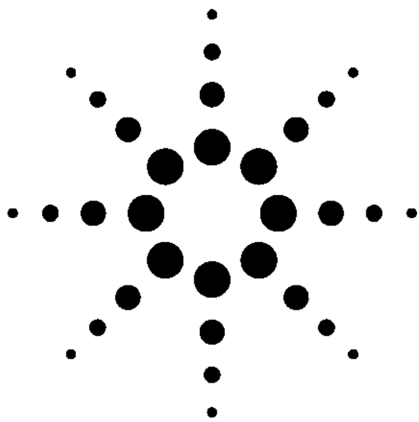

“Plug & Play” Fast and Ultra-Fast Separations Using 3.5-µm Rapid Resolution and 1.8-µm Rapid Resolution High Throughput Columns

Application

Pharmaceutical

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Abstract

Over a half dozen isocratic liquid chromatography methods, originally developed with 4.6 mm × 150-mm, 5-µm columns, were easily converted to high-throughput applications solely by substituting the original column with a 4.6 mm × 50-mm, 1.8-µm, Rapid Resolution High Throughput (RRHT) column, or a 4.6 mm × 100-mm, 3.5-µm, Rapid Resolution (RR) column. The variety of methods include USP methods, high pH separations, and extracts of complex matrices such as medicated syrup, a medicated tablet, and a sunblock lotion.

New instrumentation is not necessary. Redeveloping or optimizing the methods is not needed. No flow rate adjustments are needed since the HPLC system pressure remains in the normal operating parameters of the Agilent 1100 pumps. There is an increase of sensitivity as measured by signal-to-noise ratio (S/N) for identical amounts injected on the shorter RRHT columns versus the longer 5-µm columns. An example is made for selecting the best L7 bonded phase for fenprofen calcium according to USP requirements.

Introduction

To remain competitive and viable, there is an ongoing trend to demand higher productivity and better economy from analytical laboratories. One way labs can improve productivity is to shorten analysis time. Liquid chromatography (LC) separations less than 10 minutes and less than 1 minute are popularly known as fast and ultra-fast separations, respectively. Redeveloping methods to improve an analysis can be costly in terms of the time required for development and validating robustness. New instrumentation may also be required.

A simpler, cost-effective way to achieve fast and ultra-fast analyses for isocratic methods is to use the original method, and only modify it by simply substituting the original LC column (and perhaps some connecting tubing) with a shorter column containing smaller particles of the same bonded phase. Scaling down from a 4.6 × 150 mm, 5-µm column to 4.6 × 100 mm, 3.5 µm or 4.6 × 50 mm, 1.8-µm columns can reduce analysis time by up to two-thirds, while leaving all the other method parameters intact. Similar to the simplicity of connecting a “Plug & Play” peripheral to a personal computer for immediate utility, substituting short RR or RRHT columns for longer columns can produce a faster analysis without compromising the quality of the separation.

Agilent Technologies

In this study, gradient methods were not considered because other method parameters, besides the column substitution, need adjustment. It is possible to convert gradient methods developed with 4.6 × 150 mm, 5-µm columns to fast and ultrafast methods using RR and RRHT columns. The gradient time must be adjusted, however, to compensate for the change in column length. Otherwise, the relative peak positions would change and the analysis time would not decrease proportionately with the shorter column. The equation below provides an easy way to determine how to adjust the gradient time when switching to shorter columns with the same internal diameter:

$t_{G2} = t_{G1} \times (L_2/L_1)$

Where:
t_{G1} = Gradient time with original column
t_{G2} = Gradient time with column 2
L_1 = Length of original column
L_2 = Length of column 2

Column re-equilibration time should be reduced also (the column will re-equilibrate faster, because it is shorter). Although these changes are straight forward, these additional modifications eliminate gradient methods from being considered "Plug & Play".

Experimental Conditions

Columns

A variety of isocratic pharmaceutical analyses were made faster following pre-existing methods. Some of these were chosen from the United States Pharmacopeia (USP) compendia of standards. Table 1 lists the original columns used in each method and other RR and RRHT column choices available to achieve fast analyses.

Table 1. Column Options

Column	Dimension (mm)	Particle size (µm)	Agilent p/n
ZORBAX Extend C18	4.6 × 150	5	773450-902
ZORBAX Extend C18	4.6 × 100	3.5	764953-902
ZORBAX Extend C18	4.6 × 50	1.8	722975-902
ZORBAX Eclipse XDB-C18	4.6 × 150	5	993967-902
ZORBAX Eclipse XDB-C18	4.6 × 100	3.5	961967-902
ZORBAX Eclipse XDB-C18	4.6 × 50	1.8	922975-902
ZORBAX Eclipse XDB-C8	4.6 × 150	5	993967-906
ZORBAX Eclipse XDB-C8	4.6 × 100	3.5	961967-906
ZORBAX Eclipse XDB-C8	4.6 × 50	1.8	922975-906
ZORBAX StableBond-C18	4.6 × 150	5	883975-902
ZORBAX StableBond-C18	4.6 × 100	3.5	861953-902
ZORBAX StableBond-C18	4.6 × 50	1.8	822975-902
ZORBAX StableBond-C8	4.6 × 150	5	883975-906
ZORBAX StableBond-C8	4.6 × 100	3.5	861953-906
ZORBAX StableBond-C8	4.6 × 50	1.8	822975-906

Instrumentation

All separations were carried out using an Agilent Technologies 1100 Liquid Chromatograph (LC) comprised of a:

G1379A Degasser
G1312A Binary Pump
G1367A Well Plate Autosampler (WPALS)
G1316A Column Compartment
G1315B Diode Array Detector (DAD)

Tubing between the WPALS outlet and the detector flow cell was flexible stainless steel, 0.12-mm id. A 150-mm length connects the 3-µL heat exchanger in the column compartment to the WPALS valve, and a 70-mm length connects the heat exchanger outlet to the column inlet. The majority of the LC analyses were carried out without elevated temperatures, however, the 3-µL heat exchanger remained installed to demonstrate that the only change in the method is a column substitution. The column outlet was plumbed directly to the diode array detector (DAD) micro flow cell. The Agilent 1100 is versatile, and available in a wide variety of configurations. Figure 1 represents the configuration described and used here.

Samples and Mobile Phases

Three high pH separations
Tricyclic antidepressants, antihistamines, and alkaloids, were carried out with the following mobile phases: Mobile phase A is pyrrolidine buffer, 50 mM, made by adding 4.17 mL of pyrrolidine to 1 L of deionized water. Mobile phase B was HPLC grade methanol (Burdick & Jackson, Muskegon, MI). The percent organic varied for each method and was entered in the pump parameters in the ChemStation software. All analytes were dissolved in 40% methanol. The above analytes, and all those used in the following analyses, were obtained from Aldrich, Milwaukee, WI, unless otherwise stated.

USP assay for loratadine tablets
One Claritin tablet (Schering Corporation, Kenilworth, NJ) was pulverized by mortar/pestle and transferred to a 25-mL volumetric flask. Ten mL of 0.05 N hydrochloric acid was added and sonicated for 3 minutes. Methanol: acetonitrile (1:1) diluent (7.5 mL) and 0.6 M dibasic potassium phosphate (2.0 mL) was added and sonicated 3 minutes, then diluent was added to the 25-mL mark. After mixing, 1 mL was passed through a 0.45-µm glass-fiber syringe filter into an autosampler vial for injection.

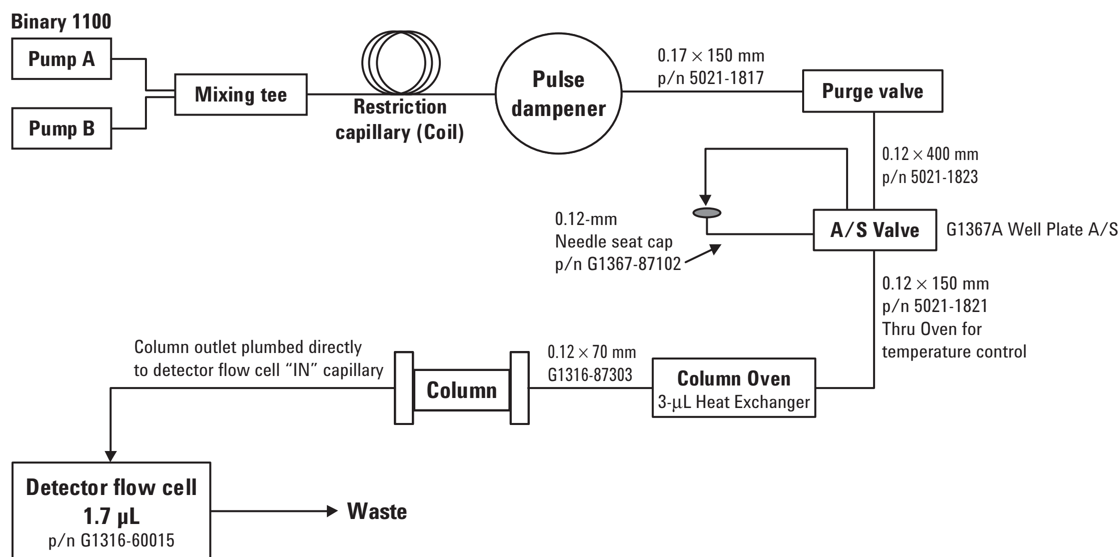

Figure 1. Example of Agilent 1100 LC plumbing for RRHT 4.6-mm id columns.

Mixture of sunscreens
Individual solutions of ultraviolet (UV) ray blockers were made by adding 1 mL of methanol to 5 mg of 2-hydroxy-4-methoxybenzophenone (HMBP), 100 µL of 2-ethylhexyl trans-4-methoxycinamate (EHMC), 100 µL of ethylhexyl salicylate (EHS), and 50 µL of padimate-O, (USP, Rockville, MD). A stock mixture was then prepared by combining 100 µL of HMBP, 10 µL of EHMC, 10 µL of padimate-O, and 15 µL of EHS with 800 µL of methanol followed by 100 µL of water. The injection sample was made by combining 100 µL of stock mixture with 700 µL of methanol and 200 µL of water into an autosampler vial. Mobile phase was delivered by two channels: A - deionized water, and B - methanol. The binary pump via ChemStation combined the two to the desired organic strength.

Additionally, a retail sunblock lotion was extracted and analyzed. One and a half grams of "Banana Boat Sport" sunblock lotion (Sun Pharmaceuticals, Delrey Beach, FL) was added to a 20-mL vial and 5 mL of methanol was added. The vial was briefly vortexed and then sonicated for 5 minutes. About 1.5 mL of this milky suspension was clarified by filtering it through a 0.45-µm syringe filter. This extract was then diluted by combining 10 µL of it with 690 µL of methanol and 300 µL of water in an autosampler vial for injection.

USP assay for ibuprofen oral suspension
The sample was prepared by adding 1.5 mL of Children's Motrin (McNeil, Ft. Washington, PA) to a 25-mL volumetric flask and filling to the mark with diluent (acetonitrile: water [1:1]). To make the internal standard (ISTD), 80 mg of benzophenone was added to a 25-mL volumetric flask and filled with acetonitrile. The injection sample was made by combining 10 mL of the oral suspension solution with 2.5 mL of ISTD into a 25-mL volumetric flask and filling to the mark with diluent. No filtering was done, even though directed to do so in USP procedure. The flask was shaken to resuspend particulates before transfer to the autosampler vial for injection. The mobile phase was 630 mL of acetonitrile, 370 mL of water, and 1.8 mL of H_3PO_4. This USP method specified a flow rate of 2 mL/min. Although smaller particles cause higher system pressure, it was not necessary to reduce flow for the RRHT columns for any of these examples in this application note.

USP assay for fenprofen calcium
The sample was prepared by combining 25 mg of fenprofen calcium and 25 mg of gemfibrozil into a 25-mL volumetric flask and adding about 10 mL of diluent (70% methanol), sonicating until dissolved, then filling to the mark with diluent.

USP assay for terazosin
The sample was prepared by combining 12.5 mg of terazosin with 25-mL mobile phase. This was then diluted 50:1. The mobile phase consisted of 6.0 g of sodium citrate dihydrate, 14.25 g of anhydrous citric acid, and 1 L of water, pH 3.2.

Results

The first three examples use a high pH buffer. High pH is advantageous for separating basic compounds above their pKa in free base form. This often results in less peak tailing and better retention. ZORBAX Extend-C18 was specifically developed to operate in the high pH range (up to pH 11.5). It has a unique bidentate structure and double-end capping that resists silica dissolution at high pH, permitting long column life under high pH conditions.

Figure 2A shows an assay of tricyclic antidepressants on a ZORBAX Extend-C18 4.6 × 150 mm, 5-µm column. These compounds often show poor peak shape and long retention due to interaction with ionized silanols. However, at high pH (above their pKa's), satisfactory peak shape and retention is achieved. The first peak, doxepin, is an E, Z-isomer mixture (82% E); Z is detected as a shoulder. The other four peaks are symmetrical, indicating minimal ionized silanol interactions influencing the chromatography. Run time is easily shortened threefold, by substituting the typical "analytical" column with a column one-third the length, containing 1.8-µm particles of the same bonded phase. Figure 2B shows the same method performed with a 4.6 × 50-mm column packed with 1.8-µm ZORBAX Extend-C18. No chromatographic quality is lost, the Z-isomer shoulder is still detected, and peak shape is still excellent.

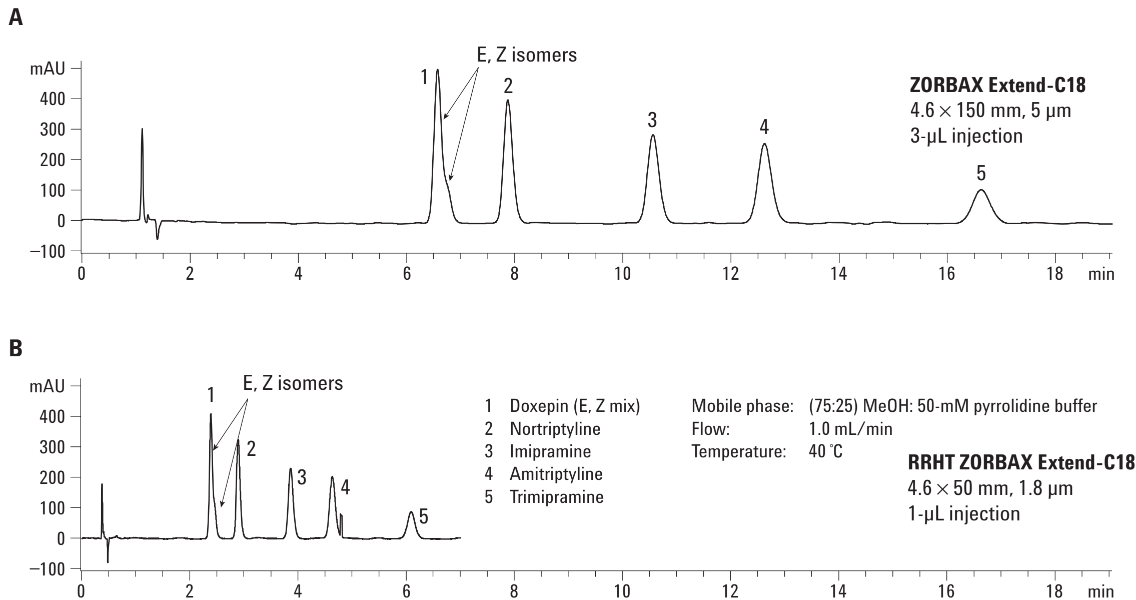

Figure 2. Assay of tricyclic antidepressants (TCA's) at high pH on Extend-C18.

A second example of a robust separation at high pH is shown in Figures 3A and 3B. Six antihistamines are resolved with good peak shape. High pH is preferred here, because at lower pH, scopolamine and pseudoephdrine are not well retained and co-elute near the void volume. The other amine-containing compounds can excessively tail at a pH less than their pKa, due to the positive charge of the protonated amine interacting with ionized silanols on the column packing surface. These obstacles are overcome when analyzed at a pH above their pKa. By simply changing the longer 5-µm column to a shorter RRHT column, the anaylsis time is shortened substantially, and chromatographic performance is also maintained (Figure 3B).

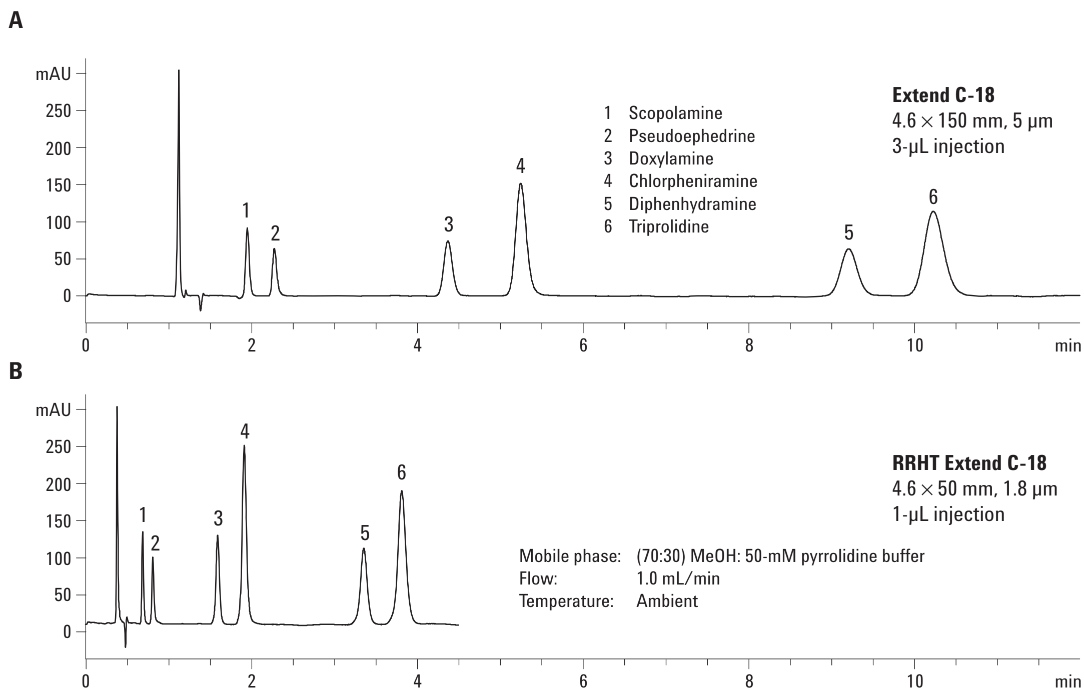

Figure 3. Assay of antihistamines at high pH on Extend C-18.

Scopolamine and atropine are belladonna alkaloids, natural compounds derived from *Atropa Belladonna*, or deadly nightshade, a highly poisonous plant. In nonpoisonous doses, they have several medicinal uses, including treatment of motion sickness and cardiac arrest. Alkaloids are highly water soluble and are not retained well at low pH. Using high pH mobile phase, however, the alkaloids are retained, with good peak shape (Figure 4A). A RRHT Extend column (Figure 4B) quickly turns the 5-minute analysis into a fast, less-than-two-minute run.

The next example, Figure 5A, is a USP method for the assay of loratadine tablets. It specifies an L7 column such as ZORBAX Eclipse XDB-C8. Loratadine is a long lasting antihistamine, the active ingredient in the all-day allergy-relief products Claritin and Alavert. USP methods have specific chromatographic conditions that must be met. In this case, the capacity factor (k') must be greater than 3.5 and the tailing factor (Tf, measured at 5% peak height) must be less than 1.7. The 4.6 × 150 mm, 5-µm Eclipse XDB-C8 column easily exceeds the USP requirements with a k' = 7, or twice the retention required, and satisfactory peak shape, Tf = 1.1. The runtime is about 40 minutes, due to tablet excipients that elute well after the loratadine. Substituting the original "analytical size" column with a shorter 4.6 × 100 mm, 3.5-µm RR column shortens the run to about 25 minutes, while still having the same chromatographic performance (Figure 5B). When an even shorter 4.6 × 50 mm, 1.8-µm Eclipse XDB-C8 RRHT column is installed (Figure 5C), runtime is further reduced to 14 minutes, again providing good peak shape and about twice the capacity (k') needed for the USP requirements. Increasing organic strength can further reduce analysis time. The high k' of loratadine and excipients on ZORBAX Eclipse XDB-C8 allows the option of using a higher organic strength, resulting in even faster elution, but this was not pursued in this study.

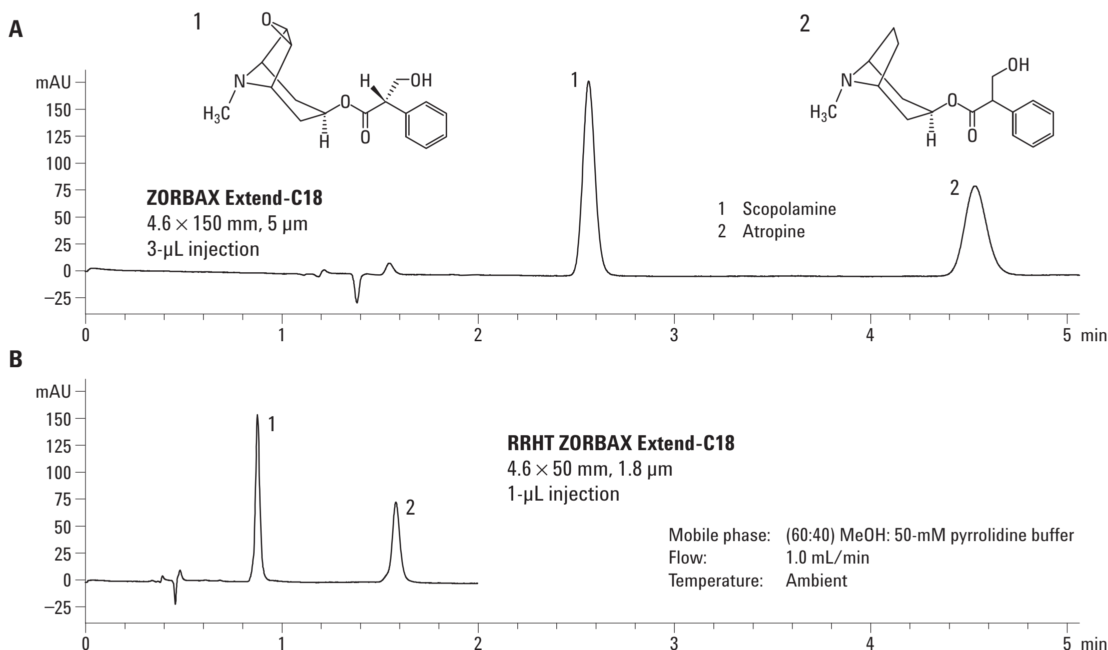

Figure 4. Assay of belladonna alkaloids at high pH on Extend-C18.

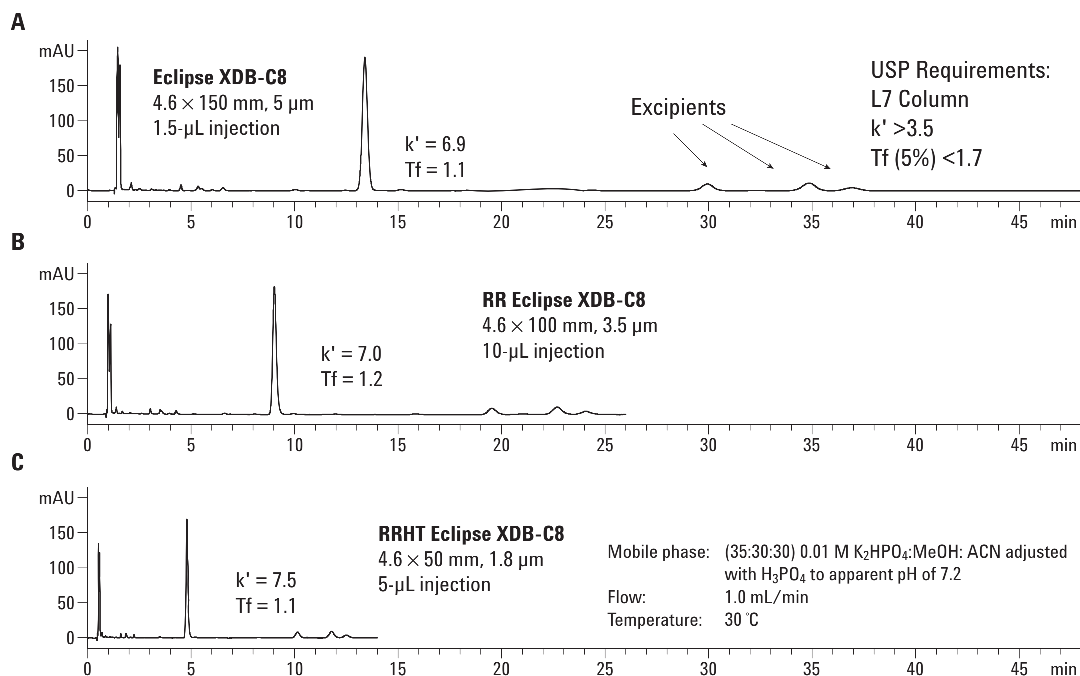

Figure 5. USP Assay for loratadine tablets on Eclipse XDB-C8.

The next RRHT analyses are sunscreen active ingredients. The original method performed on a ZORBAX Eclipse XDB-C18 takes about 15 minutes, as seen in Figure 6A. Substituting the appropriate RR or RRHT column reduces run time. See Figures 6B-C. These UV blockers were also separated using SB-C18 with an acetonitrile mobile phase. In this method, the original run time is only about 2.5 minutes. The same sequential increase in throughput is demonstrated with the appropriate StableBond RR and RRHT columns, resulting in a final analysis time of less than 1 minute (Figure 7A-C). Comparing Figure 6 to Figure 7, it is clear that the elution order differs between the Eclipse XDB-C18/methanol separation, and the StableBond-C18/acetonitrile separation. Difference in selectivity is a powerful method development tool.

Using RRHT columns for initial method development is practical and an excellent way to improve productivity. Figure 8 shows a stacked plot of a sunscreen lotion extract and the reference mix, indicating the robustness of the method, including the column. The smaller particles are as rugged as the 5-µm particles.

One purpose of this note is to demonstrate high-throughput using pre-existing methods and equipment; therefore, injection amounts for all analyses described were reduced by one-third or two-thirds, proportional to the length of the column used, to maintain the same sensitivity as the original method. If the original, larger amount was injected on a 4.6 × 50 mm, 1.8-µm RRHT column, an increase in sensitivity (S/N) of about a factor of 2, was noted. Improving sensitivity is another reason, in addition to reducing analysis time, for upgrading existing methods to fast or ultra-fast methods (Figure 9).

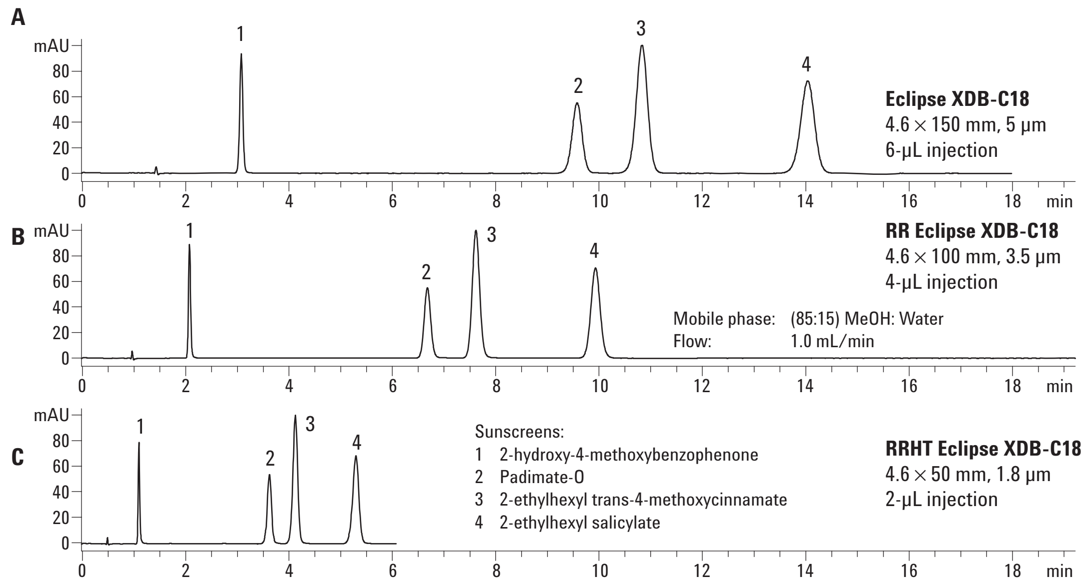

Figure 6. Sunscreens on Eclipse XDB-C18, MeOH method.

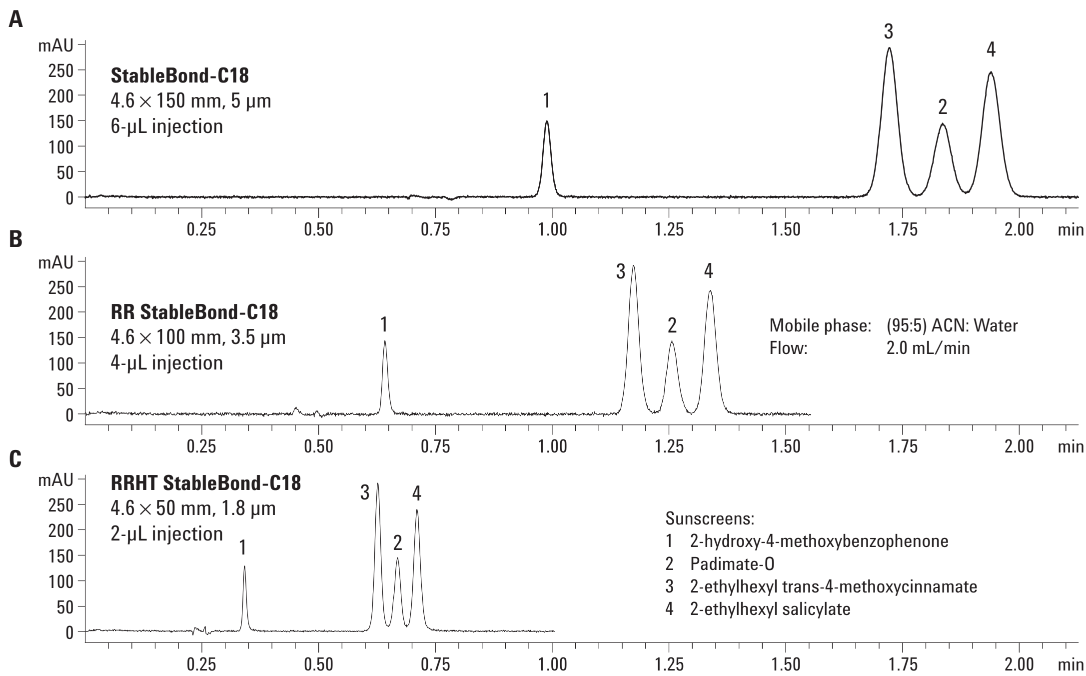

Figure 7. Sunscreens on SB-C18, ACN method.

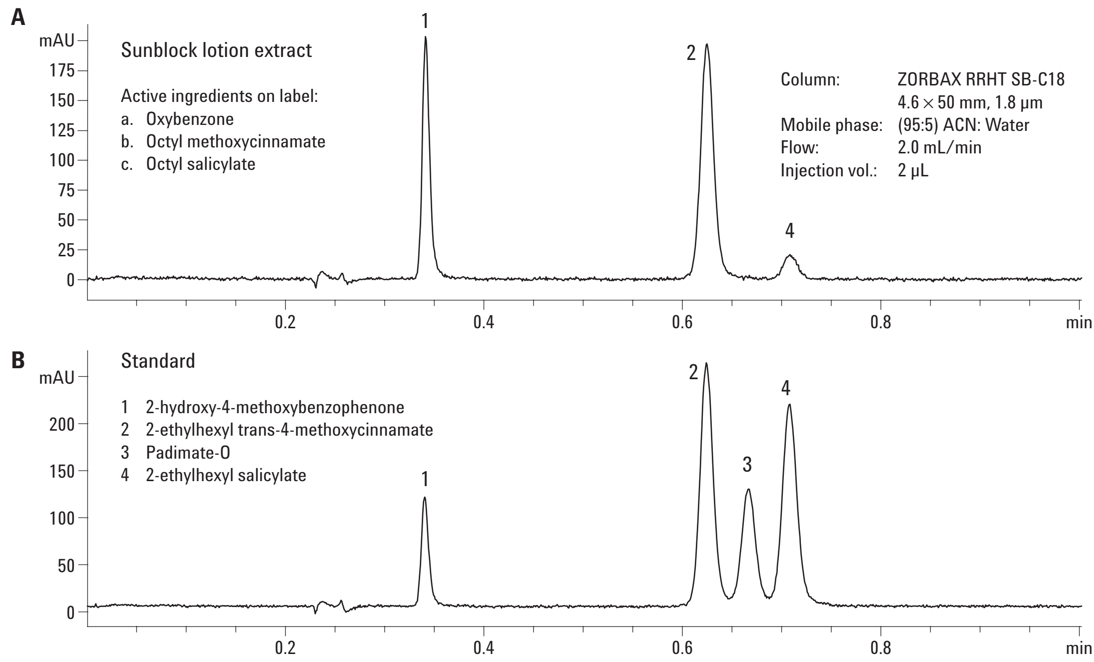

Figure 8. Ultra-fast analysis of sunblock lotion on SB-C18.

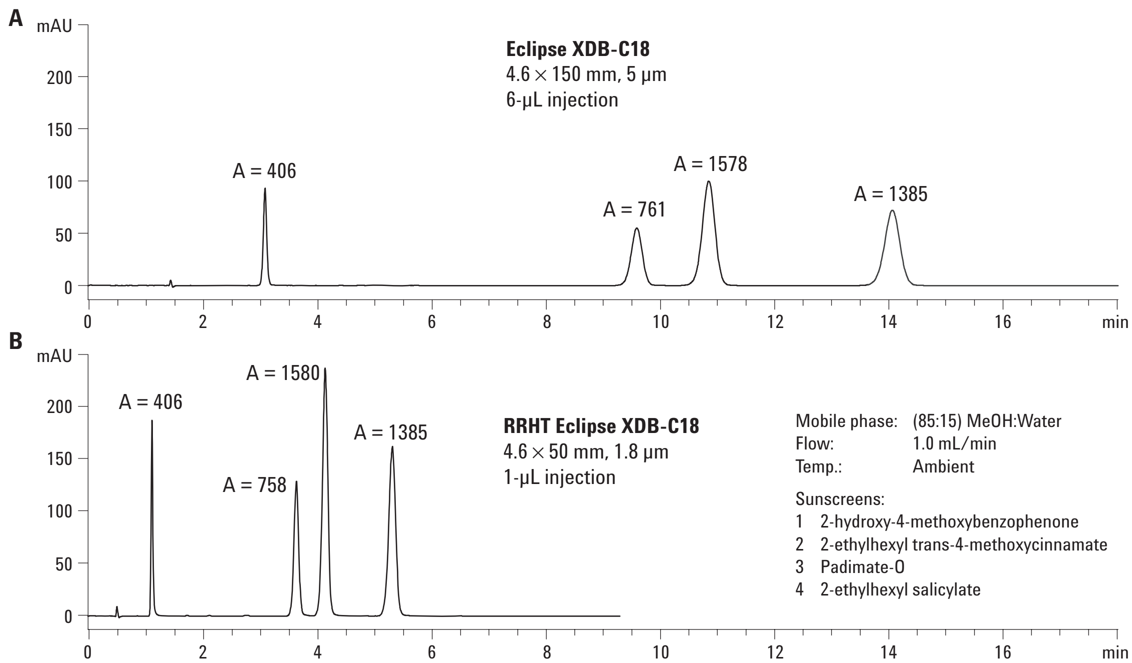

Figure 9. Increase in sensitivity (S/N) for RRHT.

Several USP examples that specify low pH mobile phase and L7 columns, such as ZORBAX Eclipse XDB-C8 or StableBond-C8, were reproduced and then made faster by installing a RR or RRHT column. Again, chromatographic performance was maintained, and analysis time is significantly reduced due to the smaller 3.5 µm and 1.8-µm particle size contained in the shorter RR and RRHT columns.

The USP assay for ibuprofen oral suspension specifies chromatographic conditions of resolution, R_s >1.5 between ibuprofen and benzophenone, (ISTD) and a Tf <2.0 for each peak. Analysis using a ZORBAX Eclipse XDB-C8 4.6 × 150 mm, 5-µm column easily provided about seven times the resolution required, as well as satisfactory peak shape (Figure 10A). This medicated syrup extract had several other components that were resolved before the peaks of interest. Substituting a 4.6 × 100 mm, 3.5-µm RR column (Figure 10B) or 4.6 × 50 mm, 1.8-µm RRHT column (Figure 10C) cut analysis time by one-third and two-thirds respectively, while still producing about seven times the resolution required and a USP Tf under 1.15. The unidentified early eluting peaks are still resolved.

Fenprofen, like ibuprofen, is a nonsteroidal anti-inflammatory (NSAID). The USP assay for fenprofen calcium has an additional chromatographic condition, theoretical plates, N. The fenprofen peak must have N >3000, or 20,000 N/meter, when using a 4.6 × 150 mm, 5-µm column. Figure 11A shows the method performed with a 4.6 × 150 mm, 5-µm SB-C8. Resolution, theoretical plates, and Tf all easily surpass the USP specifications. Although this analysis is robust and has a relatively short analysis time compared to ibuprofen oral suspension, the same principle of “Plug-&-Play” applies, to reduce retention time (RT). Figures 11B-C show the same analysis cut by one-third and two-thirds when performed with RR or RRHT columns. The plate counts for the shorter columns are about the same as the 150-mm columns since the particle size is reduced proportionally with the column length. No loss of resolving power is observed with the RR or RRHT columns.

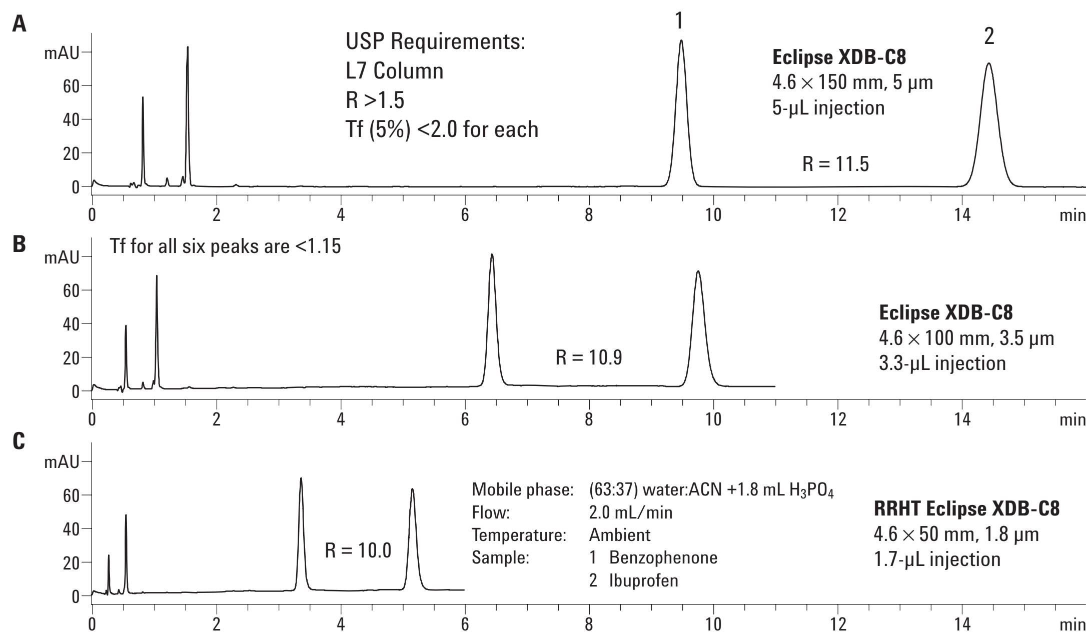

Figure 10. USP Assay for ibuprofen oral suspension on Eclipse XDB-C8.

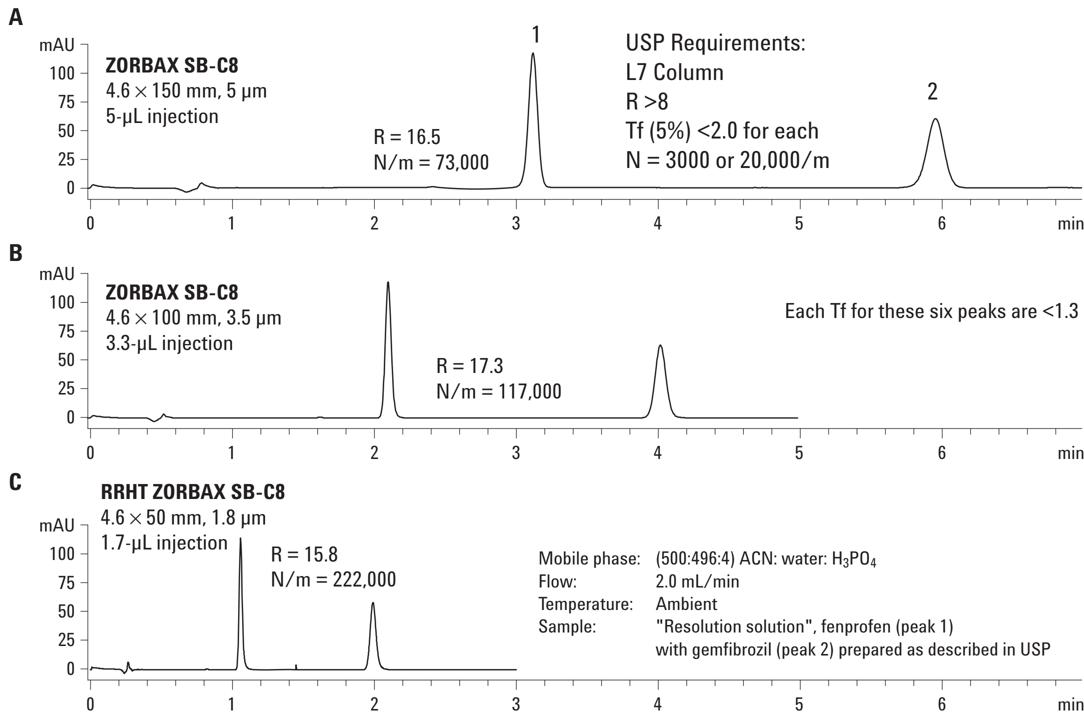

Figure 11. USP Assay for fenprofen calcium on SB-C8.

The USP assay for fenprofen calcium was also performed with a ZORBAX Eclipse XDB-C8 column (Figure 12A). The assay was subsequently made faster, and more efficient by using the shorter Eclipse XDB-C8 RR and RRHT columns (Figures 12B–C), just as it was with the shorter SB-C8 RR and RRHT columns in Figures 11B–C.

Either RRHT L7 column is suitable for improving the method; however, if improving throughput is the primary objective, Eclipse XDB-C8 is the better choice for this particular method because of its greater resolution. Optimization of the USP method by increasing organic strength would lower k', but would have higher resolution than SB-C8 under the same conditions.

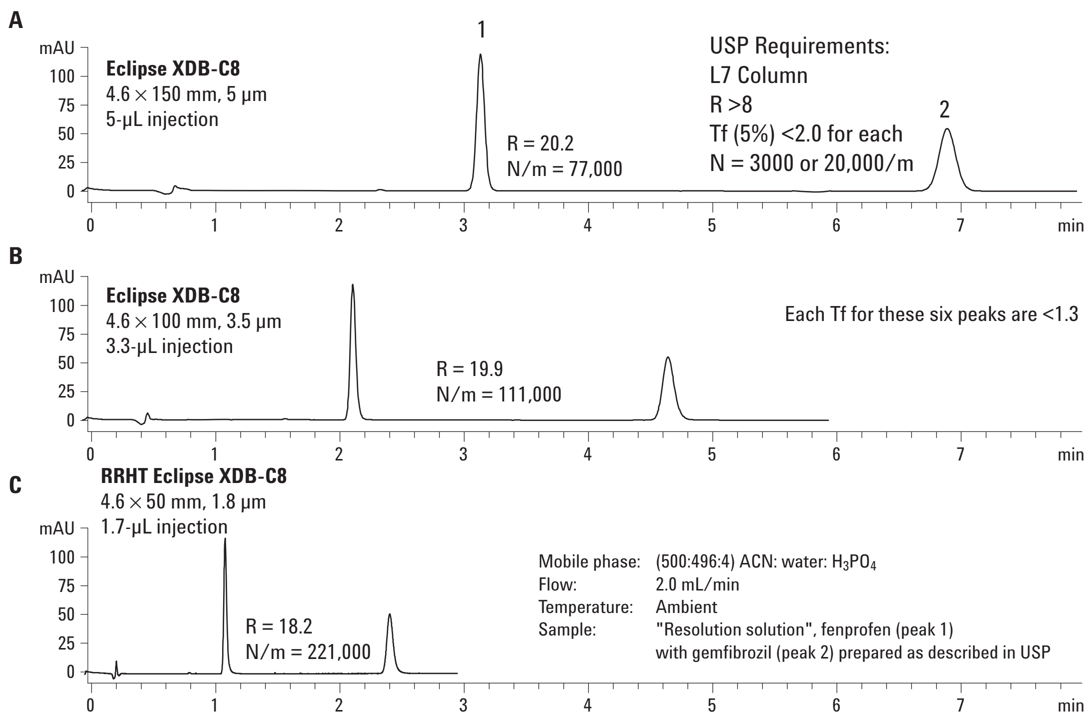

Figure 12. USP Assay for fenprofen calcium on Eclipse XDB-C8.

This USP method also specifies a flow of 2 mL/min. The Agilent 1100 LC pump operating range is up to 400 bar. Every RRHT analysis performed in this study exhibited pressures within that range. The flow rate in this case was not a major concern because the 4.6-mm id is unchanged, and the shorter column length helps offset the higher system pressure from smaller particles.

Finally, Figure 13A shows the USP assay for terazosin HCl, an alpha blocker. This drug blocks specific receptors in arteries and smooth muscle. This relaxes the blood vessels and leads to an increase in blood flow and a lower pressure for the control of hypertension. In the urinary tract, the relaxation enhances urinary flow to alleviate an enlarged prostate. The analysis is easily scaled down a third by changing to a 4.6 × 100 mm, 3.5-µm RR ZORBAX StableBond-C8 and scaled down by another third by substituting the 3.5 µm, 100-mm column with a 1.8 µm, 50-mm RRHT ZORBAX StableBond-C8 column (Figures 13B–C).

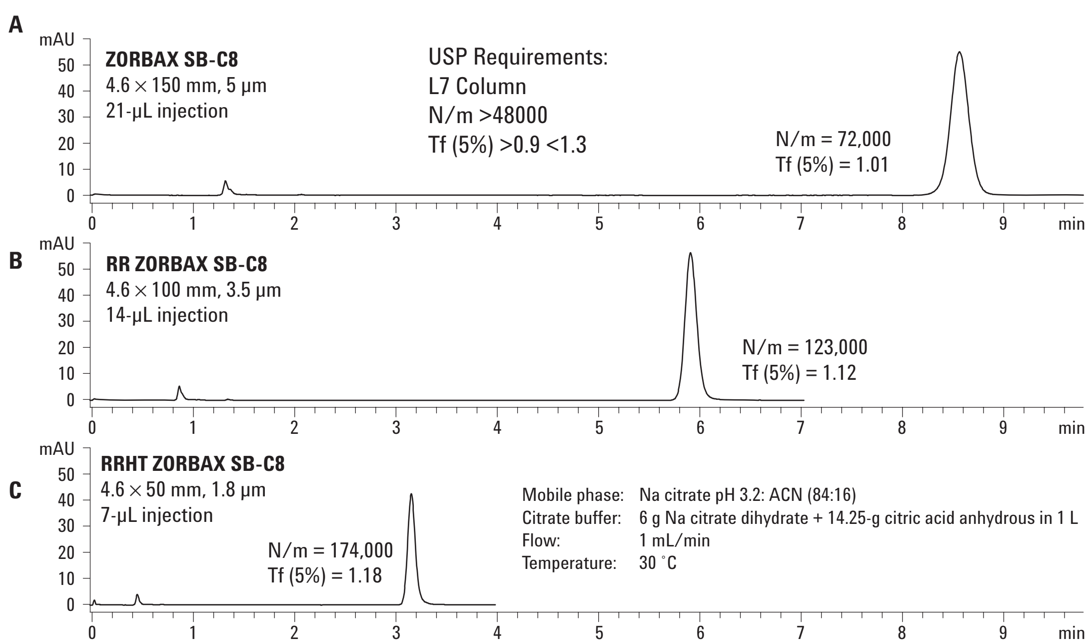

Figure 13. USP Assay for terazosin HCl on SB-C8.

Conclusions

Countless pre-existing isocratic methods already developed use “analytical-size” columns, typically 4.6 × 150 mm, with 5-µm particles. Many of these can easily be converted to high-throughput applications solely by substituting the original column with newer column technology. Smaller particle-sized columns (1.8–3.5 µm), combined with shorter lengths (50, 100 mm) of the same diameter and bonded phase, provide similar good chromatographic results as their longer 5-µm sized predecessors, in a fraction of the time. Column efficiency and resolution are maintained due to the particle size decreasing proportionally to the column length.

In this application note, over a half dozen isocratic methods originally developed with 4.6 × 150 mm, 5-µm columns were easily converted to fast or ultra-fast methods by simply substituting the original column with a 4.6-mm id, RR (100 mm) or RRHT (50 mm) column. The variety of analyses included USP methods, high pH separations, and extracts of complex matrices, such as a medicated syrup, a medicated tablet, and a sunblock lotion. New instrumentation was not necessary. Redeveloping or optimizing the method was not necessary. This included no adjustment of flow rate. System pressure for all analyses was within normal operating range of the Agilent binary pump.

Gradient methods are possible to convert to fast and ultra-fast separations, but were not included in this study because several parameters besides the column substitution would need adjustment.

Another benefit of using RR and RRHT columns, besides faster analyses, is higher sensitivity. There is a higher S/N ratio when injecting the same amounts on a shorter RR or RRHT column versus a longer 5-µm column. RR and RRHT columns are available in many configurations, including bonded phases specifically designed to exploit a wide pH range for the most rugged analyses. They are ideally suited for labs seeking to quickly improve productivity and economy without spending time or incurring high costs of new method development.

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