Regeneration of Tetrabutylammonium Ion-Pairing Reagent Distribution in a Gradient Elution of Reversed Phase Ion-Pair Chromatography

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Abstract

The regeneration of ion-pairing reagent distribution on liquid chromatography columns after gradient elution has been well recognized as the cause for long column equilibration time, a major drawback associated with gradient elution reverse phase ion-pair chromatography. To date, the majority of studies have focused on optimizing the separation conditions to shorten the equilibration time. There is limited understanding of the ion-pairing reagent distribution process between the mobile phase and stationary phase in the course of gradient elution, and subsequent column re-equilibration. The focus of this work is to gain a better understanding of this process. An ion-pair chromatographic system, equipped with a YMC ODS C_{18} column and a mobile phase containing tetrabutylammonium (TBA) hydroxide as the ion-pairing reagent, was used in the study. The TBA distribution profile was established by measuring its concentration in the eluent fractions collected during the gradient cycle using different column equilibration times with an ion chromatographic method. Furthermore, the analyte retention time was evaluated as the function of the column equilibration time and TBA concentration in the mobile phase. The column equilibration and its impact on the method robustness will also be discussed.

Introduction

Ion-pair chromatography has been widely used for the separation of ionic compounds which cannot be adequately retained or separated using conventional reverse phase high-performance liquid chromatography (RP–HPLC) techniques. The retention mechanism in ion-pair chromatography is based on the interactions formed between the ionic solutes and ion-pairing reagent adsorbed on stationary phase (1). The retention process, which is affected by both ion exchange with the counter ions and adsorption of the ionic solutes on the charged stationary phase, is primarily a function of the ion-pairing reagent concentration on the stationary phase (2–6). Numerous studies have evaluated parameters which affect ion-pair reagent adsorption behaviors (7–11). These studies have shown that the extent of the adsorption on the stationary phase is a function of the ion-pairing reagent concentration in the mobile phase, its hydrophobicity, and the concentration of the organic solvent present in the mobile phase. In addition, eluent pH and ionic strength were shown to play important roles in ion-pairing reagent adsorption as well (2,12)

While isocratic ion-pair HPLC has been widely utilized for retaining and separating ionic species, the use of gradient elution ion-pair chromatography has been limited. This has been largely due to the need for a long column equilibration time associated with the ion-pairing reagent re-distribution after gradient elution. Typically, 15–20 column volumes of the mobile phase are used to re-establish the ion-pair distribution in the stationary phase of a column (13). As a result, column regeneration sometimes takes a longer time than the analytical separation itself. Despite this limitation, the application of gradient ion-pair chromatography should not be overlooked since it may offer unique separations with robust resolution and selectivity for samples containing multiple ionic solutes (14–16). Studies have suggested that several approaches can be implemented to reduce the column regeneration time after a gradient elution (17,18). Based on these studies, shorter column regeneration times can be achieved by increasing the column flow rate during equilibration, using an ion-pairing reagent concentration gradient, or optimizing ion-pairing reagent concentration.

As opposed to a well-established ion-pair equilibrium in an isocratic ion-pair chromatography method, the distribution of an ion-pairing reagent on a column changes constantly during a gradient elution as a result of the changing organic content of the mobile phase. The goal of this study was to gain insight into the dynamic process of the ion-pairing reagent distribution between the stationary and mobile phases during an organic solvent gradient to further understand how the column is regenerated after a gradient elution, and what effect this has upon the ruggedness issues, such as the retention time shift associated with gradient elution ion-pair chromatography. Sodium ascorbate (SAB) and sodium formaldehydesulfoxylate (SFS), two commonly used antioxidants in the pharmaceutical industry, were selected as the analytes of interest in the reversed-phase chromatographic system, equipped with an YMC ODS C_{18} column and a mobile phase containing tetrabutylammonium (TBA) as

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the ion-pairing reagent. To gain a fundamental understanding of the TBA regeneration process on the column at different equilibration times, the ion-pairing reagent distribution profile was monitored under gradient elution and subsequent equilibration. An ion chromatographic method was used to measure the TBA concentration in the eluent fractions collected from the column eluent.

The research has not found any publications to date on a quantitative determination of the ion-pairing reagent distribution in gradient elution ion-pair chromatography. The results from this study provide a better understanding to some commonly observed issues, such as retention time shift, long equilibration time, etc. associated with gradient elution ion-pair chromatography. In addition, the methodology established in the study can be applied to other ion-pairing reagents and chromatographic conditions to investigate the interactions between ion-pairing reagents with mobile phases and stationary phases.

Experimental

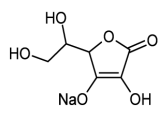
Materials

HPLC-grade water used was from a Milli-Q system (Millipore, Bedford, MA). HPLC-grade methanol was purchased from Burdick & Jackson (Muskegon, MI). Tetrabutylammonium hydroxide, 55% in water, was purchased as the ion-pairing reagent from SACHEM, Inc. (Austin, TX). Methylsulfonic acid (> 99.5%), SAB, and SFS (Figure 1), were purchased from Aldrich Chemicals (Milwaukee, WI).

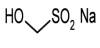
Gradient ion-pair chromatography conditions

A reversed-phase gradient ion-pair HPLC method was developed to analyze SAB and SFS. All separations were performed on a chromatographic system that consisted of a Waters Alliance 2690 separation module equipped with a 2486 dual lambda absorbance UV detector (Waters, Milford, MA). A LAC–E32 acquisition server (Waters) was connected to the detector. A Millennium32 chromatography data management system from Waters was employed for instrument control, data acquisition, and processing.

An YMC ODS-A column (250 mm × 4.6 mm, 5 um particle size, from Waters) was used for all separations. The column temperature was maintained at 25°C. After an initial hold of 20% eluent B for 3.5 min, a linear gradient was employed by ramping to 100% eluent B in 14.5 min. The system was then returned to the initial conditions in 2 min. The flow rate was held constant at 1 mL/min for both the gradient elution and the column equilibration.



Sodium Ascorbate (SAB)



Sodium Formaldehydesulfoxylate (SFS)

Figure 1. Analytes of interest. The pKa values for SAB and SFS are 4.17 and 1.65.

The effect of the TBA concentration on the analyte retention time was evaluated in the range from 0.05 mM to 60 mM of the TBA concentration. The concentration of 5 mM was chosen to evaluate the effect of the column equilibration time from 2 to 60 min. The equilibration time in this paper is defined as the time the column is held at the initial gradient condition after the previous gradient elution until the next sample injection. The equilibration time is synonymous with the column regeneration time.

For the 5 mM TBA concentration in the mobile phase, the eluent A was prepared by transferring 5.0 mL of 55% TBAOH aqueous into 2 L of water. Eluent B was prepared by adding 5.0 mL of 55% TBAOH aqueous into 2 L of a premixed solution of water–methanol (4:6). The final concentration of TBA in both eluents was 5.0 mM, and the pH of both solutions was adjusted to 6.4 with phosphoric acid. A 0.1 mg/mL solution of SAB and SFS was prepared by dissolving 10 mg of each compound in 100 mL diluent (water–methanol 4:1). The injection volume was 10 µL. The UV signal was monitored at a wavelength of 230 nm.

Fraction collection of eluent

A Spectra/Chrom CF^{-1} Fraction Collector (Spectrum Chromatography in Houston, TX) was employed to collect the eluent at different time points during the gradient elution and column regeneration. The fraction collector was connected to the outlet of the UV detector. Fractions were collected based on the elution time.

Before collection, the system was equilibrated by running the initial mobile phase conditions overnight at a low flow rate (0.1 mL/min), which corresponded to ~30 column volumes. The flow rate was increased to 1 mL/min for 1 h prior to the injection. No analytes were injected in the fraction collection. Fractions of the column eluent were collected during the 20 min gradient elution and the subsequent column re-equilibration. To study the effect of equilibration time, fractions were collected using equilibration times of 0, 10, 20, and 100 min, respectively. For the 100 min equilibration time, a 2 mL eluent fraction was collected for every two min in the first 60 min of the gradient run and then every 10 min afterwards at a 1 mL collection size. An eluent fraction was collected every min for all other runtimes. For every equilibration time, duplicate experiments were conducted, and the average value of the two measurements is reported.

Ion chromatography conditions for the analysis of TBAOH

A Dionex ICS-3000 IC system with conductivity detection (Sunnyvale, CA) was used for the analysis of the TBA ion in the elution fractions. The IC system was controlled by Chromeleon software (Dionex, Sunnyvale, CA), and the conductivity signal was collected using Empower 2 software through a SAT–IN module (an analog to digital converter from Waters) that was connected to a LAC–E32 acquisition server from Waters. An isocratic elution was used and the flow rate was at 1.5 mL/min. The mobile phase was water–acetonitrile (55:45), containing 5 mM methylsulfonic acid. The analytical column, Dionex IonPac CS17 (250 mm × 4 mm), was used and the column temperature was maintained at 35°C. The injection volume was 20 µL. The IC method was calibrated with TBAOH over the concentration range of 0.1 to 25 mM. A linear regression was established with

an R^2 value of 1.0 and the RSD of 8 replicate injections was 1.1% at the concentration of 1.0 mM.

Results and Discussion

Effect of equilibration time on retention time

Representative separations of SAB and SFS were obtained using a 5 mM ion-pairing reagent concentration at different equilibration times, and are shown in Figure 2. Without the addition of TBAOH, no separation was achieved between SAB and SFS as both eluted in the void volume (~2.5 mL, data not shown). The effect of varying the column equilibration time on the retention time is demonstrated in Figure 3. As the equilibration time increased from 2 to 60 min, the retention time of the SAB peak initially increased, and then the retention times of both components decreased drastically. This decrease was more pronounced for the SFS peak. Since the effect of the equilibration time on the separation is compound specific, the resolution of the separation changes as the equilibration time varies. As shown in Figures 2 and 3, the resolution of the two analytes decreases when the column equilibration time increases, until the two components eventually coeluted at 40 min equilibration time. Further increases in the equilibration time leads to the reversal of elution order. It appears that the optimal resolution and peak shape occurred at 10 min equilibration time at 5 mM TBA concentration. The data in Figure 3 also suggests that the effect of the equilibration time on the retention time becomes insignificant after 40 min, which indicated that for this ion-pairing system a 40 min equilibration was the critical time needed to reestablish the equilibrium of the ion-pair reagent in the column.

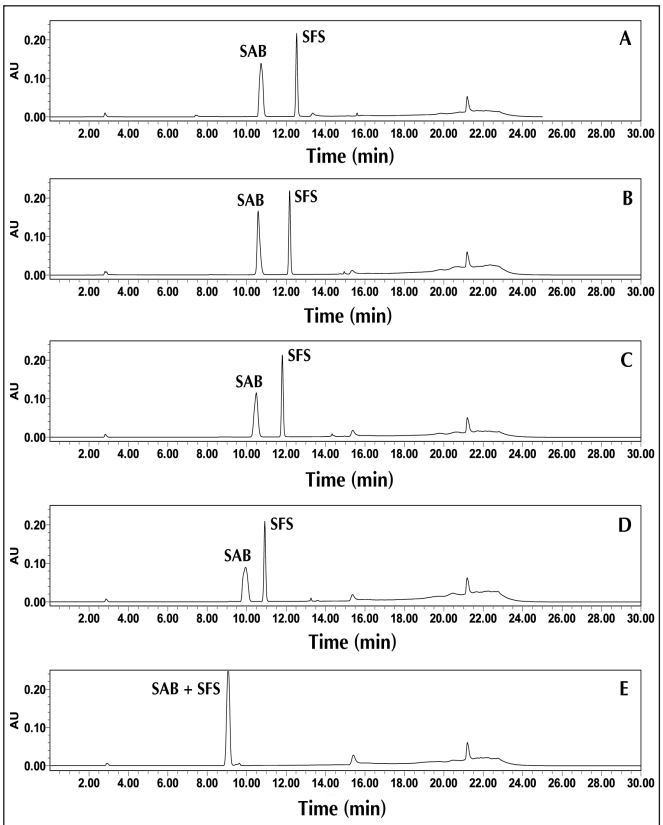


Figure 2. Gradient elution ion-pair chromatograms with different equilibration times using 5 mM TBAOH in the mobile phase. The peaks that are not labeled are the system peaks. The trace (A) represents equilibration time of 5 min, (B) 10 min, (C) 15 min, (D) 25 min, (E) 40 min. The two analytes coeluted at an equilibration time of 40 min.

Effect of the TBA concentration on retention

The effect of the TBA concentration on the analyte retention time was studied over the concentration range of 0.05 to 60 mM using a constant equilibration time of 10 min. The results of this study are shown in Figure 4. In Figure 4A, a slight increase in the retention times of the analytes was observed as the concentration of TBA increased over the range of 0.05 to 0.5 mM, reaching a maximum around 0.5 mM. As the TBA concentration was further increased, retention times began to decrease, similar to the effect that was observed when the equilibration time was increased. Loss of selectivity occurred at a concentration of ~30 mM

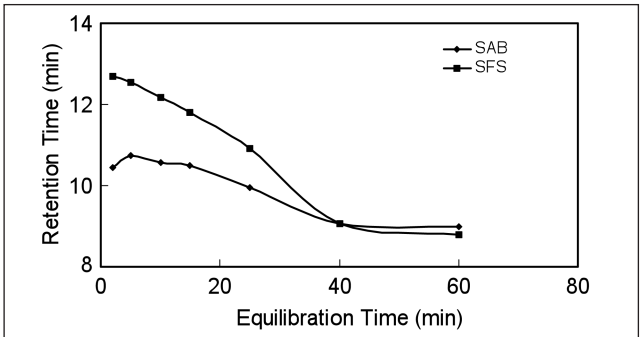


Figure 3. Effect of the equilibration time on the retention time of the analytes at 5 mM TBA concentration.

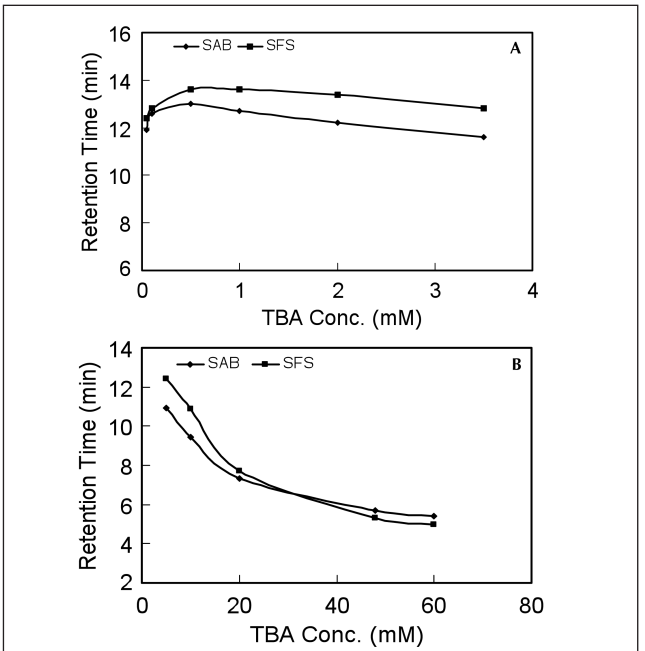


Figure 4. Effect of the TBA concentration in the mobile phase on the retention time at the equilibration time of 10 min. (A) TBA concentration is from 0.05 to 3.5 mM, (B) TBA concentration is from 5 to 60 mM.

(Figure 4B), and further increases in the ion-pairing reagent concentration resulted in an elution order reversal similar to what had been observed with increasing equilibration time.

These observations are consistent with the literature where retention time reduction was reported as increasing with the ion-pairing reagent concentration (3–5,10,11). Several possible mechanisms, including micelle formation of the ion-pairing reagent, desolvation, and counter ions from the ion-pairing reagent, have been proposed in these papers. A more comprehensive discussion and review of the effect of the counter ions, pH, and other variables can also be found elsewhere (19). The change in the retention time as a result of the increasing ion pairing reagent concentration observed in this work appears related to the counter ion effect in the mobile phase. As suggested by previous studies, although more counter ion enhanced adsorption of the ion pairing reagent on the stationary phase, it hindered the ion exchange to form ion pairs with the solute ions, which resulted in a reduction of the retention time (10,11). Figure 5 shows the retention times of the analytes as a function of the TBA concentration when a constant equilibration time of 40 min was utilized. The retention profile was slightly different from the profile observed when an equilibrium time of 10 min was used as shown in Figure 4B. The overall retention time profile of the two analytes over the entire ion-pairing reagent concentration range investigated shifted downwards with the increase of the equilibration time. The equilibration time had less impact on the retention time at TBA concentrations greater than 20 mM. At 48 mM, the retention times for the two analytes at 10 min and 40 min equilibration times were essentially the same (e.g., 5.71 and 5.69 min for SAB, and 5.29 and 5.27 min for SFS). This result suggests that shorter equilibration times are required to achieve column equilibrium when higher ion-pairing reagent concentrations are used. Of interest to note, the elution order at the 40 min equilibration time did not invert as it had been observed using a 10 min equilibration time. The selectivity between the two compounds remained essentially the same over the entire ion-pairing reagent concentration range studied for the 40 min equilibration time.

Selectivity of ion-pair separations is a function of the specific interactions between the solute ions and the ion-pairing ions in the stationary phase, which can be affected by the ionic strength and the pH of the eluent that can affect the charge state and charge densities of the solutes and the stationary phase. Studies have shown that certain mobile phase conditions can trigger the transition from one retention mechanism to another, or to mixed retention mechanisms during elution in ion-pair chromatography (23). The changes in selectivity observed in this work were likely caused by competing retention mechanisms.

Elution order reversal was also reported in Deelder's work on retention of amines using dodecane-1-sulphonate as an ion-pairing reagent (6). A more specific study to understand the interactions between the analytes and the pairing ions during the gradient elution would be required to interpret the elution order inversion observed. This was not included within the scope of the current study.

TBA distribution profile

Previous studies showed that in general, increasing organic modifiers in an eluent reduces the ion-pairing reagent adsorption on the stationary phase (8–10). However, no study has been reported to describe the effect of a continuous change in the organic composition of the eluent on the ion-pairing reagent distribution between the mobile and stationary phases during the gradient and column equilibration steps. In this study, fractions were collected over the entire HPLC run time including the equilibration time and were analyzed using an IC method to determine the TBA concentration in the eluent. The concentration of the ion-pairing reagent in the eluent was used as an indication of its distribution in the stationary phase.

Shown in Figure 6, the bar plot demonstrates the ion-pairing reagent concentration observed in each column eluent fraction collection for the 10 min equilibration time. A continuous smooth line was used to present the results obtained at different equilibration times as shown in Figure 7. The TBA concentrations at the three different regions of significance in the observed TBA gradient profile (labeled initial, peak, and valley), along with the actual TBA concentration in the mobile phases, are listed in the attached table. The results indicate that the concentration profile of TBA in the eluent as it exited the column varied significantly over the course of the chromatographic run, even though the TBA concentration in the mobile phase when it entered the column was held constant. The initial inspection of the four curves indicated three main differences. First, the value of TBA in the initial eluent fraction for the four equilibration times varies drastically, with nearly a 30-fold difference observed between the 10 and 100 min equilibration time. Second, the

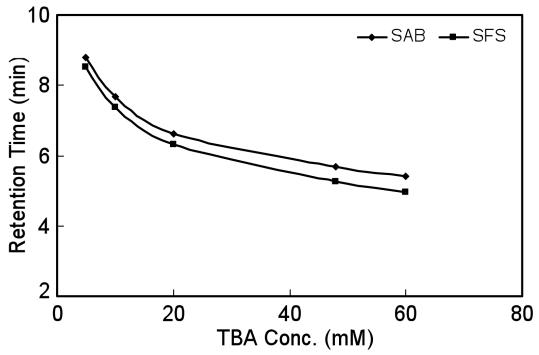


Figure 5. Effect of the TBA concentration on the retention time at an equilibration time of 40 min.

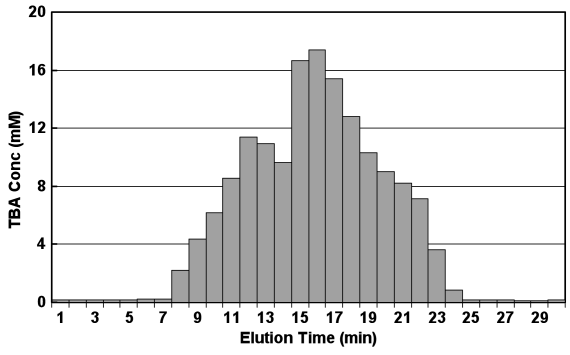
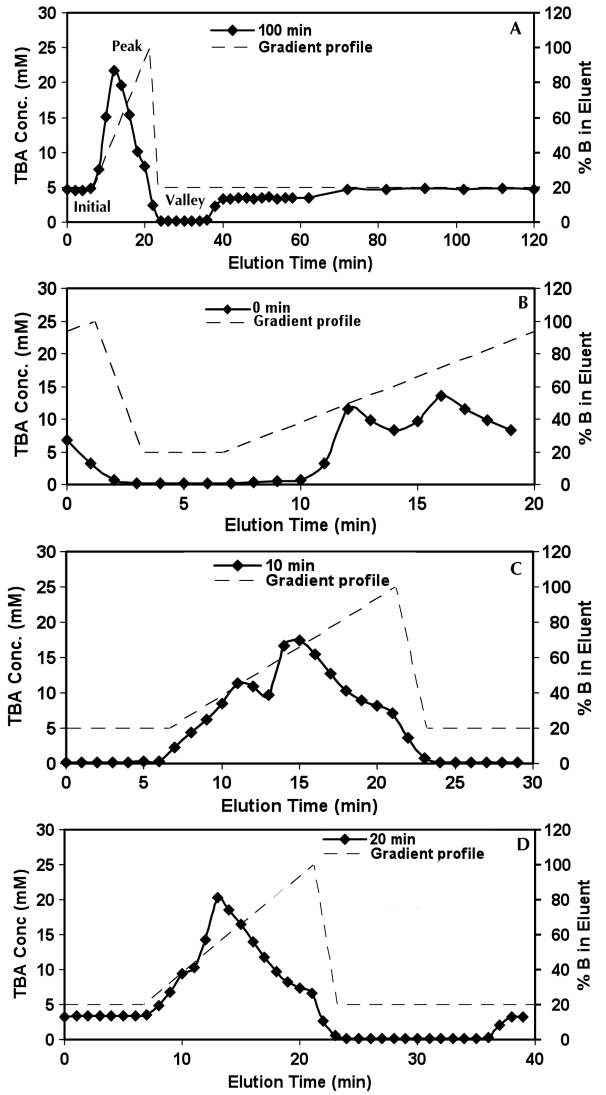


Figure 6. Bar graph of the TBA concentration obtained from each 1 mL fraction collected for a system using a 10 min equilibration time.

0 and 10 min equilibration time traces exhibited clearly resolved double maximum responses. Once the equilibration time increased to 20 min these two maxima essentially merged as the traces exhibited a single maximum at 12 to 13 min. In all cases except the 0 min equilibration time, the TBA concentration showed a minimum valley between 23 to 40 min after which the concentration increased and reached a plateau at around 40 min.



	TBA Conc. (mM)			
Equilibration Time (min)	0	10	20	100
Initial	6.74	0.14	3.26	4.61
Valley	N.A.	~ 0.12	to	~ 0.16
Peak	11.6 & 13.5	11.4 & 17.4	20.3	21.7
Mobile Phase*	4.92			

*Average of A & B

Figure 7. TBA concentration profile in the eluent fractions collected from the gradient elution at 0, 10, 20, and 100 min equilibration times. The TBA concentration in the mobile phases is 5 mM. (A) 100 min equilibration time, (B) 0 min, (C) 10 min, (D) 20 min. The TBA concentrations in the eluent fractions of interest are listed in the attached table.

Superimposed on the ion pairing concentration profiles in Figure 7 is the percent B mobile phase in the eluent. The HPLC system used in this study had a gradient delay volume of ~0.7 mL, and a column void volume ~2.5 mL. The total gradient delay observed as the eluent exited the column is ~3.2 min. The gradient profile was plotted as the function of time that the eluent exitd the column, which included the 3.2 min delay time to reflect the true percent B in the mobile phase at the moment the fraction was collected. In all other equilibration times evaluated, except 0 min, the fractions collected in the first 6.7 min consisted of a 20% mobile phase B. During this period, the TBA concentration detected in the fractions collected for the 10, 20, and 100 min equilibration times all remained constant, yet different from each other despite the fact that the TBA concentration in the mobile phase was held the same in all cases. As the mobile phase B increased linearly from 20 to 100%, the response of the TBA in the eluent initially lagged behind the gradient due to the system gradient delay volume (~3.2 mL), and then rapidly increased and reached a maximum value (peak) prior to the completion of the gradient profile at 18 min. The increase in the TBA concentration in the eluent was consistent with its increased solubility in the mobile phase, which effectively "washed" the TBA off of the column. The elution time at which the response reached a maximum varies, from 12 to 16 min depending on the equilibration time. After reaching a maximum, the TBA response measured in the fraction decreased, although it remained significantly higher than the concentration in the mobile phase, until ~22 min, as shown in Figures 7A, 7C, and 7D, after which time the concentration of TBA plunged below the level of the mobile phase. This indicates the "wash-off" process was completed at ~22 min, which is coincident with the sum of the gradient time (18 min) and the system delay time (3.2 min). The system begins to re-adsorb TBA in the stationary phase when the mobile phase composition returned to the initial gradient conditions, resulting in a decrease in the observed TBA concentration. A shoulder appears between 19 to 22 min, corresponding to the sharp gradient switch from 100% B back to the initial conditions. As the gradient is held at the initial gradient condition and allowed to re-equilibrate, the TBA concentration in the eluent eventually begins to increase again at around 35 min, and reached a plateau after ~40 min (shown in Figure 7A). The higher response of TBA in the initial fraction obtained for the run with 0 min equilibration time was attributed to the carry-over of TBA in the eluent that was washed-off prior to the injection. Since the system was not allowed to equilibrate prior to the injection, the TBA concentration reached a minimum ~3 min after the injection.

Interestingly, the first plateau in the TBA response at ~40 min (20 min, or ~8 column volume equilibration) for the 100 min equilibration time was approximately 27% lower than the baseline response at time zero. A return to the baseline response was not achieved until ~70 min, ~20 column volumes in total. This suggests that the complete equilibration of the column involved sites of at least two levels of access or energy. Once the sites with easier access are filled (~40 min), the ion-pairing reagent begins to occupy the sites of the next accessible level in the column. The two-adsorption levels can be explained using the two main mechanisms that govern the adsorption behavior of the basic compounds on the C_{18} stationary phase, hydrophobic interaction

with the alkyl chains and ion-exchange with the acidic silanols (21,22,24). The adsorption initially occurs on easily accessible C_{18} chains then eventually penetrates to the sub-layer of the silanol groups. Note that the mobile phase, pH of 6.4, used in this study favors the ionization of the residual silanol groups in the column. Although the column used has a highly end-capped surface structure, in general there are still a significant number of uncapped silanol groups remaining on the surface. Based on a simple "area under the curve" comparison (the difference in the response multiplied by the elution time) of the two equilibration areas: the initial hydrophobic interaction (from 22 to 38 min) appeared to outnumber the electrostatic adsorption (38 to 70 min) by approximately 3 to 2, which suggests that about 40% of the overall adsorption is due to the silanol functional group. This estimate does not represent the actual ratio of end-capped and uncapped silanols of the stationary phase, because the amount of the adsorption in the column prior to the elution is unknown. Previous studies suggested that the ion-pairing reagent adsorbed on stationary phases bonded with free silanol groups does not act an ion-exchange support like those that are hydrophobically adsorbed (19). Therefore, the analyte retention is simply not proportional to the total amount of the ion-pairing reagent absorbed on a stationary phase.

Another distinction observed in the TBA concentration profile shown in Figure 8 is that two peaks are obtained in the TBA profile with 0 and 10 min equilibration times as opposed to only one peak when longer equilibration times are used (e.g., 20 and 100 min). The two-peak phenomenon is likely related to the incomplete equilibration in the column as it is unique to the shorter equilibration times. The first peak is derived from the ion-pairing reagent adsorption on the stationary phase prior to the start of the gradient elution. When the column is not fully equilibrated, the distribution of the ion-pairing reagent across the column is not uniform. The farther from the column inlet, the less TBA is adsorbed in the stationary phase. During the gradient elution, although desorption was dominant, the re-adsorption of TBA in the end of the column cannot be overlooked due to the fact that the column was not fully equilibrated with TBA in short equilibration time. The TBA profiles shown in Figure 7A–7D are therefore the net result of the desorption and re-adsorption process going on throughout the column. The second peak is likely associated with the wash-off of the re-adsorbed TBA as the result of the further increase in organic solvent in the eluent. The experiment was repeated several times on two different columns, and the same TBA profile was obtained.

Column Equilibration

The TBA concentration profiles shown in Figure 7 illustrate the effect of varying the equilibration time on the retention time when the concentration of the ion-pair reagent is kept constant. Not surprisingly, this same effect was also observed when the equilibration time was kept constant while changing the ion-pairing reagent concentration as the retention time is correlated to the amount of ion-pairing reagent in the stationary phase. Since the ion-pairing reagent distributed across the stationary phase is based on the fixed number of available interaction sites within the column, a constant number of moles of ion-pairing reagent can be added to the column between injections by either increasing equilibration time, flow rate or ion-pairing reagent concentration during equilibration to generate similar retention and selectivity. This effect was observed in Figure 3, when the TBA concentration was held constant and the equilibration time was varied; and in Figure 4 when the equilibration time was held constant and the TBA concentration was varied.

Complete column regeneration creates a static ion-pairing reagent distribution between the mobile phase and stationary phase that will not change with further equilibration. It is a lengthy process and is a major limitation for ion-pair methods using gradient elution. In this study, the equilibration time needed to regenerate the column was varied from ~40 min (~16 column volumes) to 10 min (~4 column volumes) for the TBA concentrations of 5 mM and 48 mM, respectively. It was also found, however, that the retention and response of the two analytes were very reproducible as long as the equilibration time was held constant. The optimal separation for the analytes of interest was achieved when using a dynamic ion-pairing reagent distribution achieved with a 10 min equilibration time at 5 mM TBA. The method was successfully validated for linearity, precision, and accuracy using a 10 min equilibration time. Shown in Table I, the injection precision achieved at the analyte concentration of 0.1 mg/mL and linearity from 70% to 130% of this concentration using the gradient elution with an equilibration time of 10 min. Furthermore, this dynamic equilibrium system provided superior selectivity over the static equilibrium system created using complete column regeneration (~40 min equilibration times). It is evident that an optimal separation condition in gradient elution ion pair chromatography involves variables such as analytes, ion-pairing reagent concentrations, equilibration times, and stationary and mobile phase choices.

Conclusion

This work established the TBA concentration profiles in an ODS C_{18} stationary phase system during a gradient elution ion-pair separation. The results demonstrated that the ion-pairing reagent distribution in the stationary phase undergoes drastic changes during a gradient elution. The shift in the analyte retention times at different equilibration times is attributed to the significant difference in the stationary phase distribution profile of TBA as the equilibration time is varied. The study also demonstrated that a full equilibrium of the ion-pairing reagent on the column may not be necessary in developing a robust and selec-

Table I. Linearity and Injection Repeatability of the Gradient Elution Ion-Pair Chromatographic Separation at 5 mM TBA with 10 min Equilibration Time

		Injection repeatability (n = 10)	
	Linearity R^2	Retention time (% RSD)	Peak Area (% RSD)
SAB	0.9996	0.8	0.6
SFS	1.0000	0.2	1.0

tive gradient elution ion-pair chromatographic method. As long as the equilibration time is held constant between injections, to allow the system to maintain dynamic equilibrium of the ion-pairing reagent distribution, a robust method can be achieved.

The study has revealed the complexity of the ion-pairing reagent distribution process during a gradient elution. As suggested by the results, a very different column regeneration process and ion pairing reagent distribution profile can result when choosing different ion pairing reagents, using different ion-pairing reagent concentrations, and a different mobile phase and stationary phase. Although only the equilibration time and the ion-pairing reagent concentration were considered in the current study, the approach established in this work is applicable to other ion pairing separation systems.

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