[d] Standard deviation in retention time for the 16 runs from eluent batches 1–4.

Table 4
Day-to-day precision in retention time[a]

Solute number	Day[b]			Average[c]	S.D.[d]
	1	2	3		
1	1.490 ± 0.000	1.490	1.491	1.490	0.000
2	2.224 ± 0.001	2.224	2.225	2.224	0.000
3	4.198 ± 0.001	4.199	4.202	4.200	0.002
4	6.217 ± 0.000	6.217	6.219	6.218	0.001
5	7.390 ± 0.001	7.390	7.392	7.391	0.001
6	8.261 ± 0.000	8.261	8.262	8.262	0.001
7	9.023 ± 0.001	9.022	9.022	9.022	0.000
8	9.699 ± 0.001	9.699	9.699	9.699	0.001
9	10.296 ± 0.000	10.295	10.296	10.295	0.000

[a] Data was collected using condition 12 (see Table 1).
[b] The average retention time for four experimental runs ($t_{re,15}$) is reported and the standard deviation, as shown for eluent day was always less than ±0.001 min for all days.
[c] Average retention time for the 12 experimental runs from days 1–3.
[d] Standard deviation in retention time for the 12 experimental runs from days 1–3.

Table 5
Precision of retention using the system with a reduced flush-out volume[a]

Solute number	Control[b]			Experimental[c]		
	t_R (min)	S.D.	%R.S.D.	t_R (min)	S.D.	%R.S.D.
1	0.1541	0.0009	0.57	0.1543	0.0002	0.10
2	0.2055	0.0004	0.21	0.2053	0.0001	0.05
3	0.6406	0.0006	0.10	0.6411	0.0008	0.12
4	0.8131	0.0003	0.04	0.8127	0.0000	0.00
5	0.9046	0.0004	0.04	0.9045	0.0001	0.01
6	0.9631	0.0004	0.04	0.9636	0.0007	0.08
7	1.0224	0.0012	0.12	1.0156	0.0061	0.60

[a] Data was collected using the conditions described in Figs. 1 and 16.
[b] Average and standard deviation of six control runs.
[c] Average and standard deviation of three experimental runs for $t_{re,0.25}$.

errors from the instrument are not significant, which appears to be the case.

To obtain a better estimate of instrument performance under conditions that allow fast gradient cycle times, we measured the standard deviation and %R.S.D. of retention time of each peak on a narrow-bore column using the reduced flush-out system. Table 5, shows the average, standard deviation and %R.S.D. of retention times for each solute when the re-equilibration time was either 15 s or 2 min. There is no significant difference between the average retention times under these conditions, and the %R.S.D. of retention times from run-to-run are just as good as those measured for the longer column despite the tremendous difference in the absolute timescales of the separations.

6. Conclusions

Using the methods described here we have greatly improved the speed of gradient elution RPLC. There is now no reason to prefer isocratic elution to gradient elution based only on overall run time. Extremely short re-equilibration of the stationary phase with one column volume of eluent provides run-to-run repeatability in gradient retention time similar to that demanded in isocratic elution (<0.002 min) for all chromatographic conditions investigated. This is a somewhat surprising, but very practical and significant finding because it suggests that one need not allow 10–15 column volumes of initial eluent to pass through the column to obtain repeatable results. We stress that this particular finding is only applicable to non-ionizable solutes on the stationary phases used in this work; ultimately, the user must determine the limits of their particular chromatographic system. However, we have no reason to believe that the repeatability of retention time from run-to-run will be widely variable and highly dependent on the stationary phase and solute type; investigations are currently underway in this laboratory to assess the limits of gradient elution with ionizable solutes such as tryptic peptides and basic amines as the test solutes.

Despite the fact that very reproducible results can be obtained, full equilibration of the column using an instrument with a conventional flush-out volume can require much more than 10–15 column volumes of initial eluent as commonly believed; however, re-equilibration occurs more rapidly when the composition of the initial and final eluent are similar, as expected. Larger pore size stationary phase particles appear to improve the speed of full equilibration. Also, the process of full equilibration is limited by thermodynamic,

not kinetic processes, based on the fact that flow rate has a significant effect on the speed of equilibration whereas column temperature has only a minor effect. This result is usually mentioned in the literature without reference or experimental confirmation [13,29].

As suggested by Dorsey and coworkers, the addition of an ancillary solvent such as *n*-propanol to the eluent has a significant effect on the time required for full equilibration. We have found that the addition of as little as 1% *n*-butanol to the initial and final eluents of a typical gradient profile (see condition 2 in Table 1) provides for astoundingly short (i.e. 2–3 column volumes) re-equilibration. Furthermore, because the required re-equilibration times are so short when using these modified eluents, one must consider the flush-out volume of the instrument, particularly when the HPLC column volume is small. A novel instrument configuration has allowed us to reduce the time required for full equilibration of a short narrow-bore column from 1.5 min to 15 s, resulting in a 50% reduction in the gradient cycle time when the gradient time is 1 min.

Based on our results, we feel it is necessary to define new guidelines for reduction of the cycle time in the gradient elution separation of non-ionizable solutes. When obtaining reproducible retention times is the main objective, replicate injections allowing for a minimum volume of initial eluent equivalent to one HPLC column volume plus the instrument flush-out volume to flush the column will provide acceptable run-to-run repeatability. However, when one demands an absolute value of retention time independent of the re-equilibration time (i.e. full equilibration), addition of an ancillary solvent to the eluent before method development and/or reduction of the system flush-out volume will provide a means of reducing the required number of column volumes of initial eluent to re-equilibrate the column. Using the appropriate eluent composition and novel instrument modification outlined above, we believe full equilibration of any column is possible using only 1–2 column volumes of initial eluent. The most important finding of this study is that the re-equilibration time is no longer a limiting factor to improving the speed of gradient elution when using the proper instrument and eluent composition. In many cases, the instrument, not the other operating conditions, limits full equilibration. Future work will involve investigation of the limits of cycle time in gradient RPLC of pharmaceuticals and biological samples using buffered eluents.

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