

## UPLC versus UHPLC: Comparison of Loading and Peak Capacity for Small Molecule Drugs

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### APPLICATION BENEFITS

- Higher loadability and peak capacity can be obtained for basic drugs using UPLC® Technology when compared to superficially-porous particle columns run on a UHPLC instrument.
- The higher pressure capability of UPLC allows the use of higher flow rates to maximize peak capacity for basic drug separations.
- UPLC Technology gives 40% higher peak capacity for basic drugs in 5X less time than superficially-porous particle columns linked together on a UHPLC system.

### WATERS SOLUTIONS

ACQUITY UPLC® columns  
ACQUITY UPLC system

### KEY WORDS

Mass loading, UPLC, UHPLC,  
peak capacity, pH, temperature,  
basic drugs

### INTRODUCTION

Recent advances in both stationary-phase chemistry and analytical instrumentation have caused chromatographers in the pharmaceutical industry to reassess how their current methods are performing, as well as how to develop new methods. Over the last 6 years, this has led to the adoption of one of three possible approaches to increase the separation power for small molecule drug compounds:

- (1) the use of superficially-porous particle columns with traditional HPLC instrumentation (5,800 psi or 400 bar)
- (2) the use of superficially-porous particle columns with UHPLC instrumentation. Here, this is defined as a system capable of operating up to 8,700 psi (600 bar)
- (3) UPLC Technology- defined as the use of sub-2  $\mu\text{m}$  particle columns in combination with low dispersion, high pressure (15,000 psi or 1034 bar) instrumentation

For conventional HPLC analysis of small molecule drugs, which tend to be more basic in chemical nature, the benefits of using fully-porous particle columns rather than superficially-porous particles have already been addressed.<sup>1</sup> The subject of this application note is to compare the use of superficially-porous particle columns on UHPLC instruments and UPLC Technology with regards to mass loading and peak capacity for small molecule drug separations.

## Sample Description:

For isocratic loading studies, amitriptyline and diphenhydramine were prepared at concentrations of 2, 10, 20, 50, 100, and 200 ng/ $\mu$ L in 50:50 acetonitrile/water.

The mixture of basic compounds contained 10  $\mu$ g/mL each of uracil (void marker) and prednisone, 20  $\mu$ g/mL each of pindolol, quinine, and diltiazem, 50  $\mu$ g/mL of amitriptyline, and 100  $\mu$ g/mL of labetalol.

## Method Conditions:

### LC conditions:

UHPLC experiments were performed on a system capable of operation up to 600 bar (~8,700 psi). The system was configured to give the lowest possible gradient delay volume and dispersion by the manufacturer. The gradient delay volume was 170  $\mu$ L. This was measured using a previously published protocol.<sup>2</sup>

UPLC experiments were performed on an ACQUITY UPLC system equipped with an ACQUITY® PDA detector. The system was used as received with no modifications to tubing, sample loops, etc. The measured gradient delay volume for this system was 104  $\mu$ L.

Other conditions can be found in the figure captions.

### Data management:

Empower™ 2 CDS

## RESULTS AND DISCUSSION

### Loading Capacity

Maximizing the amount of sample that is loaded onto a chromatographic column has implications for impurity profiling and stability indicating methods, where a large amount of an active pharmaceutical ingredient (API) is injected in order to detect low-level impurities in the sample. As more material is loaded onto the column, the peak becomes wider, thereby decreasing column efficiency and resolution. The loading capacity of fully- and superficially-porous particle columns was compared using two common basic drugs (Figure 1). For both amitriptyline and diphenhydramine, the fully-porous particle columns have 2- to 3-times higher loading capacity than the superficially-porous particle column under the same mobile-phase conditions. The difference between the 1.7  $\mu$ m fully-porous particle column and the 2.7  $\mu$ m superficially-porous particle column is even greater (almost 4-fold) at low mass loads, indicating that the superficially-porous particle column is even becoming overloaded at the very low mass loads used for routine analysis of these drugs. In order to maximize the loading capacity for these basic compounds, a high pH mobile phase was used with the 1.7  $\mu$ m ACQUITY UPLC BEH C<sub>18</sub> column. Since the analytes are neutral at high pH, they have greater retention and better peak shape. As shown in Figure 1, this column shows a marked increase in efficiency at high pH, and essentially maintains the same efficiency even at high mass loads. Running at high pH is not possible with superficially-porous silica columns, which are only stable between pH 2 and 9.

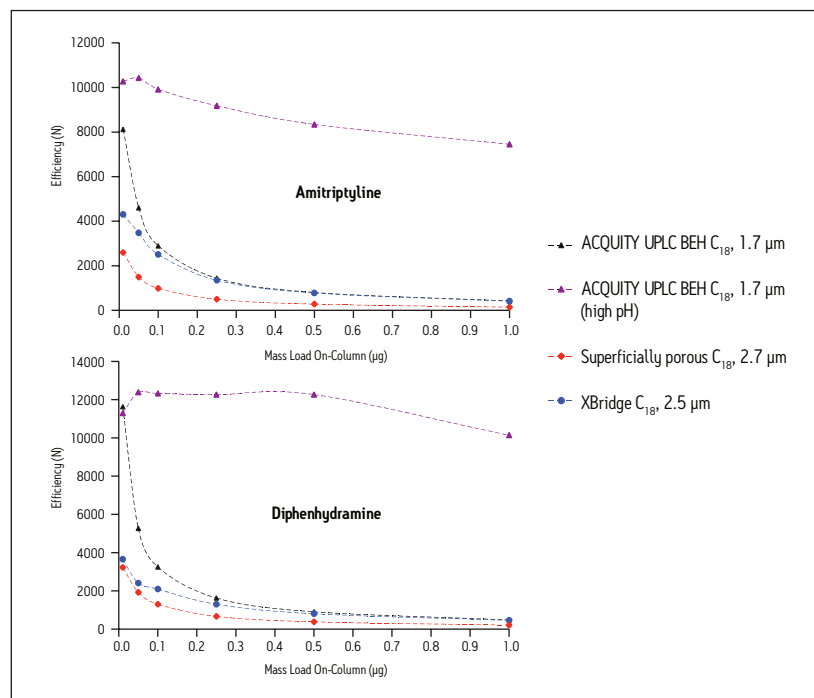


Figure 1. Efficiency of fully- and superficially-porous particle columns as a function of mass load. Efficiency was calculated using the peak width at 4.4% peak height (5 $\sigma$ ). Data were corrected for extra-column band spreading. The isocratic mobile phase was either 32% acetonitrile, 68% 10 mM ammonium formate, pH 3.17 or 57% acetonitrile, 43% 10 mM ammonium bicarbonate, pH 10. Flow rate was 0.5 mL/min. Column temperature was 30 °C. Injection volume was 5  $\mu$ L, and UV detection was performed at 240 nm. Sampling rate was 40 Hz with no filter time constant. Thiourea was used as the void marker.

## Combining the column and the system

The separation of small molecule drugs does not depend just on the column and mobile-phase conditions being used, but also depends on how the column and chromatographic system work together to achieve the desired separation. Highly efficient, small particle columns cannot be run on conventional LC instrumentation and produce the performance predicted by theory. They need to be run on systems specifically designed to have low system dispersion and high pressure capability. To illustrate this, a mixture of basic drugs was separated using UPLC (sub-2  $\mu\text{m}$  particle column on an ACQUITY UPLC system) and UHPLC (2.7  $\mu\text{m}$  superficially-porous particle column on a UHPLC system). The ACQUITY system was used without modifications, and the UHPLC system was modified to achieve the lowest possible system dwell volume per the manufacturer's recommendations. Figure 2 shows the comparison of UPLC and UHPLC for the separation of some common small molecule drugs. The UPLC separation results in about a 54% increase in peak capacity over the UHPLC separation. In addition, the UPLC separation appears to have 3- to 4-fold higher signal intensity.

The reasons for these differences in performance are numerous. First, as shown in Figure 1, superficially-porous particle columns become overloaded, even at very low mass loads for basic compounds, whereas fully-porous particles do not overload as quickly. Second, in order to achieve the low gradient delay volume on the UHPLC system, certain components of the system must be by-passed, including the pump pulse dampener, injection loop, etc. This is most likely the reason for the disturbances in the baseline seen early in the UHPLC chromatogram. In order to achieve the low system delay volume on the UHPLC system, the sample loop is taken off-line after the injection. It is likely that the baseline disturbances seen in the UHPLC separation are a result of the injector valve cycling between the load and inject positions during this process. Finally, there are differences between the detector cells and settings of the two systems.

Since the instrument and column have to work together to produce high quality data, sacrificing the performance of one will ultimately lead to sub-optimal performance of the entire system. For the data shown in Figure 2, it is clear that better separation performance can be achieved with UPLC when compared to UHPLC, especially for basic drugs. This was confirmed by running the 1.7  $\mu\text{m}$  fully-porous particle column on the UHPLC system under the same conditions and observing a 38% loss in peak capacity, which is simply due to the contributions of the chromatographic system (data not shown).

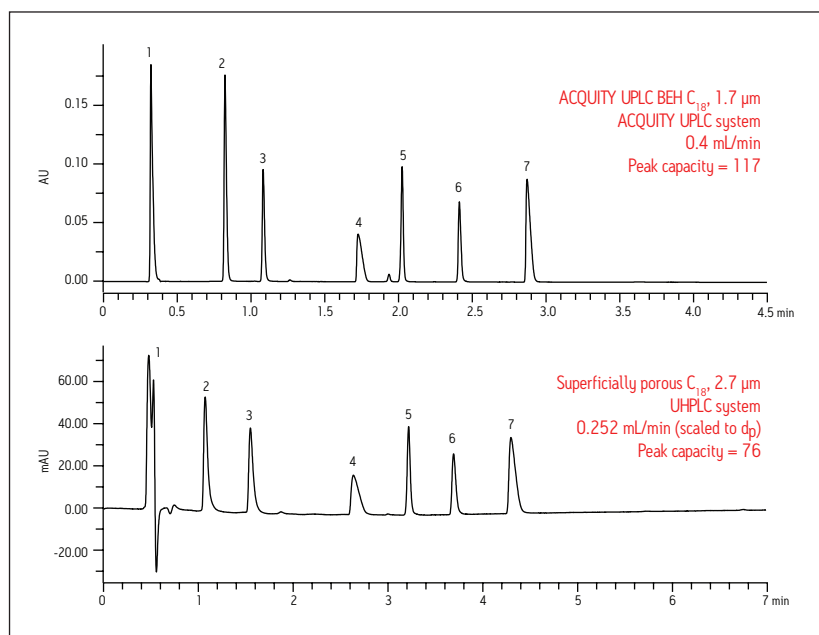


Figure 2: Peak capacity comparison between UPLC and UHPLC for basic drugs. Mobile phase A was 10 mM ammonium formate, pH 3. Mobile phase B was 100% acetonitrile. The UPLC gradient was 15-65% B in 4.6 minutes. The flow rate was scaled to the particle size ( $d_p$ ) for the superficially-porous particle column. The gradient was then recalculated to maintain constant column volumes during the separation. Peaks: (1) uracil, (2) pindolol, (3) quinine, (4) labetalol (racemic), (5) prednisone, (6) diltiazem, (7) amitriptyline. Separation temperature was 30 °C. All columns were 2.1 x 50 mm. UV detection at 260 nm. Injection volume was 2  $\mu\text{L}$ .

## Increasing Peak Capacity

One way to increase the separation performance and obtain higher peak capacities for small molecule drug separations is to link more than one column together in series. This basically takes advantage of the additional theoretical plates, on which the peak capacity has a square root dependence. In other words, if you double the number of theoretical plates in a separation, you increase the peak capacity by about 40%. Since the back pressure of superficially-porous particle columns is ~ 50% of 1.7  $\mu\text{m}$  BEH columns, it has been proposed that these columns can be linked together to achieve “UPLC-like” performance without the need for high pressure instrumentation.<sup>3</sup> Figure 3 shows the separation of a mixture of basic drugs using such an approach. In this case, three 150 mm superficially-porous particle columns were linked together to achieve a peak capacity of 261 on a UHPLC system. This separation was optimized at a temperature and flow rate that gave the highest peak capacity for this set of compounds under these mobile-phase conditions. Based on theory, we should expect a 3-fold increase in peak capacity on the 450 mm length column when compared to the 50 mm length column in Figure 2. However, we actually observe a bigger gain in peak capacity than what is expected (~3.5 times). This could be due to a couple of factors, including the use of higher temperature, a massive gain in gradient time (which is used to calculate peak capacity), and/or a smaller contribution from the extra-column volume since the column volume is increased by 9X. The main limitation of this approach is the maximum operating pressure of the chromatographic system.

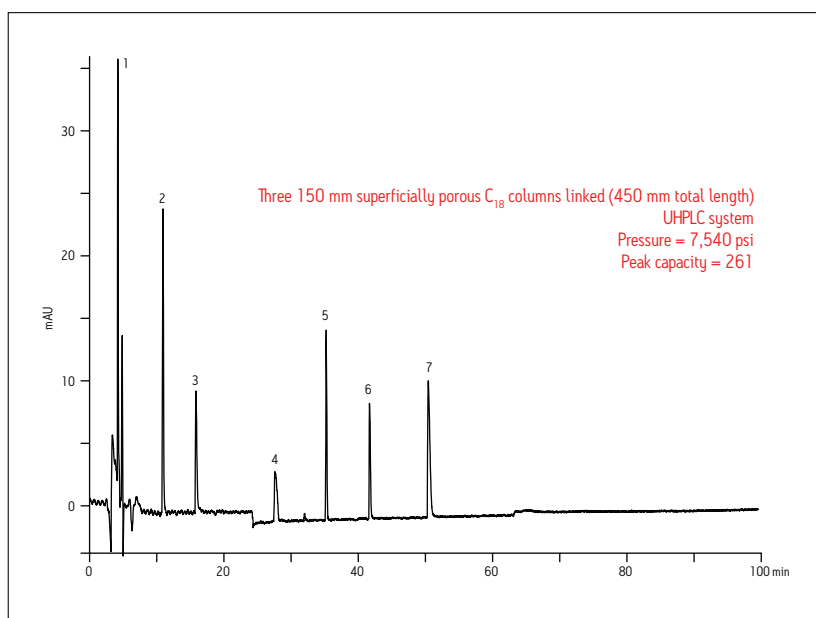


Figure 3: Increasing peak capacity in UHPLC. Three 150 mm (2.7  $\mu\text{m}$ ) superficially-porous particle columns were linked together in series and run on the UHPLC system. Mobile phases and peak ID's are identical to Figure 2. Gradient was from 15-65% B in 104 minutes. Separation temperature was 50  $^{\circ}\text{C}$ . UV detection at 260 nm. Flow rate was 0.22 mL/min. Injection volume was 2  $\mu\text{L}$ .

Another way to increase peak capacity is to increase the flow rate while maintaining the same gradient time. In Figure 4, the peak capacity for a mixture of basic drugs is compared as a function of flow rate for three different columns. The 3.5  $\mu\text{m}$  fully-porous and 2.7  $\mu\text{m}$  superficially-porous particle columns give essentially the same peak capacity up to a flow rate of about 0.6 mL/min. Above this, the superficially-porous column gives about 10% higher peak capacity compared to the 3.5  $\mu\text{m}$  fully-porous particle column, most likely due to the smaller contribution of diffusion-related band broadening at higher flow rates. Both the 2.7  $\mu\text{m}$  and 3.5  $\mu\text{m}$  particle columns are limited to flow rates of about 1.2 mL/min due to the pressure limitations of the columns. Since the ACQUITY UPLC system and 1.7  $\mu\text{m}$  UPLC columns can both be operated at pressures up to 15,000 psi, 20% higher peak capacities can be achieved for small molecule drug separations using this approach when compared with superficially-porous particle columns.

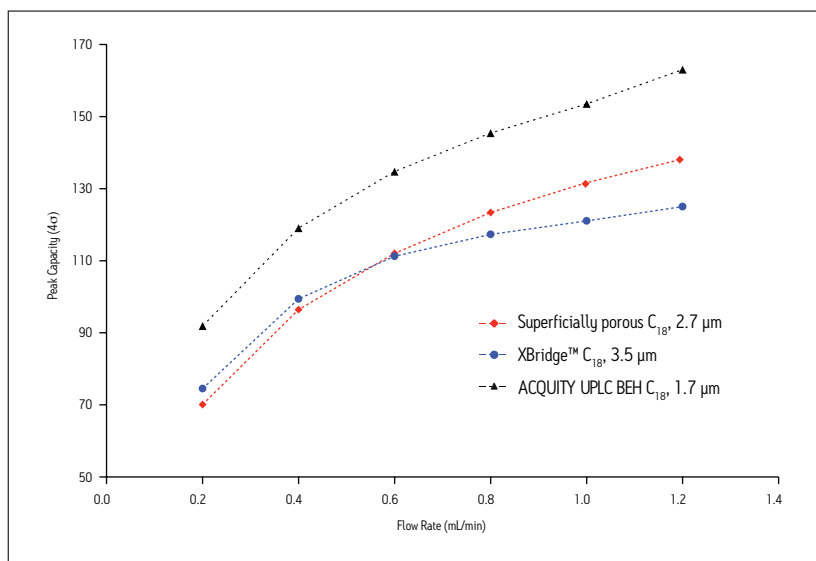


Figure 4: Peak capacity for basic compounds as a function of flow rate for three different columns. Column dimensions were 2.1 x 50 mm. Gradient time remained constant at 5 minutes. The peak capacity is an average of all peak widths from the compounds used in Figures 2 and 3. All other conditions are identical to Figure 2.

Finally, an increase in peak capacity can be achieved by combining high pressure, high temperature, and elevated mobile-phase pH in UPLC. Since most small molecule drugs are basic compounds, they are ionized at low pH, which causes them to overload on the stationary phase even at mass loads that are typical for drug analysis.<sup>4</sup> To improve peak shape and retention for basic compounds, a high pH mobile phase must be used. When combined with elevated temperature and pressure on 1.7  $\mu\text{m}$  particles that are stable under these conditions, a dramatic increase in peak capacity can be observed (Figure 5). When compared with linked columns containing superficially-porous particles on a UHPLC instrument (Figure 3), UPLC can give 40% higher peak capacity for basic drugs in 5-fold less time under optimized pressure, temperature, and mobile-phase conditions. In addition, having the ability to use mobile phases with extreme pH on bridged-ethylene-hybrid (BEH) particles allows for alternate selectivity during small molecule drug development.



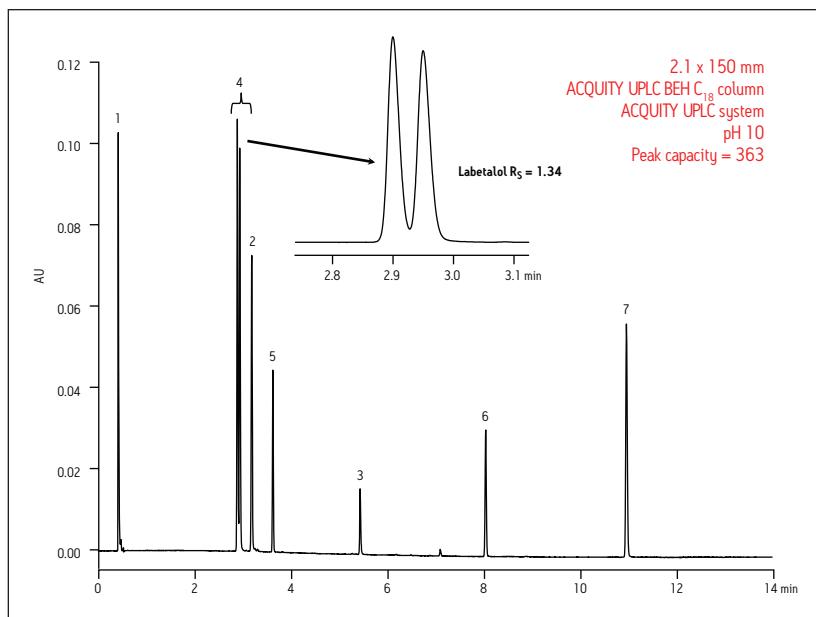


Figure 5: Separation of basic compounds at pH 10 on a 2.1 x 150 mm, 1.7  $\mu$ m ACQUITY UPLC BEH C<sub>18</sub> column. Mobile phase A was 10 mM ammonium bicarbonate, pH 10. Mobile phase B was acetonitrile. The gradient was 15-65% B in 11.74 min. The flow rate was 0.75 mL/min. Separation temperature was 60 °C. Injection volume was 2  $\mu$ L. See Figure 2 for peak ID.

## CONCLUSIONS

- UPLC using fully-porous particle columns gives 3 times higher loading capacity for basic drugs than superficially-porous particle columns on a UHPLC system.
- The loading capacity for basic compounds in UPLC can be further increased (~10-fold) using high pH mobile phases.
- UPLC gives higher peak capacity separations for bases than UHPLC.
- The most effective way to increase peak capacity for basic drug separations is to combine high pressure, high temperature, and elevated mobile-phase pH on UPLC columns and instrumentation.

## References

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