Reduction of Total Analysis Time in Gradient Elution, Reversed-Phase Liquid Chromatography

The major disadvantage associated with gradient elution, reversed-phase liquid chromatography is the lengthy column reequilibration time required between chromatographic runs. Conventional wisdom holds that analysts may need to use as much as 20 column volumes of the original mobile phase for the reequilibration process. Cole and Dorsey previously showed that column reequilibration time can be reduced dramatically with the addition of 3% 1-propanol to each mobile-phase component to ensure a constant composition of propanol during the gradient. This article describes the addition of 3% 1-propanol to the mobile phases of three types of selected separations. The authors calculated and compared the total analysis time with the original separations. They also examined retention order, retention time, and resolution of the separations with and without 3% 1-propanol. In each separation, total analysis time was significantly reduced without adversely affecting the separation parameters.

Gradien elution liquid chromatography (LC) is one of the most effective means of separating samples that contain several analytes with different relative retention times. It also provides a solution to the general elution problem, which is prevalent in isocratic separations (1-4). The general elution problem is defined as poor resolution of peaks appearing early in a chromatogram and broadening of peaks at longer retention times. In reversed-phase LC, gradient elution is accomplished by increasing the percentage of organic modifier with respect to the aqueous solvent component of the mobile phase as the chromatographic run progresses. The use of gradient elution allows long-retained compounds to be eluted quickly and short-retained compounds to be eluted later than they would be under isocratic conditions. The overall effect is a compacted, well-spaced chromatogram with improved peak shape and lower detection limits. Although gradient elution provides the advantages of reduced analysis time and improved peak shape, this technique has a distinct disadvantage: the time required to reequilibrate the column with the initial mobile phase at the end of a chromatographic run (1,2,4).

In gradient elution, chromatographers continuously increase the solvent strength of the mobile phase over time to achieve the desired effect. Methanol, acetonitrile, or any organic modifier acts as any small organic molecule and partitions to the stationary phase. This process changes the solvation of the bonded alkyl chains in the stationary phase during a chromatographic run. When the mobile phase reaches its final composition, which contains a high percentage of organic modifier, the alkyl chains have dramatically different solvation characteristics than they had at the initial mobile-phase composition. Before analyzing another sample, the bonded alkyl chains in the stationary phase must be returned to their original composition by flushing the column with 15-20 column volumes of the initial mobile phase (2,4). Unfortunately, this column reequilibration time can be as long as the sample elution time (2).

Researchers have published several procedures to reduce column reequilibration times. Frenz and Horváth (5) recommend flushing the column with various solvents in decreasing order of their affinity for the stationary phase. This method reduces regeneration time only slightly and is very labor intensive and impractical for routine analysis. Dolan (4) suggests using a reverse gradient, but this approach seems to have no time advantage over the conventional method. After studying these attempts, we decided that the best way to reduce column reequilibration time was to control the solvation of the bonded alkyl chains throughout the chromatographic run.

Scott and Simpson (6) showed that 3% 1-propanol provides near monolayer coverage of the C18 surface, although as much as 50% methanol may be required for monolayer coverage of some stationary phases. This report was the impetus for many studies showing the effectiveness of adding low concentrations of propanol as a wetting agent to highly aqueous mobile phases (7-9). Using this concept, Cole and Dorsey (10) showed that reequilibration time can be reduced dramatically by the constant addition of 3% 1-propanol to the mobile-phase components. Although reequilibration time can be reduced by as much as 75% for a gradient from 100% aqueous solvent to 100% organic solvent, reequilibration time still can be reduced considerably using this technique even when the gradient range is smaller (10).

Because of the wetting effect provided by the 1-propanol, analysts also may observe higher column efficiencies at the beginning of chromatograms with highly aqueous initial mobile-phase compositions (7,8).

Although Cole and Dorsey (10) demonstrated that the use of propanol reduces reequilibration time, no studies of the effects of 3% 1-propanol has on various chromatographic parameters have been published.

Another question that remains unanswered is the applicability of this technique to practical separations. We selected three chromatographic separation types normally performed using gradient elution, reversed-phase LC and applied Cole and Dorsey's method of reducing column reequilibration time by adding 3% 1-propanol to each mobile-phase component. To demonstrate that this technique is applicable to real separations and chromatographic conditions, we studied the following separations:

- 17 dansyl-l-amino acids, 5 phenols, and 6 polyanromatic hydrocarbons (PAHs). These separations allowed us to investigate 18 columns in two lengths (15 and 25 cm) and different mobile
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We separated 17 dansyl-L-amino acids on a 25-cm C18 column using buffer and acetoni trile as the mobile phases; the initial mobile phase contained 10% organic modifier. In the analysis of five phenols, we used a 25-cm column with water and methanol as the mobile phases; the initial mobile phase was 100% water. The PAH separation had an initial mobile-phase composition of 40% organic modifier, and we used a 15-cm column with water and acetoni trile as the mobile-phase components.

In this article, we will present evidence that resolution, retention times, elution order, and separation times are similar for separations with the traditional mobile phase and for the separation with the constant addition of 3% 1-propanol to each mobile phase. We also will report time differences, including separation time and reequilibration time, between a traditional reequilibration scheme and the reequilibration procedure recommended by Cole and Dorsey (10). Then we will calculate the total analysis time required for each chromatographic run and compare it with the traditional method.

EXPERIMENTAL

Instrumentation: The chromatographic system consisted of two Spectroflow 400 solvent delivery systems and a Spectroflow 783 programmable absorbance detector–gradient controller (both from Kratos–Applied Biosystems Inc., Foster City, California); an Altex high-pressure mixer (Beckman Instruments, Fullerton, California); a Rheodyne injector with a 10-μL sample loop (Rheodyne, Cotati, California); and a PE Nelson Turbochrom data acquisition system (PE Nelson, San Jose, California).

We separated sample mixtures using 15 or 25 cm × 4.6 mm, 5-μm d₄, Supelcosil LC-18 C18 columns (Supelco, Inc., Bellefonte, Pennsylvania). The columns were maintained at 25 °C using a water jacket and a model 73 Isotemp immersion circulator (Fisher Scientific, Fair Lawn, New Jersey).

We degassed all of the mobile phases with helium sparging during use.

Chemicals and reagents: We used the following chemicals without additional purification: dansyl-L-aspatic acid, dansyl-L-aspartagine, dansylglycine, dansyl-L-proline, dansyl-L-phenylalanine, N,N’-didansyl-L-cystine, N-dansyl-L-serine, dansyl-L-alanine, dansyl-L-valine, dansyl-L-methionine, dansyl-L-isoleucine, N-dansyl-trans-4-hydroxy-L-proline, N-dansyl-L-threonine, Nα-dansyl-L-tryptophan, dansyl-L-norvaline, dansyl-L-leucine, dansyl-L-glutamic acid, 1,2-benzanthracene, and α-phenylenediamine (all from Sigma Chemical Co., St. Louis, Missouri); acenaphthene, p-cyanophenol, p-iодophenol, pyrene, and o-tet-butylnaphthalene (Eastman Kodak Co., Rochester, New York); acetic acid and ammonium acetate (both from Fisher Scientific Co.); and phenanthrene (from Matheson, Coleman and Bell, Cincinnati, Ohio).

We used the following high performance liquid chromatography (HPLC)–grade solvents without additional purification: acetoni trile, methanol, and 1-propanol (Fisher Scientific Co.).

Before use, we purified distilled water using a Nanopure II system (Barnstead, Dubuque, Iowa).

Chromatographic conditions: Amino acids: For the separation of 17 dansyl-L-amino acids, we used conditions modeled loosely after the work of Reit sma and Yeung (11): a 25-cm C18 column maintained at 30 °C, UV-absorbance detection at 254 nm, and a flow rate of 0.6 mL/min. We used a gradient from 0–40% mobile phase B in 75 min (0.53% B/min). Next we changed the flow rate to 2.0 mL/min and rinsed the column for 7 min with 100% acetoni trile (approximately five column volumes) to remove any remaining impurities.

In the reference separation, mobile phase A was 90% 10 mM ammonium acetate buffer adjusted to pH 6.8 with acetic acid and 10% acetoni trile. Mobile phase B was 100% acetoni trile. We reequilibrated the column by running mobile phase A through the system at 2.0 mL/min for 21 min. In the separation with 3% 1-propanol, mobile phase A contained 87.3% buffer, 9.7% acetoni trile, and 3.0% 1-propanol, and mobile phase B contained 97.0% acetoni trile and 3.0% 1-propanol. We reequilibrated the column by running mobile phase A through the system at 2.0 mL/min for 3 min.

Phenols: We modeled our separation of five phenols loosely after the work of Jandera, Chur racek, and Colin (12). We used a 15-cm C18 column maintained at 25 °C. The detection wavelength was 270 nm, the flow rate was 1.0 mL/min, and the gradient program was 0–100% mobile phase B in 60 min (1.67% B/min). Then we changed the flow rate to 2.0 mL/min and rinsed the column for 4 min with 100% methanol (approximately five column volumes) to remove any remaining impurities.

In the reference separation, mobile phase A was 100% water, and mobile phase B was 100% methanol. We reequilibrated the column by running mobile phase A through the system at 2.0 mL/min for 16 min. In the separation with 3% 1-propanol, mobile phase A contained 97% water and 3% 1-propanol, and mobile phase B contained 97% methanol and 3% 1-propanol. We achieved reequilibration by running mobile phase A through the system at 2.0 mL/min for 2 min.

PAHs: Using a 15-cm C18 column maintained at 25 °C, we modeled our separation of six PAHs on U.S. Environmental Protection Agency (EPA) Method 610 (13). The detection wavelength was 280 nm, the flow rate was 1.0 mL/min, and the gradient program was 40–100% mobile phase B in 60 min (1.0% B/min). We next changed the flow rate to 2.0 mL/min and rinsed the column for 4 min with 100% acetoni trile (approximately five column volumes) to remove any remaining impurities.

In the reference separation, mobile phase A was 100% water, and mobile phase B was 100% acetoni trile. The column was reequilibrated by running mobile phase A through the system at 2.0 mL/min for 8 min. In the separation with
3% 1-propanol, mobile phase A contained 97% water and 3% 1-propanol. Mobile phase B contained 97% acetonitrile and 3% 1-propanol. We achieved reequilibration by running mobile phase A through the system at 2.0 mL/min for 2 min.

RESULTS

Separation, reequilibration, and total analysis times: Separation time simply is the time required for all solutes to be eluted from the column and detected. Total analysis time, however, is the time required for separation and reequilibration. The number of column volumes of initial mobile phase used to reequilibrate the column in each original separation was intended to represent closely the amount that most operators would use, according to general guidelines. Traditional guidelines recommend 15–20 column volumes for gradient separations with 0–100% organic modifier (2,4). If the initial mobile-phase concentration contains at least 10% organic solvent modifier, the reequilibration time normally would be reduced slightly.

Using these modified guidelines, the number of column volumes used to reequilibrate the column was 10 for the original PAH separation, because the initial organic solvent modifier concentration was 40%. The number of column volumes used for the dansyl-l-amino acid analysis was 15, because the initial organic modifier concentration was 10%. We used 20 column volumes for reequilibrating the column in the phenol separation, because it was performed over the range of 0–100% organic modifier. The number of column volumes recommended for all separations containing 3% 1-propanol in both mobile phases was two (10). We measured volumes of 2.82 and 1.62 mL for the 25- and 15-cm columns, respectively.

As seen in Table I, all separations performed with the addition of 3% 1-propanol had a total analysis time that was considerably shorter than that of their original counterpart. As expected, we observed a greater decrease in total analysis time for separations that required a greater volume of initial mobile phase for reequilibration. In the analysis of the amino acids, the separation time was reduced by 4 min in the propanol separation because of a higher percentage of organic modifier present in the initial mobile phase. Reequilibration time, however, was decreased by 18 min with the addition of the propanol. Therefore, total analysis time in this separation was reduced from 92 min to 70 min, which is a decrease of 22 min or 24%.

In the separation of the phenols, the separation time was reduced by 3 min, and the reequilibration time was decreased by 14 min. The total analysis time went from 66 min to 49 min, yielding an overall time savings of 17 min or 26%.

The separation time of the PAHs diminished by 2 min, and reequilibration time decreased by 6 min with the addition of the propanol. This change reduced the total analysis time from 54 min to 46 min, which corresponds to an 8-min or 15% decrease.

Flow rate also is an important parameter in reequilibration-time studies. In all three of our separation types, we increased the flow rate used for reequilibration of the column from the flow rate used for analysis to 2.0 mL/min. Obviously, we increased the flow rate to decrease the reequilibration time substantially and, therefore, decrease total analysis time. This practice, however, can be impractical or impossible for repetitive separations that are automated and do not use flow programming. Small-particle-diameter columns also preclude the use of higher flow rates for reequilibration because of increased back pressure.

Table II illustrates the effect of flow programming on total analysis time. In the dansyl-l-amino acid separation, the percent decrease in total analysis time goes from 24% to 45% if the same flow rate is used for analysis and reequilibration. In the separation of five phenols, the percent decrease changes from 26% to 38%. Finally, in the case of the PAH separation, the percent decrease in total time increases from 15% to 23%. If the analysis flow rate is used for reequilibration of the column, the addition of 3% 1-propanol becomes even more important as a time-saving practice.

Resolution and elution order: Our analysis of the 17 dansyl-l-amino acids is a good example of a complex separation in which it is difficult to resolve all components. The chromatogram from the original separation (Figure 1a) shows that although 17 solutes were present, we obtained only 15 peaks. Despite our manipulation of the chromatographic parameters, two pairs of solutes were coeluted, namely N-dansyl-trans-4-hydroxy-L-proline and dansyl-L-asparagine and dansyl-L-norvaline and dansyl-L-methionine. When we added 3% 1-propanol to each mobile phase, all 17 amino acids were nearly baseline resolved (Figure 1b). Both pairs of solutes that previously were coeluted, were nearly baseline separated. In this case, the addition of the propanol enhanced, rather than degraded, the resolution of the analytes. The elution order of the solutes that were not coeluted in the original separation remained constant despite the addition of the propanol.

As we expected, the higher percentage of organic modifier in the mobile phase of the separation containing 3% 1-propanol caused a slight decrease in the retention times of all 17 analytes versus the original (Table III). The more hydrophilic analytes exhibited a more pronounced decrease in retention, and the more hydrophobic amino acids showed less change in retention time. In the separation of 17 amino acids, the overall effect of adding 3% 1-
propanol was better resolution, constant elution order, and decreased retention times.

The separation of the five phenols is an example of a fairly simple separation in which all five of the components should be resolved easily. As we predicted, all of the components — p-cyanophenol, p-chlorophenol, p-iodophenol, o-phenylphenol, and o-tert-butylphenol — were baseline resolved in the original separation (Figure 2a). The addition of 3% 1-propanol did not affect the resolution adversely (Figure 2b); all five phenols remained baseline separated. The elution order remained constant for this separation with and without the addition of the propanol. Retention time data showed a slight decrease in retention time for all solutes as expected (Table IV), with the more hydrophilic analytes showing a more pronounced effect. In this separation, the resolution is uncompromised, the elution order remained constant, and the retention times decreased.

In our analysis of the six PAHs only four of the six analytes were resolved in the original separation (Figure 3a). Naphthalene, phenanthrene, pyrene, and 1,2-benzanthracene were resolved, but acenaphthene and fluorene were coeluted. Adding 3% 1-propanol failed to resolve the two solutes that were coeluted, but the other four components remained baseline separated (Figure 3b). The failure to resolve these compounds probably was a function of column chemistry. (Sander and Wise [14,15] studied separations of PAH compounds extensively and found that polymeric column chemistry provides the highest shape selectivity for these rigid solutes.) As with the other separations, the elution order of resolved components remained constant. Once again, the retention times were somewhat longer in the original separation than in the separation using 3% 1-propanol (Table V). In this case, however, all of the analytes were very hydrophobic, so similar decreases in retention times were observed between analytes. In the separation of the six PAHs the resolution remained constant, the elution order was unchanged, and the retention time decreased.

**CONCLUSIONS**

Adding 3% 1-propanol to each mobile-phase component in gradient elution reversed-phase liquid chromatography greatly reduces the total analysis time without affecting resolution adversely. In the three representative separation types we chose, the retention and total analysis times were slightly shorter for the separations performed with the addition of 3% 1-propanol than in the original separations. The elution order of the analytes in the separations...
with and without the addition of 1-propanol remaining constant in all three separation types.

As can be seen from the three separation types chosen for study, total analysis times were reduced significantly with the addition of 3% 1-propanol to each mobile phase, and the parameters associated with the separations remain favorable.

The addition of 3% 1-propanol to each mobile-phase component in gradient elution, reversed-phase LC seems to be a viable solution to reducing column reequilibration time. These results also are encouraging because this method of reducing reequilibration time can be applied to many of the separations routinely performed in the chemical industry to save hours of analysis time and liters of solvent every day.

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