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Investigation of retention on bare silica using reversed-phase mobile phases at elevated temperatures

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Abstract

The use of unbonded silica as a stationary phase in reversed-phase HPLC is described as a useful alternative to bonded phase columns for polar, lipophilic amines. Using four lipophilic amines, the role of temperature is shown to favorably impact both efficiency and selectivity, which is not universally seen when using bonded phases. As temperature is raised, retention drops on the silica column. The temperature behavior appears to support the hypothesis that retention is dependant upon electrostatic and adsorptive forces.

Keywords: Temperature effects; Van't Hoff plot; Silica; Retention behavior

1. Introduction

In reversed-phase HPLC, it is generally believed that the excessive retention and tailing of lipophilic amines on bonded stationary phases is due to the interaction of the analytes with the underlying, unbonded silanols on the packing. Over the years much effort has gone into preparing bonded phases where residual silanols are minimized and/or eliminated. Reports continually appear which investigate the tailing of existing bonded phases and the newer, so-called "base-deactivated packings" [1]. Unfortunately, several issues cloud the universal understanding and interpretation of the testing. First, there is no standard, agreed-upon accepted test, second each report investigates a limited number of packings and third, chromatographic effects are compound dependant. Additionally, a column superior in one application may appear inferior in another application. This all contributes to a situation where a true comparison of the properties of all reversed-phase columns is virtually impossible [2].

So far, the total elimination of the silanol effect in bonded phase columns has been unsuccessful. This may reflect the observation suggested in an early work that sterically it is impossible to completely bond 100% of all available silanols [3]. In other words, if complete coverage is obtained with a primary reaction, a secondary endcapping reaction does not reduce the concentration of surface silanols due mostly to the steric inability to react all of the surface hydroxyls [3]. Thus, in many instances, investigators have added amine additives to the mobile phase to "block" analyte interaction with the underlying silanols. Nevertheless, work will continue on attempting to prepare stationary phases which will eliminate the silica contributions to reversed-phase HPLC and, hence, eliminate the need for mobile phase additives.

One report demonstrated that contrary to popular belief, it is not just the presence of the silanols that is important in influencing retentive behavior but the accessibility of the silanols [4]. In that same report, it was also shown that instead of vigorously attempting to eliminate the silanols, it is possible to exploit the silanols in a positive manner and use bare, unbonded silica as a stationary phase with aqueous/organic mobile phases [4]. In this mode, retention of some basic, organic amines under reversed-phase conditions depends upon electrostatic and adsorptive (mild hydrophobic) contributions. Many separations of lipophilic amines that were shown to be difficult on bonded phase packings are easily accomplished with good peak shapes on bare silica [4,5].

The surface of a hydrated silica surface has been shown to contain three general types of silanol groups [6–9]. These

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different types of silanols can be identified and estimated by magic-angle-spinning (MAS) NMR [8] and diffuse infrared spectroscopy with Fourier Transform (DRIFT) [9]. Of the three types of surface silanols, the free, isolated are believed to be more acidic and cause broad tailing peaks for basic samples. These free silanols are believed to exist in low concentrations. Geminal and vicinal (or associated) silanols are believed to make up the highest concentration of surface silanols. Geminal silanols are believed to be less acidic than isolated silanols and can be considered more "friendly" to bases. The vicinal (or associated) are believed to be in the highest concentration and are the most desirable for retaining basic solutes. Perhaps, this is because the hydroxyl groups are hydrogen-bonded to the nearest neighbor are the least acidic of the three types. While much has been written about the role of these three types of silanols in reversed-phase separations on bonded phase columns, little has been written about their role on bare silica stationary phases [2]. Perhaps, this is due to the situation that, in the unbonded state, the relative role and population of each silanol type is less significant to attaining reproducible chromatography when using bare unbonded silica in the reversed-phase mode.

Over the years following that initial report of using typical reversed-phase mobile phases with bare silica stationary phases, many successful separations using this technique have appeared [5,10–12]. Also, recently it has been shown that using a silica stationary phase with aqueous/organic mobile phases significantly enhances LC–MS–MS method sensitivity [13,14] As a result, the use of underivatized silica columns with aqueous/organic mobile phases is becoming an attractive alternative to bonded-phase columns for MS detection [15].

Most of the investigations and reports on the use of bare silica as a stationary phase with aqueous/organic mobile phase have been carried out at room temperature and found to be quite adequate for the separations. And, while room temperature is suitable for some applications, for many, temperature can be a significant variable that assists in improving the reproducibility of analysis and in attaining high-throughput separations. Thus, temperature should be considered an important variable in the modern HPLC method-development process. Therefore, since the increasing interest in using silica as a stationary phase in modern separations, we decided to investigate the role of temperature.

Of course, as has been learned when using bonded phases in the reversed-phase mode, if the analyte or stationary phase degrades at elevated temperatures, lower system backpressure and other benefits of higher temperature are not achievable. Obviously, the critical item, if elevated temperature is to be used, is that both the analyte and stationary phase must be stable. However, many compounds encountered in HPLC can be exposed to elevated temperature during separation because the compound is only at the higher temperature for the short period of time when it is on the column. However, if a compound is thermally labile, the column temperature should be controlled at a lower temperature value where minimal or no degradation occurs. Sometimes conformational changes of solutes can occur. In this case a temperature should be chosen where a single confirmation is favored. An example of this is from the biological area, where it was reported for temperatures up to 85 °C, changes in the secondary structures of biomolecules could be monitored [16].

The stability for bonded phase columns is quite different. Many of the typically prepared bonded-phase columns have limited lifetimes at elevated temperatures [17]. Those bonded phases prepared by bonding a dimethylalkylsilane to the silica particle through a surface hydroxyl group tend to not be stable and are degraded at high temperatures and especially at pH values of 3 and below [18]. The degradation mechanism is believed proceed through acid hydrolysis of the silane bond of the attached bonded phase and has led to the development of specially bonded stationary phases like the sterically protected side groups on the bonded silane [18].

With silica as the stationary phase, there is no stationary phase to hydrolyze. However, silica may slowly dissolve during operation under some mobile phase conditions. If any degradation of the silica occurs there is, in essence, no loss of the stationary phase—when a layer of silica is lost, silica remains. Retention remains constant as only the silica surface remains. Eventually, however, the silica may dissolve enough for the column to no longer be of use. When this occurs it should be a quick loss in performance not a slow change or gradual loss in retention.

Chromatographers have long known that carefully controlling temperature can be a valuable means of controlling the reproducibility of a separation. Although as a rule of thumb, a 1 °C increase will result in a 1–2% reduction in retention time, creation of a van't Hoff plot will allow a more precise understanding of the temperature dependence of each analyte in a mixture. The influence of temperature on retention is a function of free energy changes when the analyte interacts with the stationary phase, as defined by the equation:

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \Phi$$

where k' is the retention factor of the compound, ΔH is the enthalpy change for the retention interaction, R is the molar gas constant, ΔS is the entropy change for the retention interaction and Φ is the phase ratio of the column. The term Φ is constant for a given column and mobile phase. The plot of k' versus $1/T$ is referred to as a van't Hoff plot and is commonly used in the assessment of enthalpies and entropies of transfer from mobile phase to stationary phase.

In ion-exchange HPLC, enthalpy changes are generally large and dominate the retention interactions. Enthalpy changes can be either exothermic or endothermic, and in ion-exchange, generally cause significant changes in retention. Thus, separations run at higher temperatures in ion-exchange are quite commonly found in the literature reports. In reversed-phase HPLC, retention interactions are usually exothermic with relatively small changes in enthalpy. This

usually manifests itself in only modest decreases in retention time as temperature increases in contrast to what happens in ion-exchange chromatography. Of course, what is a modest change to one person may be a dramatic change to another. It has been pointed out that the retention enthalpy can be temperature dependent for analytes that have secondary equilibria and/or are retained due to mixed mechanisms [19–22]. Nevertheless, the best way of evaluating the temperature effect is to prepare a van't Hoff plot, a plot of temperature in reciprocal degrees Kelvin versus the natural log of the retention factor (k').

This plot can be easily prepared on many modern HPLCs systems and is also quite helpful when developing a method and when determining its robustness [23]. Perhaps, because there has been a lack of interest in temperature as a variable for improving chromatographic separations, only recently have studies of temperature and retention have been appearing using reversed-phase systems. Even less is known about the role of temperature on the quality of separations using bare silica in the reversed-phase mode. Therefore, an additional purpose of this paper is to examine the thermodynamic contributions to retention using bare, unbonded silica as the stationary phase by preparing van't Hoff plots for representative organic amine test probes.

2. Experimental

2.1. Instrumentation and reagents

All experiments were performed on an Agilent 1100 liquid chromatograph (Wilmington, DE) equipped with quaternary pump, autoinjector, a heated column compartment and a diode array detector. Flow rate was set at 2.0 ml/min and the detector was set to 254 nm. Injections of 1 μl were done for each sample. Version 9.03 Chemstation (Agilent Technologies Inc.) was used. Macros that reside in the Chemstation were used to calculate plate counts and tailing factors. The plate count was determined using the full-width-at-half-height method and the tailing factor used the USP procedure measured at 5% peak height. In this work, thermocouples were attached to both ends of the column in order to assist in assuring that the column had come to equilibrium at each temperature before analysis were begun. Injections were only made after the column had come to temperature equilibrium as demonstrated by temperature measurement at the head and the exit of each column being within 0.1 °C. The equilibrium column temperature was the average temperature of the two values from the thermocouples.

Columns used in these experiments were Zorbax Rx-Sil, 4.6 mm × 150 mm column packed with 3.5 μm or 5 μm particles, as noted in the text and figure captions, for high efficiency (Agilent Technologies Inc.). Columns were used as shipped from the manufacturer in hexane and flushed with tetrahydrofuran to remove the shipping solvent. The reversed-phase mobile phases were then introduced and equilibrated to a stable detector baseline and to a reproducible retention time of the specific analytes. If the columns were stored for an extended time, the columns were stored in acetonitrile/water (50/50, v/v). The void volume was determined using sodium nitrate which agreed with a uracil peak and a baseline deflection of an injection of the analytes. The organic solvents used were HPLC grade (Burdick and Jackson, Phillipsburg, NJ). All chemicals and buffers were from Sigma–Aldrich (Milwaukee, WI). The aqueous potassium phosphate buffer was prepared using HPLC grade water from a Milli-Q system (Millipore, Bedford, MA), pH adjusted and then diluted with an appropriate amount of organic solvent. All test samples were prepared by taking 1 mg/ml solutions of each individual component (Sigma–Aldrich) in the mobile phase buffer and mixing 0.5 ml of each solution to make the final test sample. When an over the counter formulation was used, it was purchased from a local store and the liquid was diluted 1:1 in mobile phase. For the lifetime study, test the column at 50 °C in methanol/water (70/30) containing 20 mM sodium citrate at pH 6. The test compounds were injected periodically while continuing to run solvent at 1 ml/min.

3. Results and discussion

Typical examples of separations achievable using bare silica in the reversed-phase mode are shown in Fig. 1 where several common, over-the-counter cough suppressant formulations were analyzed for their content. The relative retention of the analytes is considerably less than would be obtained when using a bonded phase column and the peak shapes are much improved over what would be attained using a typical bonded phase column. This type of chromatogram is typical of what was earlier reported for less efficient packings [4,5]. The two later eluting compounds, pseudoephedrine and dextromethorphan, were chosen to be representative of model compounds for the rest of this study. In addition to these two compounds, two additional compounds, amitriptyline

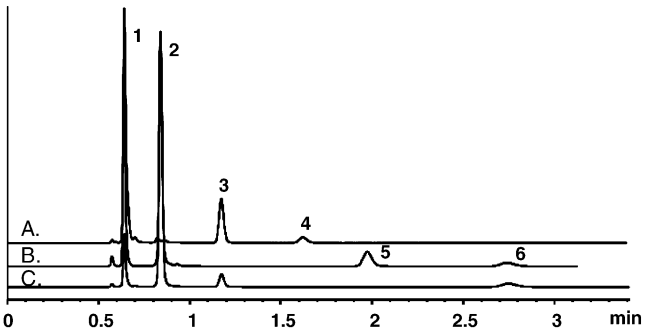

Fig. 1. Separation of three cough and cold formulations. Column: Zorbax Rx-Sil; mobile phase: methanol:water (69:31) containing 8 mM dipotassium phosphate at pH 7; flow: 2 ml/min, detection at 217 nm and temperature: ambient. Samples were three over-the-counter cough and cold medications. Components are (1) saccharin, (2) guaifensin, (3) phenylpropanololamine, (4) bropheniramine, (5) pseudoephedrine and (6) dextromethorphan.

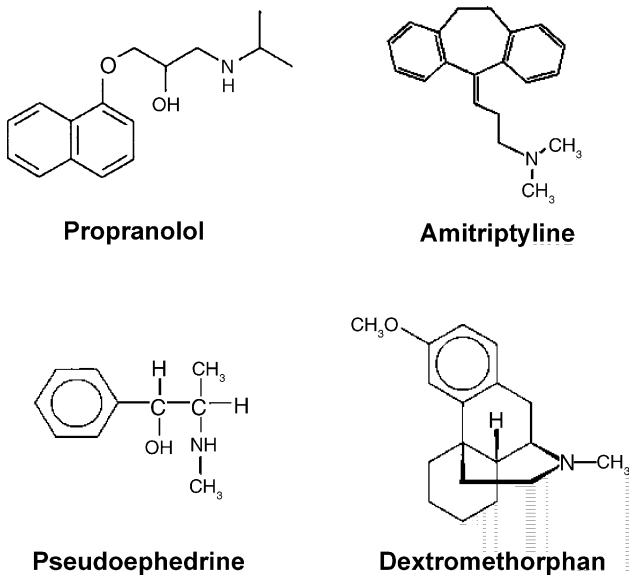

Fig. 2. Structures of compounds used in this study.

and propranolol, were used because these have been often used to test tailing on bonded phases [23]. All four of these compounds, whose structures are shown in Fig. 2, exhibited the typical reversed-phase behavior with respect to solvent strength as demonstrated by a linear relationship of $\ln k'$ versus percentage organic with earlier elution occurring at higher organic content.

When elevated temperature is discussed in chromatographic circles mention is usually made to the resulting reduction in system backpressure with it various operational benefits of lower backpressure. Lower pressure causes less stress on the hardware (valves, seals, etc.) and also enables the use of higher flow rates, if desired. This becomes important when using some of the modern columns packed with smaller sized stationary phase particles that have higher backpressures than the more commonly used 5 μm material. Using elevated temperature will reduce the system backpressure because the backpressure is directly proportional to the viscosity of the mobile phase. For our investigations, we found that the backpressure decreases approximately in half from 23 °C to 80 °C in a curvilinear fashion. This drop in backpressure follows the predicted behavior that viscosity decreases by 1% per 1 °C increase in temperature between 10 °C and 70 °C [24]. In our experiments, the backpressure reaches a lower value at approximately 70 °C beyond which increased temperature has little additional effect in reducing the backpressure.

3.1. Temperature effect upon efficiency

Increasing column temperature in reversed-phase chromatography is generally believed to improve column efficiency because of the reduction in eluent viscosity and an increase in solvent diffusivity [25]. While this would appear true for ion-exchange separations, results have been mixed in reversed-phase with the literature reporting improved efficiencies, no effects and even decreased effects [25]. A thorough study of the full van Deemter curve across a range of temperatures in reversed-phase HPLC concluded that contrary to expectations, an increase in temperature led to a higher HETP value at all flow rates [25]. A key section of that report shows an overall upward shift in van Deemter curves reflecting the effect upon the eddy diffusion contribution (A term). In the 0.3–0.5 ml/min range, the plate count is 20% lower at 65 °C than at ambient temperature. For a wider diameter column, this effect was even greater. This negative impact of temperature on HETP has been attributed to thermal gradients that seem to exist for commercially available column thermostating devices. This may indeed be the result of modern heaters being less effective due to radial or axial thermal heat gradients complicated by resistive heating in the column [26–29]. A recent report demonstrated that for several solutes on polystyrene-coated zirconia, as temperature is raised, the HETP improves at high velocities, but worsens in the low-velocity region of the plot [30]. So, while theory predicts that there should be improved efficiencies in reversed-phase chromatography as temperature increases, practical barriers may exist which keep the system from realizing this theoretical gain. Therefore, the choice of elevated temperature should be based upon the goal of improved selectivity, faster analysis or improved precision of retention time; and should not be based on the need for increased efficiency.

To study the effect of temperature on the quality of the separations on bare silica, four compounds were chosen and their structure is shown in Fig. 2. These compounds generally exhibit tailing on bonded phase columns. The effect of temperature on the chromatography of two of the compounds, propranolol and amitryptyline, at the three selected values on a bare silica stationary phase is shown in Fig. 3. As seen in the figure, the characteristic shift to lower retention time as temperature is increased occurs as it does on traditional bonded phases. Also, the peak tailing was reduced as temperature

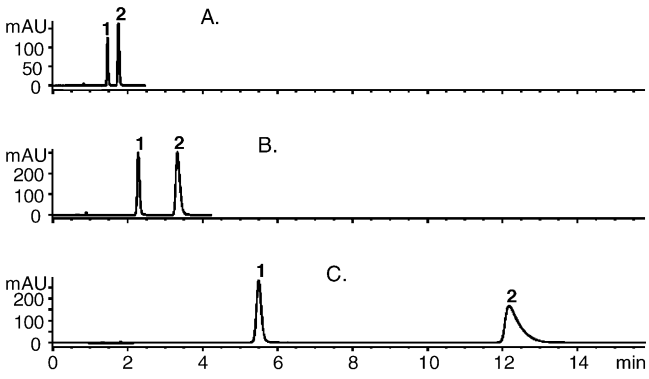

Fig. 3. An example of the effect of temperature on chromatography. Conditions the same as Fig. 1. Components: (1) propranolol and (2) amitriptyline. Chromatogram A is at 75 °C, chromatogram B is at 50 °C and chromatogram C is at 24 °C.

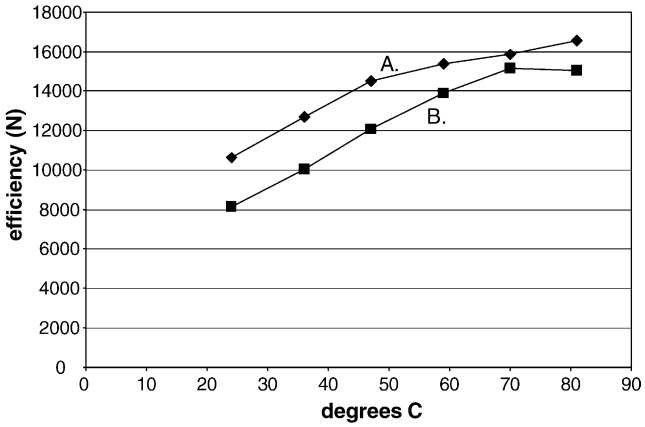

Fig. 4. Effect of temperature upon efficiency. Column: Zorbax Rx-Sil; mobile phase methanol:water (70:30) containing 6 mM dipotassium phosphate at pH 7. Compound A is propranolol and B is amitriptyline.

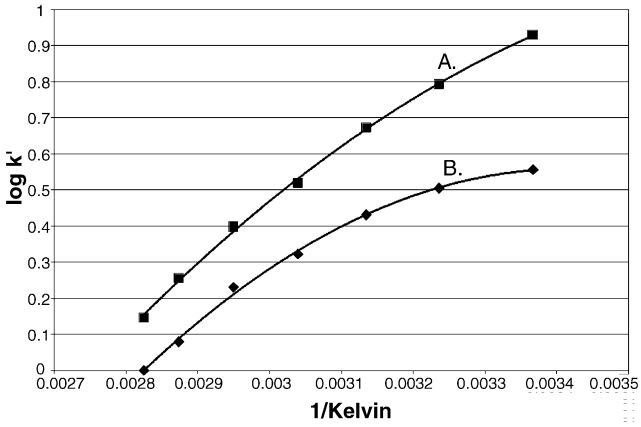

Fig. 6. Effect of temperature upon retention. Column: Zorbax Rx-Sil; mobile phase methanol:water (70:30) containing 6 mM dipotassium phosphate at pH 7. Compound A is dextromethorphan and B is pseudoephedrine.

increased. In a similar but different mobile phase, adjusted to obtain a reasonable retention of pseudoephedrine and dextromethorphan, the same relative type of behavior as shown in Fig. 3 was exhibited. In order to quantitate the apparent improvement in efficiency that was occurring, plots of efficiency versus temperature were made as shown in Figs. 4 and 5. It is interesting to note that dextromethorphan seems to have a continual increase in efficiency up to the highest tested temperature of 80 °C. Propranolol appears to have a sharp initial increase followed by a gradual lessening of the effect. Pseudoephedrine and amitriptyline, on the other hand, seem to have a steep rise but reach a maximum efficiency, one at 40 °C and the other at 70 °C, after which efficiency remains constant. This behavior may reflect the fact that the temperature is influencing the adsorptive and electrostatic forces contributing to retention. Thus, the retention of pseudoephedrine may be due more to adsorptive contributions while retention of the other compounds are more governed by electrostatic forces.

3.2. Temperature effect upon retention

Several reports on bonded phase columns have observed, in general, linear relationships in the van't Hoff plot for neutral molecules [24,31] and of basic pharmaceuticals [24,31,32]. Specifically retention of both amitryptyline and propranolol on a bonded C18 stationary phase were shown to have a linear van't Hoff plot [23]. Using silica stationary phase, on the other hand, did not show a linear van't Hoff plots for the lipophilic amines as seen in Figs. 6 and 7. In fact none of the relationships are linear. On silica, the largest curvature is seen with propranolol and dextromethorphan. In reversed-phase HPLC using bonded phases, the explanation of curvature has sometimes been that there was a phase transition [33] to a more-ordered and extended phase. Generally, when non-linear van't Hoff plots are observed, it is assumed that the enthalpy and entropy change with temperature. But, as recently pointed out by Chester and Coym, if changes in the phase ratio are considered, a non-linear behavior may or may not be due to changes in enthalpy or entropy [34].

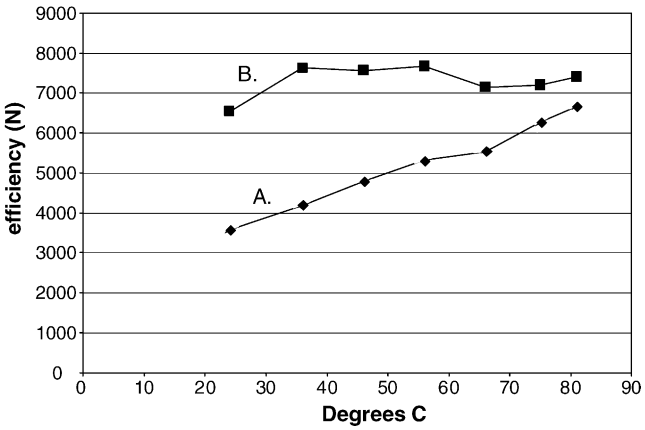

Fig. 5. Effect of temperature upon efficiency. Column: Zorbax Rx-Sil; mobile phase methanol:water (70:30) containing 6 mM dipotassium phosphate at pH 7. Compound A is dextromethorphan and B is pseudoephedrine.

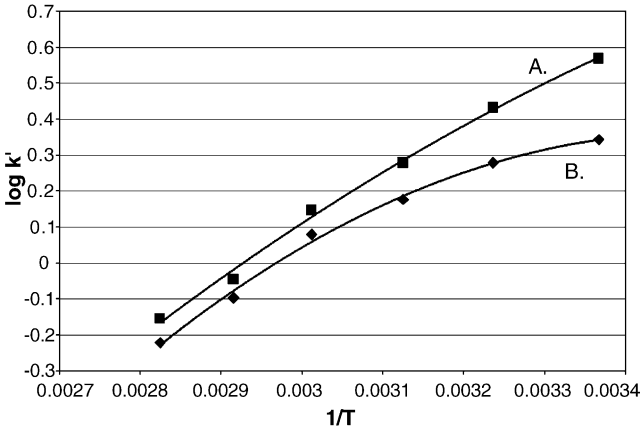

Fig. 7. Effect of temperature upon retention. Column: Zorbax Rx-Sil; mobile phase methanol:water (70:30) containing 6 mM dipotassium phosphate at pH 7. Compound A is amitriptyline and B is propranolol.

There are two main probable explanations of the van't Hoff behavior. One explanation is that in the case of using bare silica, there is no bonded stationary phase; therefore, the explanation is unlikely to be rationalized as due to a phase transition. Additionally, in the case of a bare silica stationary phase, it is unlikely that changes in the phase ratio are an issue. Therefore, it is more likely that the transition is due to a difference in the dominant force responsible for retention. As temperature is changed, the major mechanistic contributions to retention changes from one dominate force to another. All of the compounds have similar curvature and the type of curvature implies that for these compounds the retention enthalpy increases with increasing temperature. Thus, the shape of the van't Hoff plots would be the result of a mixed retention mechanism where multiple factors are in play [20–23]. Viewing these data in light of the earlier hypothesis that electrostatic and adsorptive forces are responsible for retention on silica in the reversed-phase mode, one might suggest that at lower temperature both electrostatic and adsorptive forces contribute to retention; while at the elevated temperature, the electrostatic contribution is mitigated less than the adsorptive force.

However, it is still possible that the non-linear van't Hoff plot could arise from the thermodynamics of temperature if there is adsorption of mobile phase onto the silica surface and if this amount of adsorbed solvent changes with temperature. In essence, the adsorbed solvent on the silica surface would be acting like a pseudo-phase ratio. And, of course, a combination of both possible explanations could rationalize the shape of the van't Hoff plot. Before it is possible to offer a definitive hypothesis of what interactions change and how, more quantitative van't Hoff information needs to be gathered, especially in light of the complex nature of the situation. For instance, a comparison of the transfer of enthalpies on silica could be compared to those on an alkyl-bonded phase. Interpreting the cause of curvature from a van't Hoff plot is a tricky subject [34].

3.3. Column lifetime

It was earlier mentioned that many stationary phases bonded using dimethylalkyl silanes are not thermally stable. Silica, however, at low pH values is stable but at neutral and higher pH may have some issues with slow silica dissolution. Since the applications in this paper investigated elevated temperatures, it was decided to use a "bad-case scenario" to evaluate the column lifetime. The performance of using a column and of using a column with an in-line, pre-saturator silica column was compared. The use of a pre-saturator silica column has been used in the past to enhance the lifetime of the stationary phase of an analytical column using mobile phases that can erode the stationary phase's usefulness [35,36]. The in-line, pre-saturator column has also been used to enhance the lifetime 10-fold of a fused-silica capillary tube before tiny splits in the wall appear due to dissolving of the silica [37].

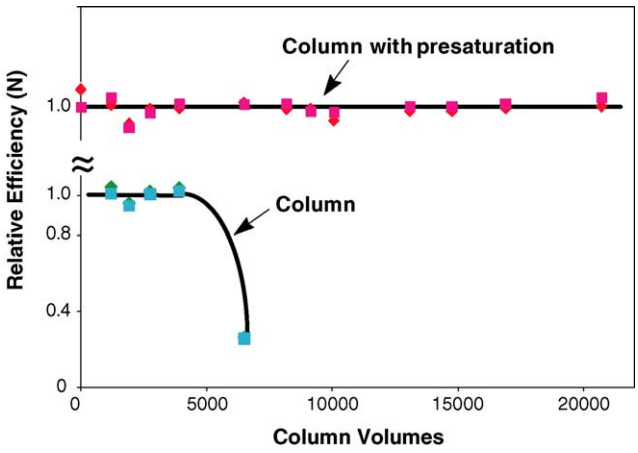

Fig. 8. Column life test. Column: Zorbax Rx-Sil; mobile phase methanol:water (70:30) containing 20 mM citrate at pH 6 and 50 °C. Propranolol (diamonds) and amitriptyline (squares) were the analytes. Data from column alone is bottom line and data from column with precolumn prior to the main column to saturate mobile phase is top line.

As seen in Fig. 8, by plotting the relative retention of the two analytes, propranolol and amitriptyline, on the bare, unbonded silica column alone lasted approximately 5000 column volumes (CVs) without any change in retention. After 5000 CVs the column had a precipitous drop in retention. These data substantiate the hypothesis that silica slowly dissolves during operation under relatively neutral pH mobile phase conditions. As degradation of the silica occurs there is, in essence, no significant loss of the active surface. When a layer of silica is dissolved, silica remains and this may explain why retention of analytes is constant as shown in Fig. 8. Eventually, the silica may dissolve enough for the pore walls to collapse and make the column no longer of use. This appears to occur as a quick loss in performance, not a slow change or gradual loss in retention. Inspection of the "dead" silica column revealed that the top of the bed structure had settled a few mm below the top of the column wall.

The retention behavior of silica under stressful conditions is in contradistinction to what happens with a bonded phase column as it degrades. With a bonded phase column the retention declines over time rather than the sharp precipitous drop observed for the silica column. The value of 5000 CVs is a reasonable useful lifetime. However, when the experiment was repeated with the use of an in-line, precolumn to saturate the mobile phase prior to entry into the column, the useful lifetime was greater than 20,000 CVs where the experiment was stopped.

From this study, it can be concluded that when using elevated temperature on silica at intermediate pH, the appropriate temperature to be used will be a compromise between attaining the desired retention time and peak shape and achieving the desired lifetime. Moderate temperature (35–45 °C) may be more appropriate for enhanced lifetime while higher temperatures (>60 °C) may favor reduced run times and higher throughput. Of course, the use of an in-line,

pre-column to "saturate" the mobile phase with dissolved silica is always an option.

4. Conclusion

The use of bare silica as a stationary phase in the reversed-phase mode is an attractive, if not preferred, approach for the separations of lipophilic amines typical of pharmaceutical compounds. Several advantages are available when using silica compared to using an alkyl C18 phase such as the use of relatively simple aqueous buffer–organic mobile phases and the opportunity to retain highly polar compounds with a reasonable retention time.

The use of temperature in reversed-phase HPLC is now becoming an important variable for improving the peak shape, reducing analysis time and improving reproducibility of the chromatography [38]. This is equally true when using a bare, unbonded silica column in the reversed-phase mode. In addition, the role of temperature using a silica stationary phase offers an additional selectivity tool for the separations of lipophilic amines that is not often available in reversed-phase systems using alkyl stationary phases. Temperature effects in reversed-phase HPLC are linear when using an alkyl phase. However, when using silica, there is curvature in the plots. Thus, when using silica, temperature becomes another variable to control selectivity when developing separations. The curvature in the temperature relationship to retention is probably result of the mixed mechanism of retention controlling the separation on the silica stationary phase. As temperature changes the two forces, hydrophobic and electrostatic, shift as the dominant force controlling retention.

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