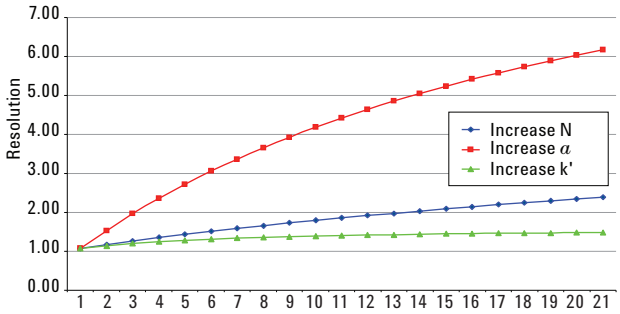
Use of Agilent Poroshell HPH-C18 Columns at Elevated pH as a Tool for Method Development

Technical Overview

Introduction

HPLC method development for chemical and pharmaceutical analysis is a challenging task. It involves screening a range of chromatographic parameters to generate sufficient resolution and robust separations. While there are many approaches to method development, such as one factor at a time, and quality by design (QbD), the goals and factors used for optimizing separations are the same. Several factors affect chromatographic resolution (RS), efficiency (N - controlled by particle size, particle morphology, and column length), retention factor (k - controlled by solvent strength), and selectivity (α - controlled by bonded phase choice and mobile phase)(Figure 1). Selectivity or α is the most powerful of these factors.



Plates:	5,000	10,000	15,000	20,000	25,000
Alpha:	1.10	1.35	1.60	1.85	2.1
k':	2.0	4.5	7.0	9.5	12.0

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$$

Figure 1. Typical method development parameters; effects of selectivity, efficiency, and retention on resolution.

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Method development involves the separation of simple and complex mixtures. Selectivity can be controlled though several factors, including the choice of stationary phase, the type of organic modifier, gradient slope, flow rate, and temperature. For ionizable compounds, the pH of the buffer is also a powerful parameter. Optimizing separation of ionizable compounds in order to find robust conditions has become an important part of method development in liquid chromatography [1]. Most pharmaceutical and biological compounds contain ionizable functions such as carboxylic or amino groups. Using pH is a very powerful selectivity tool for reversed-phase liquid chromatography (RPLC) separations. Low-pH separations involve protonated acids and bases, but these acids and bases are deprotonated at high-pH. Because retention in reversed-phase chromatography is strongly dependent upon the analyte charge, pH can be used to make large changes in selectivity. At acidic pH, acids have their maximum retention because they are neutral, but bases have their minimum retention because they are fully charged. At basic pH (above the pKa of the compound), bases have their maximum retention because they are neutral, and acids are fully ionized and have their minimum retention. For the best peak shape, retention and sample loading of basic analytes in RPLC, the mobile phase pH should be two units higher than the pKa of the compound of interest. The retention of neutral compounds is unaffected by pH. In this work, adjustment of pH was used to control selectivity using an Agilent Poroshell HPH-C18 column that is designed to be stable in high pH mobile phases.

Materials and Methods

An Agilent 1260 Infinity LC was used for this work.

- Agilent 1260 Infinity Binary Pump G1312B
- Agilent Automatic Liquid Sampler (ALS) G1367C
- Agilent 1260 Infinity Thermostatted Column Compartment (TCC) SL G1316C
- Agilent 1290 Infinity Diode Array Detector (DAD) G4220A (10-mm path, 1-µL volume)
- OpenLab version C.01.05 was used to control the HPLC and to process the data.
- Agilent Poroshell HPH-C18, 2.1 × 50 mm, 2.7 µm (p/n 699775-702) or Poroshell HPH-C18, 4.6 × 50 mm, 2.7 µm (p/n 699975-702)

In some experiments, an Agilent 6140D Single Quadrupole LC/MS was also employed.

Table 1. Compounds used in retention correlation.

Sample name
1,2-Dimethoxybenzene
1,2-Dinitrobenzene
1,2,3-Trimethoxybenzene
1,2,4-Trimethoxybenzene
1,2,5-Trimethoxybenzene
1,3-Dimethoxybenzene
1,3-Dinitrobenzene
1,4-Dinitrobenzene
2,3-Dimethylphenol
2,4-Dichlorophenol
2,4-Dimethyl benzoic acid
2,5-Dihydroxyl benzoic acid
2,5-Dimethyl phenol
2-Hydroxyhippuric acid
2-Napthalene sulfonic acid
3,4-Dimethoxybenzoic acid
3-Nitrophenol
4-Hydrobenzaldehyde
4-Hydroxybenzoic acid
4-Nitrophenol
5-hydroxy-isophthalic acid
8-Hydroxyquinoline
Acebutolol
Acetylsalicylic acid
Alprenolol
Amitriptyline
Andro
Antipyrin
APAP
Atenolol
Atorvastatin
Beta estradiol
Beclomethasone
Benzocaine
Benzoic acid
Benzophenone
Benzyl alchohol
Betamethasone
Biphenyl (DMSO)
Butacaine
Butyl benzene
Butyl paraben
Butylated hydroxy anisole
Butylated hydroxy toluene
Butyrophenone
Caffeine
Catechol
Chlorammphenicol
Corticosterone
Desimpramine
Dexametasone
Diclofenac
Diethyl phthalate
Diflunisal
Diisopropyl phthalate
Dioctyl phthalate
Dipropyl phthalate
Doxepim
Esterone
Ethinylestradiol
Ethyl-4-hydroxybenzoate
Fenprofen
Fluoxetine
Furazolidone
Hesperidin
Hydrocortisone
Irganox 1330
Ketoprofen
Labetalol
m-Nitrophenol
Mefamic acid
Naldolol
Naproxen
Nargingenin
Nisoldipin
Norethindrone acetate
Nortryptyline
p-Cresol
p-Nitrophenol
Pentachlorophenol
Phenacetin
Phenantranene
Pindolol
Piperdine
Piroxicam
Pravastatin
Prednisone
Procaine
Progesterone
Promazine
Propranolol
Protriptyline
Pyrimethamine
Quinine
Resorcinol
Salicytic acid
Salycilic acid
Sulfachloropyridazine
Sulfadiazine
Sulfadimethoxine
Sulfamerazine
Sulfamethiazine
Sulfamethiazole
Sulfamethoxazole
Sulfamethoxypyridazine
Sulfamonomethoxine
Sulfaquinoxaline
Sulfathiazole
Sulindac
Testosterone
Tetracaine
Tolemetin
Triamcinalone
Trimipramine
Ultranox 276
Uracil
Valerophenone

Mobile phases compatible with mass spectrometry, consisting of volatile buffers such as ammonium formate buffer, ammonium acetate, and ammonium bicarbonate buffer, were used. These buffers were prepared by dissolving sufficient ammonium formate or ammonium bicarbonate in water to produce 10 mM solutions, and adjusting the solutions to the desired pH with the appropriate concentrated acid (formic acid or acetic acid) or concentrated base (ammonium hydroxide). The mixture evaluated included acids (acetyl salicylic acid, and diflunisal), bases (procainamide, dipyrimadole, and diltiazem), and neutral compounds (hexanophenone and impurity (valerophenone)). Caffeine does not ionize and was also included.

Use of pH to affect selectivity

Figure 2 depicts how the elution order of a mixture consisting of acidic, basic, and neutral compounds changed as pH of the mobile phase was changed. In this work, a generic gradient was used with the organic modifier (acetonitrile) concentration changing from 10 to 90% over 4 minutes. Chromatograms at pH 3 (ammonium formate), pH 4.8 (ammonium acetate), and pH 10 (ammonium bicarbonate) are shown using buffers compatible with mass spectrometry. The flow rate was 2 mL/min.

As shown, the three chromatograms use the same gradient and column. The neutral (hexanophenone) and nonionized compounds (caffeine) remained at the same elution time. They were not affected by the change in pH. As the mobile phase pH was increased from pH 4.8 to pH 10, the acidic compounds became charged and their retention time decreased. This is depicted by the red arrows in Figure 2. As the pH is increased, the retention time of the bases increased as shown with the blue arrows. The peak elution order changed dramatically as did the spacing. In all three chromatograms, the peak shape was excellent. In this case, the spacing of the compounds was greater using the pH 10 buffer than either of the other buffers. In addition to longer retention of bases, better peak shape was also found when using high pH mobile phases as compared to low pH mobile phase.

Another way to look at selectivity is by plotting retention time using two different conditions for a group of acids, bases, and neutral compounds. A list of the compounds used in this study is found in Table 1. In this case, 117 compounds were run using the Poroshell 120 HPH-C18 column with identical gradients and two organic modifiers (methanol and acetonitrile) and at two pHs (pH 3 and pH 10). The generic gradient used here was 0.42 mL/min, starting at 5% organic and increasing to 95% organic over 4 minutes, and held at this concentration for 2 minutes.

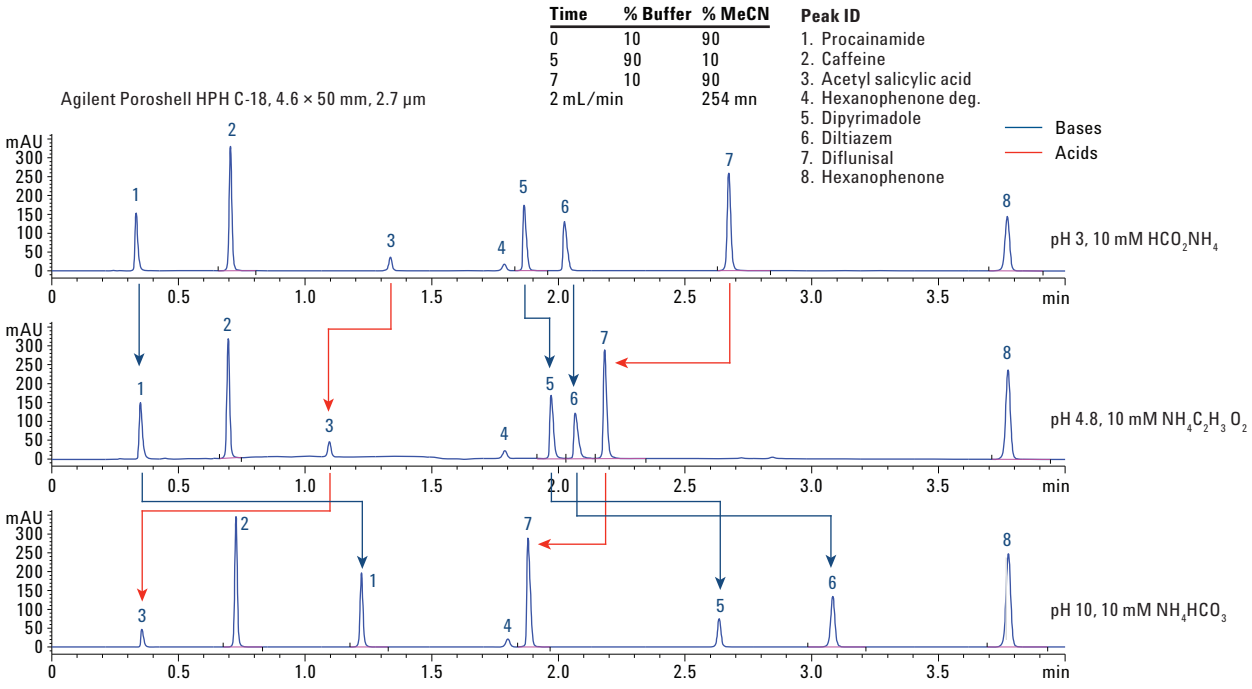

Figure 2. Selectivity control by altering pH with an Agilent Poroshell HPH-C18, 4.6 × 50 mm, 2.7 µm LC column at pH 3, 4.8, and 10.

As shown in Figure 3A, a subgroup of analytes lined up perfectly with a slope of 1. These compounds were neutral or nonionizable with methanol as the organic modifier. They include substituted benzenes, steroids, phenols, and phenones. The retention time of these materials was not affected by the pH of the mobile phase, as expected. This method was applied and discussed in previous work where two highly similar columns (Agilent Poroshell 120 EC-C18 and Agilent ZORBAX Eclipse Plus C18) were compared under similar chromatographic conditions [2]. Analytes that appear above the line are bases. At pH 3, these compounds were charged, and as they became uncharged when the pH increased to 10, the retention time increased. The correlation coefficient of retention times is a measure of the difference of the separation under two different pH conditions. A highly correlated plot would have a value close to 1. This would indicate that the chromatographic separations were very similar. Conversely, a very low correlation value (close to 0.5 or lower) indicates a more orthogonal or dissimilar separation. A second comparison is also shown in Figure 3B, where a comparison of low and high pH gradients was made using acetonitrile as the organic modifier. In this case, the correlation coefficient was smaller, than when using methanol [2,3,4].

Improved LC-MS sensitivity for basic compounds at high pH

In a third experiment, LC/MS of several bases was compared at high and low pH using a generic gradient in positive mode electrospray. Normally one expects that the ionization state of analyte molecules is dependent on the pH of the mobile phase, and that the ionization efficiency in LC/MS with electrospray in positive ion mode will be drastically lowered in high pH mobile phases since the compounds become neutral. However, many researchers investigating different types of samples (including proteins, peptides, and amino acids) have observed either an insensitivity to change of mobile phase pH or even increases.

Successful detection of basic compounds in ESI+ when using high pH buffers in the mobile phase has been reported [5-10]. High pH mobile phases do not suppress the ionization of basic compounds in ESI+; positive ions are formed abundantly, and analyte responses are often better in high pH compared to acidic mobile phases. This finding is significant as it extends the applicability of generic elution methods to the analysis of polar basic compounds previously difficult to retain.

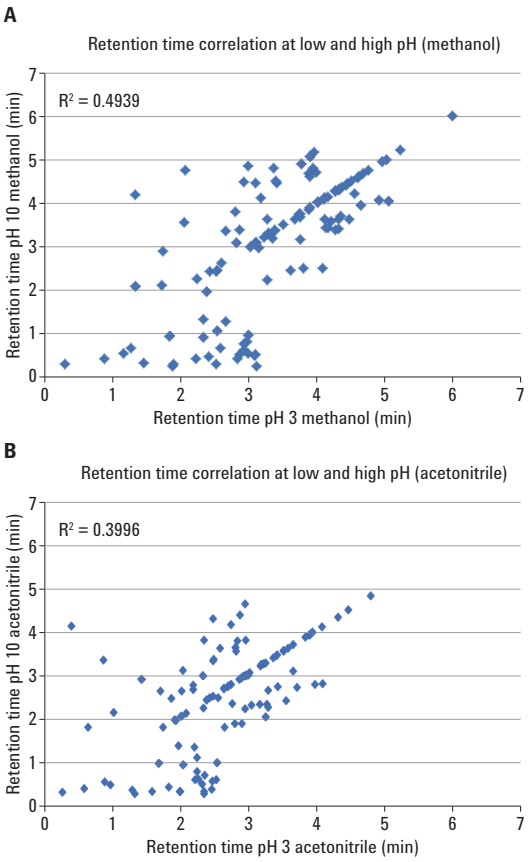

Figure 3. Retention time correlation with an Agilent Poroshell HPH-C18, pH 3 versus pH 10. A) methanol, and B) acetonitrile.

In this experiemnt, a gradient was run using acetonitrile as the organic modifier. The aqueous solvent contained 0.1% formic acid, the low pH mobile phase modifier or 10 mM pH 10 ammonium bicarbonate. In the example shown above, lidocaine was prepared in water at 0.01 mg/mL. A 1 µL injection was made. As shown, the sample was injected on a Poroshell HPH-C18 column, the lower trace shows the sample analyzed at low pH, the analyte is retained only slightly and the peak tailed. In the upper trace, the better retained analyte peak was well shaped and twice as tall. Due to the elution in a mobile phase having a higher organic content, which is beneficial for LC/MS detection, the peak area was also significantly larger. In general, ionization in the more volatile organic phase was more efficient leading to higher signal intensity.

Procainamide and diltiazem were also analyzed. The signal intensity increase of these compounds was not as dramatic as for lidocaine. Solvent evaporation rate during droplet formation is a function of the mobile phase vapor pressure. Higher volatility of a greater proportioned acetonitrile:water mobile phase favors ESI ionization.

The results in Figures 4A to 4C show that the use of high pH mobile phases for the analysis of basic compounds offered a good alternative to using low pH mobile phases in ESI+ LC/MS.

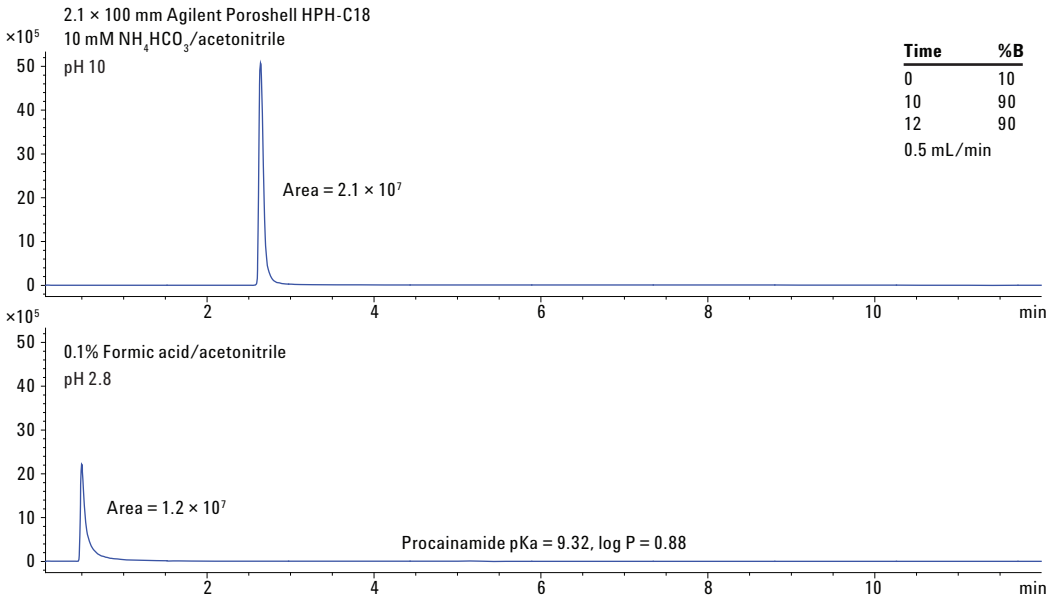

Figure 4A. Comparison of LC/MS of bases (procainamide, pKa 9.32, logP 0.88) in positive ion electrospray mode at high and low pH. Agilent Poroshell HPH-C18, 2.1 × 100 mm.

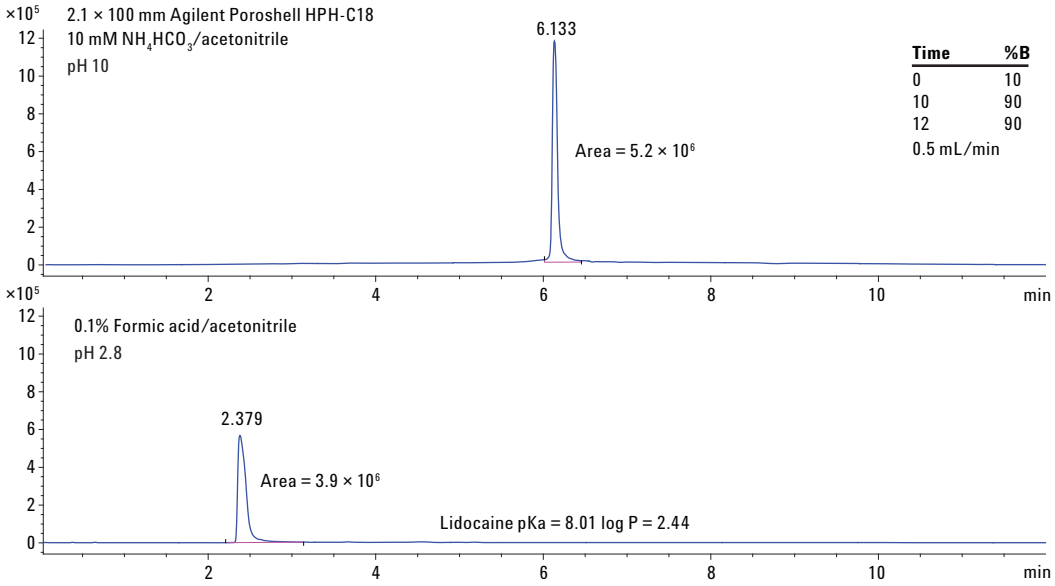

Figure 4B. Comparison of LC/MS of bases (lidocaine, pKa 8.01, logP 2.44) in positive ion electrospray mode at high and low pH. Agilent Poroshell HPH-C18, 2.1 × 100 mm.

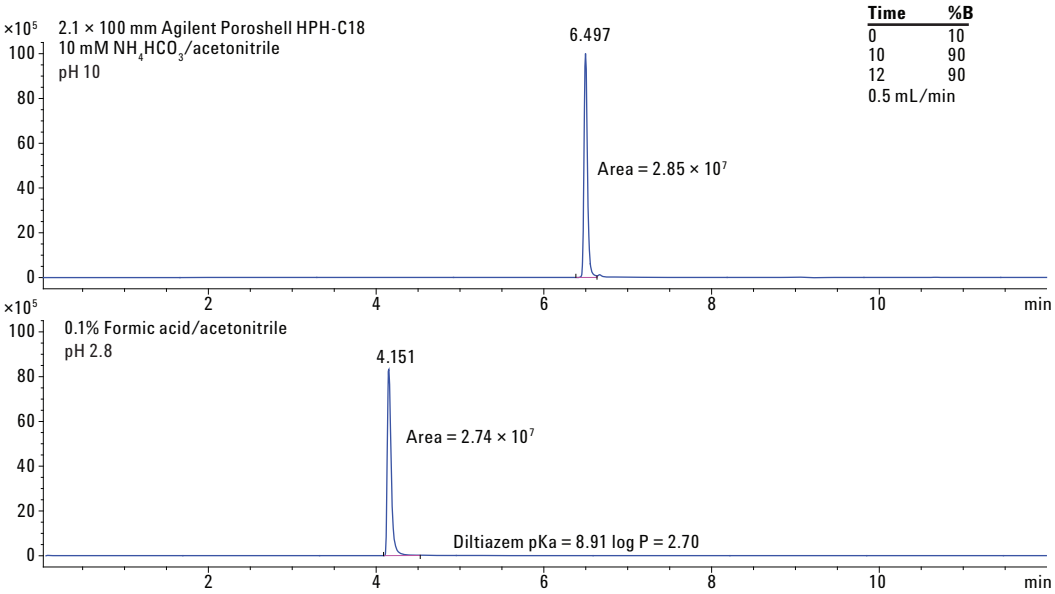

Figure 4C. Comparison of LC/MS of bases (diltiazem, pKa 8.91, logP 2.70) in positive ion electrospray mode at high and low pH. Agilent Poroshell HPH-C18, 2.1 × 100 mm.

Stability of Poroshell HPH-C18 at high pH

HPLC column stability is one of the critical factors affecting method performance and has been widely studied. Column stability can be affected by temperature, type of aqueous buffer and its concentration, choice of organic solvents, additives, and mobile phase pH. Prescreening of compounds and columns should enable scientists to arrive at successful separations more quickly. HPLC column stability is one of the critical factors affecting method performance. A robust HPLC method using a durable column leads to successful support of new clinical and manufacturing projects. A column that is not stable during method development leads to inaccurate results and frustration.

Column degradation is caused by silica dissolution, bonded-phase removal, or through the exposure of silanols by the removal of end capping (hydrolysis). Both dissolution and hydrolysis of silica columns are known to be related to pH and temperature (increased degradation rate at higher pH/temperatures). Other causes of column degradation include poor sample preparation (dirty samples) and column bed instability.

A good criterion for column stability under a given pH is 500 injections. This allows development, adjustment, and use for a column under an established method. In this section of the work, a Poroshell HPH-C18 column was evaluated in a gradient using ammonium bicarbonate and acetonitrile at pH 10. Acidic, neutral, and basic compounds were used. To evaluate columns from a variety of manufacturers, a common stress gradient was used while changing the analytes to accommodate differences in selectivity. In all cases, at least two acid, base, and neutral compounds were employed.

The protocol discussed here evaluated the impact of mobile phase modifier on column stability [11]. The impact of sample solution was minimal, as typically only a few µg of sample were loaded. The test mixture was chosen to assess column performance, not to assess the impact of the test probes themselves on column stability. A low flow rate was used to minimize column bed stability problems during development. As shown in Figure 5A, the retention time of all compounds remained stable throughout the 2,000 injections with the exception of nortryptyline. This compound, with a pKa very close to the pH of the mobile phase, moved slowly to give longer retention times.

A second column from another brand was subjected to the same experimental conditions. Most of the analytes remained at the same retention time throughout the 2,000 injections. Nortryptyline moved rapidly to later elution times. Within 500 injections, nortryptyline began to coelute with the next compound, neutral hexanophenone. The peak continued to migrate through this peak, totally coeluting by injection 2,000. This experiment indicated greater degradation of the non-Agilent column compared to the Poroshell HPH-C18 column. Differences in peak height occurred as the sample changed.

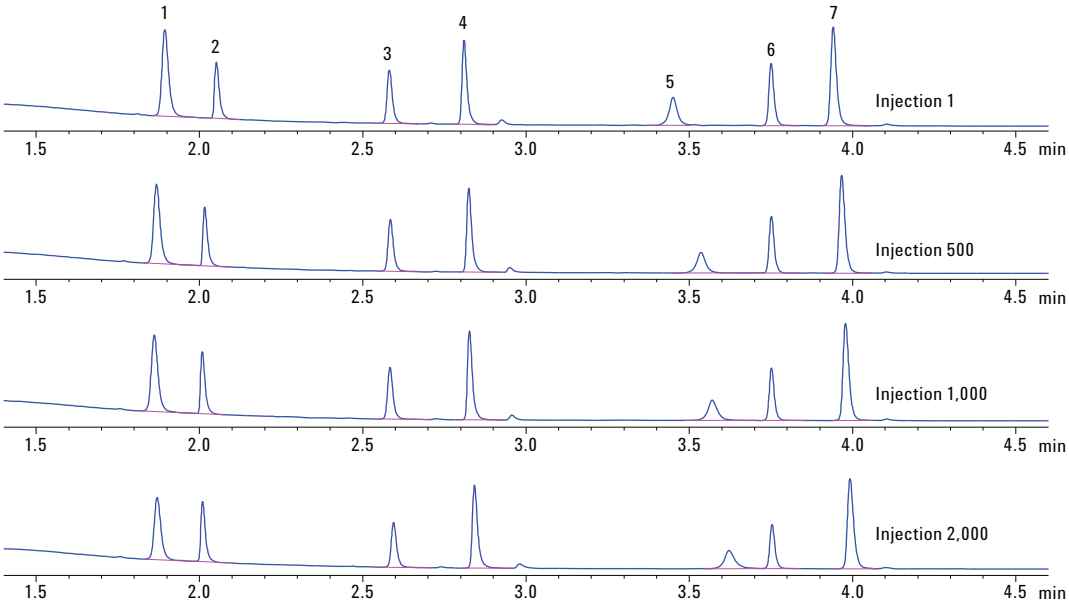

Figure 5A. Excellent retention on the Agilent Poroshell HPH-C18, 2.1 × 50 mm, 2.7 µm column even under high pH bicarbonate conditions, (total method run time = 7 minutes, flow rate 0.4 mL/min).

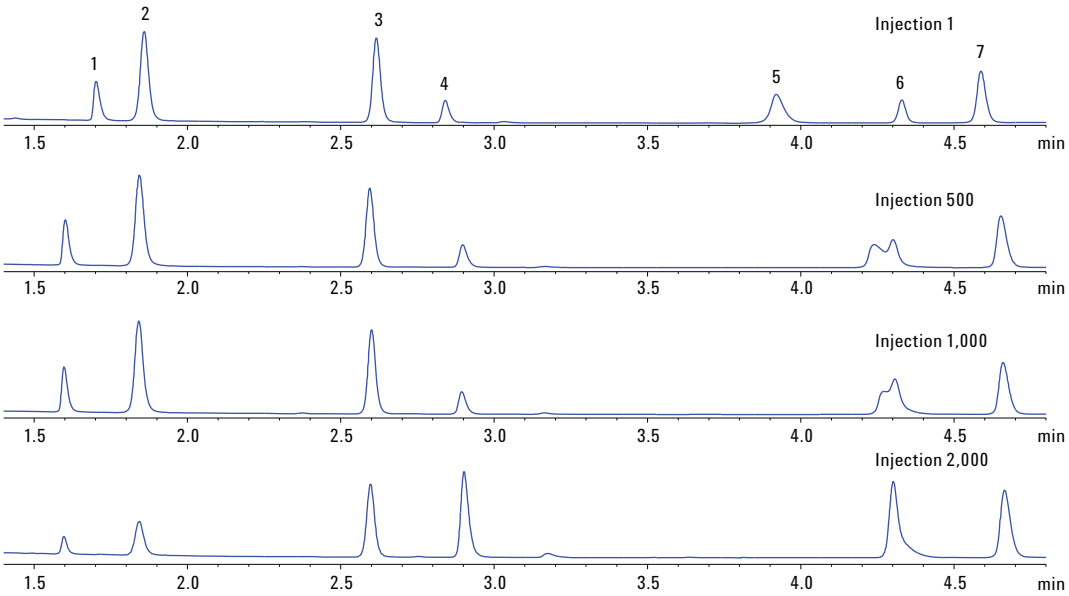

Figure 5B. A competitor 3 µm column suffered greater degradation under high pH bicarbonate.

Conclusion

Using an Agilent Poroshell HPH C18, pH can be used to adjust selectivity without sacrificing column lifetime at elevated pH. By keeping a gradient constant and altering pH, the elution order of a group of eight acid, base, and neutral compounds could be dramatically changed, and hence chromatographic resolution. In a second experiment, the correlation coefficient of the retention times was determined using a generic gradient plotted for pH 3 and pH 10. Using R^2 as a measure of orthogonality, we found that the two conditions offered different selectivity. Using pH as a method development tool was very effective, especially when the sample contained acidic or basic compounds. We also investigated positive ion electrospray mass spectrometry of several basic compounds using gradients HPLC at high and low pH. In this case, we showed that the peak shape of basic compounds improved and retention time increased. We also observed a signal increase as measured by the peak area. This effect was not the same in all cases and was likely to be compound-dependent. In no case was a signal decrease observed for bases at elevated pH. Finally, we determined that a Poroshell HPH C18 could be used for extended periods (over 2,000 injections) at pH 10 in ammonium bicarbonate at 25 °C. By using pH as a method development tool with a Poroshell HPH-C18, chromatographers can maximize flexibility in their method development and analyses, while still benefiting from the rugged and long lifetime of the Agilent Poroshell 120 family.

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