Ultra Performance LC™
Separation Science Redefined
For Pharmaceutical Confidence

From discovery to manufacturing, Waters has the tools you need to create a successful pharmaceutical product. Our ever-expanding range of technologies and services — including sample preparation, separation, detection, information management and more — helps make your process more efficient, so your products get to market faster. Add to that the dependability and accuracy you’ve come to expect from Waters and you’ll see that Waters is the only place to turn for complete solutions — and for complete confidence. Learn more at www.waters.com/pharma
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In 2004, Waters introduced a new category of LC technology that has changed separation science forever.

We call it ACQUITY Ultra Performance LC or UPLC.

Based on novel chemistry and instrumentation, UPLC delivers new levels of resolution, speed and sensitivity.

I am pleased to report that interest in UPLC from scientists worldwide has been outstanding, from those looking to drive new products through the development process faster, to those looking for a more robust technique for routine analytics, to researchers who need to push MS performance to the next level.

Scientists are now realizing they can do and see with UPLC what they couldn’t do and see with HPLC. Some of their exciting work is found in the pages of this supplement. As you will see, the benefits are real and they are compelling.

Throughout 2005, Waters will be adding to its existing portfolio of UPLC products. As recently as March, Waters introduced three new chemistries: a C8, phenyl, and a Shield RP18, as well as an evaporative light scattering detector, further expanding applications for UPLC.

Waters is committed to working with you to gain insight into your daily challenges and use this understanding to direct our technology development efforts. We remain the only company that can offer you a comprehensive set of technologies for UPLC with one goal in mind: to get you quality information — faster.

UPLC is here to stay and we are confident it will continue to have a lasting impact in labs around the globe.

Art Caputo
President, Waters Division
Waters Corporation
WE’RE A LOT MORE THAN YOU THINK
SO YOU CAN DO A LOT MORE THAN YOU THOUGHT

For Complete Confidence

Today, we have more technologies and capabilities than ever before. From Chemistry and Chromatography to Informatics, Mass Spectrometry and Support Services, we have systems and solutions that are right for you. Solutions that will give you more answers. More time to look for them. More speed and accuracy. More efficiency and productivity. More service. More uptime and less downtime. More reliability and less impossibilities. We’re a partner who will work with you to understand your needs and fuel your success. A partner who can give you more confidence in your work than ever before. To learn more, visit www.waters.com
We hope you find this special Ultra Performance Liquid Chromatography supplement to *LCGC* useful and informative. Each of the manuscripts presents a unique view and use of this exciting new technology.

The first article serves as an introduction, highlighting the theory and implementation of UPLC, including the technological strides necessary in chemistry and instrumentation in order to capitalize on UPLC’s increased speed, sensitivity, and resolution. The article goes on to describe how UPLC can be used in both drug discovery (in-vitro metabolism) and environmental applications.

The next article describes the use of UPLC in pharmaceutical development. The benefits of fast method development increasing sample throughput and laboratory productivity, and an example of an eight-fold reduction in analysis time, without compromising resolution, are reported.

The third article further investigates drug discovery applications, with UPLC coupled to orthogonal quadrupole time-of-flight–mass spectrometry (TOF–MS). The authors show significant gains afforded by UPLC over conventional capillary-scale liquid chromatography–mass spectrometry (LC–MS) for metabolite identification and MS spectral quality.

In the next article, an HPLC assay is converted and optimized for UPLC, achieving both higher sample analysis throughput and better assay sensitivity. A general strategy for method conversion is summarized, and an analysis of operation costs and sample throughput found UPLC superior to HPLC in the quality control (QC) laboratory.

The fifth article examines the use of 1 minute high-speed UPLC separations for dose formulation strength analysis. The chromatographic parameters evaluated include retention time and peak reproducibility, as well as resolution and column ruggedness in isocratic and gradient separations.

The final article provides details on the considerations taken when developing a new chromatographic particle for UPLC separations. Information on column stability under aggressive testing conditions is included. Additionally, information on several new column chemistries to provide the utmost flexibility for methods development is reported.

Also included in this supplement is an up-to-date bibliography of published UPLC references the reader can consult for more information.

At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography and obtain quality results faster, redefining separation science.

Your comments and thoughts are always welcome.

Michael Swartz, Ph. D.
Principal Scientist, Waters Corporation, and *LCGC* Editorial Advisory Board.
michael_swartz@waters.com
When quantitative analysis presents difficult challenges, Waters is the obvious solution. Combining the revolutionary ACQUITY UPLC™ System with Waters’ Quattro family of mass spectrometers, you can achieve fast, reliable, highly sensitive LC/MS/MS analyses with ease. Robust and reproducible results are assured with the Quattro micro™ and its ESCi™ multi-mode ionization capabilities. The Quattro Premier™ adds advanced T-Wave™ technology for unmatched speed, sensitivity and specificity. And data processing and method development tasks are simplified with dedicated software application managers, including QuanLynx™, QuanOptimize™ and TargetLynx™. With Waters, all the tools for success are at your fingertips. To find out more about our quantitative analysis solutions, visit www.waters.com/quan.
Ultra Performance Liquid Chromatography (UPLC): An Introduction

High performance liquid chromatography (HPLC) is a proven technique that has been used in laboratories worldwide over the past 30-plus years. One of the primary drivers for the growth of this technique has been the evolution of packing materials used to effect the separation. The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). Since particle size is one of the variables, a van Deemter curve can be used to investigate chromatographic performance.

According to the van Deemter equation, as the particle size decreases to less than 2.5 µm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rates or linear velocities. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time in gradient separations) can be extended to new limits, termed Ultra Performance Liquid Chromatography, or UPLC. The technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, with superior resolution and sensitivity.

Figure 1 shows a stability indicating assay of five related substances accomplished in under one minute, proving that the resolving power of UPLC is not compromised even at high speed. The current USP lists multiple HPLC methods for the analysis of these same compounds with run times approaching 20 min, with broad, tailed peaks.

Chemistry of Small Particles

As shown in Figure 1, smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as HPLC. In the fundamental resolution (Rs) equation:

\[ \text{Rs} = \sqrt{\frac{N}{4}} \left( \frac{1}{\alpha} \right)^{\frac{k}{k+1}} \]

resolution is proportional to the square root of \( N \). But since \( N \) is inversely proportional to particle size (dp):

\[ N \propto \frac{1}{dp} \]

as the particle size is lowered by a factor of three, from, for example, 5 µm (HPLC-scale) to 1.7 µm (UPLC-scale), \( N \) is increased by three and resolution by the square root of three or 1.7. \( N \) is also inversely proportional to the square of the peak width.

Michael E. Swartz, Ph.D.
Principal Scientist, Waters Corporation, Milford, Massachusetts, e-mail Michael_Swartz@waters.com.
This illustrates that the narrower the peaks are, the easier they are to separate from each other. Also, peak height is inversely proportional to the peak width:

\[ N \propto \frac{I}{w} \]

\[ H \propto \frac{I}{w} \]

So as the particle size decreases to increase \( N \) and subsequently \( R_s \), an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient separations, desirable for many applications, e.g., peptide maps.

Still another equation comes into play when migrating toward smaller particles:

\[ F_{opt} \propto \frac{1}{dp} \]
This relationship also is revealed from the van Deemter plot. As particle size decreases, the optimum flow \( F_{opt} \) to reach maximum \( N \) increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressures, and a system properly designed to capitalize on the efficiency gains; a system that can both reliably deliver the requisite pressures and that can maintain the separation efficiency of the small particles with tightly managed volumes.

Higher resolution and efficiency can be leveraged even further, however, when analysis speed is the primary objective. Efficiency is proportional to column length and inversely proportional to the particle size:

\[
N \propto \frac{L}{dp}
\]

Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to the smaller particles and shortening the column by one third (again due to the smaller particles), the separation is completed in \( 1/9 \) the time while maintaining resolution. So if speed, throughput, or sample capacity is a concern, theory can be further leveraged to get much higher throughput.

But the design and development of sub-2 \( \mu \)m particles is a significant challenge, and researchers have been active in this area for some time, trying to capitalize on their advantages (2–4). Although high efficiency, nonporous 1.5-\( \mu \)m particles are commercially available, they suffer from poor loading capacity and retention due to low surface area. Silica-based particles have good mechanical strength but can suffer from a number of disadvantages, which include a limited pH range and tailing of basic analytes. Polymeric columns can overcome pH limitations, but they have their own issues including low efficiencies, limited loading capacities, and poor mechanical strength.

In 2000, Waters introduced XTerra®, a first generation hybrid chemistry that took advantage of the best of both the silica and polymeric column worlds. XTerra columns are mechanically strong, with high efficiency, and operate over an extended pH range. They are produced using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups. But in order to
provide the necessary mechanical stability for UPLC, a second generation bridged ethyl hybrid (BEH) technology was developed. Called ACQUITY BEH, these 1.7-μm particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix.

Packing 1.7-μm particles into reproducible and rugged columns was also a challenge that needed to be overcome. Requirements include a smoother interior surface of the column hardware and re-designing the end frits to retain the small particles and resist clogging. Packed bed uniformity is also critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations. All ACQUITY UPLC BEH columns also include eCord™ microchip technology that captures the manufacturing information for each column, including the quality control tests and certificates of analysis. When used in the Waters® ACQUITY UPLC™ System, the eCord database is also updated with real time method information, such as the number of injections, or pressure and temperature information, to maintain a complete continuous column history.

**Capitalizing on Smaller Particles**

Instrument technology also had to keep pace to truly take advantage of the increased speed, superior resolution and sensitivity afforded by smaller particles. Standard HPLC technology simply doesn’t have the capability to take full advantage of sub-2 μm particles. Standard HPLC technology simply doesn’t have the capability to take full advantage of sub-2 μm particles. A completely new system design with advanced technology in the solvent and sample manager, auto sampler, detector, data system, and service diagnostics is required.

The ACQUITY UPLC System has been holistically designed for low system and dwell volume to minimize dispersion and take full advantage of small particle technology.

As alluded to previously, achieving small particle, high peak capacity separations requires a greater pressure range than that achievable by today’s HPLC instrumentation. The calculated pressure drop at the optimum flow rate for maximum efficiency across a 15-cm long column packed with 1.7-μm particles is approximately 15,000 psi. Therefore, a pump capable of delivering solvent smoothly and reproducibly at these pressures and that can compensate for solvent compressibility, while operating in both the gradient and isocratic separation modes is required.

With 1.7-μm particles, half-height peak

![Image](https://example.com/image.png)

**Figure 4:** Extracted ion chromatograms for major N and O dealkylated and double de-alkylation metabolites of dextromethorphan by HPLC/Tof MS. (Reproduced with permission from reference 12, copyright John Wiley and Sons Limited 2005.)
widths of less than one second can be obtained, posing significant challenges for the detector. In order to accurately and reproducibly integrate an analyte peak, the detector sampling rate must be high enough to capture enough data points across the peak. In addition, the detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2–3 times higher than HPLC separations, depending on the detection technique. MS detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting) promotes increased source ionization efficiencies (reduced ion suppression) for improved sensitivity.

Sample introduction is also critical. Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from experiencing extreme pressure fluctuations, the injection process must be relatively pulse-free. The swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to realize the increased sensitivity benefits.

The ACQUITY UPLC System consists of a binary solvent manager, sample manager (including the column heater), detector, and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure. There are built-in solvent degassing as well as solvent select valves to choose from up to four solvents. There is a 15,000 psi pressure limit (about 1000 bar) to take full advantage of the sub-2-µm particles. The sample manager also incorporates several technology advancements. Low dispersion is maintained through the injection process using pressure assist sample introduction, and a series of pressure transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and a needle calibration sensor increases accuracy. Injection cycle time is 25 s without a wash and 60 s with a dual wash used to further decrease carry over. A variety of microtiter plate formats (deep-well, mid-height, or vials) can also be accommodated in a thermostatically controlled environment. Using

**Figure 5:** Extracted ion chromatograms for major N and O dealkylated and double de-alkylation metabolites of Dextromethorphan by UPLC/Tof MS. (Reproduced with permission from reference 12, copyright John Wiley and Sons Limited 2005.)
the optional sample organizer, the sample manager can inject from samples from up to 22 microwell plates. The sample manager also controls the column heater. Column temperatures up to 65 °C can be attained. A “pivot out” design provides versatility to allow the column outlet to be placed in closer proximity to the source inlet of an MS detector to minimize excess tubing and sample dispersion.

The tunable UV–vis and PDA detectors include new electronics and firmware to support Ethernet communications at the high data rates necessary for UPLC detection. Conventional absorbance-based optical detectors are concentration-sensitive detectors, and for UPLC, the flow cell volume would have to be reduced in standard UV–vis detectors to maintain concentration and signal. However, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends; and worse: a reduction in cross-section means the light path is reduced, and transmission drops, increasing noise. Therefore, if a conventional HPLC flow cell is used, UPLC sensitivity would be compromised. The ACQUITY UPLC System detector cell consists of a light guided flow cell equivalent to an optical fiber. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10-mm flow cell path length with a volume of only 500 nL. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems.

**Applications**

Scientists are used to making compromises; and one of the most common scenarios involves sacrificing resolution for speed. With UPLC increased resolution in shorter run times can generate more information faster without sacrifices.

Higher sample throughput with more information per sample may decrease the time to market, an important driving force in today’s pharmaceutical industry.

Figure 2 illustrates a method converted from HPLC to UPLC that takes advantage of the speed of UPLC. The corresponding HPLC separation takes in excess of 12 min; UPLC accomplishes the same separation in under 30 s.

UPLC can also be used to significantly improve the success of the drug discovery process. Drug discovery is heavily dependant upon the early prediction of metabolic fate and interactions of drug candidate molecules. To prevent “poor” candidates from progressing through the discovery process, factors such as metabolic stability, toxic metabolite production, p450 inhibition, and induction are all routinely monitored. By the mid-1990s, high performance liquid
chromatography (HPLC) directly coupled to mass spectrometry (MS) was in routine use in drug metabolism laboratories for these types of studies (5–12). Enhanced selectivity and sensitivity, and rapid, generic gradients made LC–MS the predominate technology for both quantitative and qualitative analyses. However, with the ever-increasing numbers and diversity of compounds entering development, and the complex nature of the biological matrices being analyzed, new analytical procedures and technology were required to keep pace with the testing demands. Unexpected, reactive, or toxic metabolites must be identified as early as possible to reduce the very costly attrition rate. This quest for more accurate data meant improving the chromatographic resolution to obtain higher peak capacity, reducing the co-elution of metabolites, while enhancing the sensitivity and decreasing ion suppression in the MS.

The power of the ACQUITY UPLC System when used in drug discovery can be illustrated by the analysis of the in-vitro metabolism of dextromethorphan. Dextromethorphan undergoes O-dealkylation in two positions leading to three major phase I metabolites. These products can be further metabolized via conjugation with glucuronic acid to form metabolites of masses $\text{MH}^+ = 434$ and 420. The data in Figure 3a and 3b shows the HPLC–MS and UPLC–MS separations, respectively, of the in-vitro incubation of dextromethorphan with rat liver microsomes. As shown, the chromatographic performance of the ACQUITY UPLC BEH 1.7-µm particles is significantly better than that produced by the 3.5 µm material. The 1.7-µm material gives peaks of width 4 s at the base, resulting in a peak capacity of over 100, whereas with HPLC the average peak width was 20 s at the base giving a total peak capacity of just 20, resulting in a 5-fold increase in the performance of the UPLC system.

The extracted ion chromatogram $m/z = 258$ and $m/z = 244$ for the HPLC/MS analysis is shown in Figure 4.

In Figure 4, we can clearly see the two O-dealkylated metabolites of dextromethorphan $m/z = 258$, these two metabolites are resolved to about 80%, while the 244 metabolite is barely visible. These results can be compared to those obtained by UPLC, here we can see that the two 258 ions are clearly resolved and that the 244 ion is now easily detected, as illustrated in Figure 5.

This data clearly illustrates the improved resolution and sensitivity of the UPLC system. This extra resolution is particularly important when analyzing isobaric compounds such as these dealkylated metabolites. By incorporating a more efficient UPLC separation into the MS there is less ion suppression from competing compounds in the source and therefore more discreet ionization of the metabolites. Without the resolution generated by UPLC it would be possible to falsely assign the structure of a metabolite or miss a potential toxic moiety. The extra sensitivity produced by the UPLC system ensures more low concentration metabolites will be detected, helping to prevent potentially toxic compounds from progressing further into the drug discovery process. This added sensitivity is extremely important when performing MS–MS experiments as it can make the difference between obtaining an interpretable spectrum or not.

At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography.

Sensitivity, selectivity, and analysis time (sample throughput) are also some of the challenges analysts face when analyzing environmental samples such as soil and water.

Explosives residues in soil or environmental waters are of both forensic and environmental interest. These types of assays prove challenging because of the selectivity needed to resolve positional isomers. Typical HPLC analyses require viscous, buffered mobile phases operated at high temperatures, and analysis times exceeding 30 min.

Figure 6 shows the separation of a complex mixture of explosive compounds in less than seven minutes, with a much simpler, more robust mobile phase than that commonly used in HPLC assays. The simpler nonbuffered mobile phase also is ideal for MS detection if desired.

Conclusion

ACQUITY UPLC using 1.7-µm particles and a properly holistically designed system provide significantly more resolution (information) while reducing run times, and improve sensitivity for the analyses of many compound types. At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography. New ACQUITY technology in both chemistry and instrumentation boosts productivity by providing more information per unit of work as UPLC fulfills the promise of increased resolution, speed, and sensitivity predicted for liquid chromatography.

Acknowledgments

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References

The Use of ACQUITY UPLC in Pharmaceutical Development

Now more than ever, the demands of the pharmaceutical industry require companies to look for new ways to cut costs and shorten timelines in the development of drugs, while at the same time improving the quality of their products. The analytical laboratory is no exception to this trend. The development of HPLC methods for assay/purity analysis of drugs and their related substances is a time-consuming process and is often a bottleneck in analytical labs (1). Separations scientists are thus continually driven to develop LC methods with ever-shorter analysis times. The benefits of faster analyses are clear: they allow for a greater number of analyses to be performed in a shorter amount of time, thereby increasing sample throughput and lab productivity. In addition, as test experiments are performed more quickly, the overall method development time is decreased.

The approaches for fast LC method development are varied. Method development simulation software [such as ACD™ (2), DryLab™ (3), or Chromsword™ (4)] is a valuable tool for optimizing and streamlining methods. Such software allows the chemist to increase the information obtained from a limited number of runs and to predict the best possible separation conditions. Of course, the best separation achievable is restricted by the inherent performance limits of the instruments and columns in use.

Since the quality of the separation must not be sacrificed, methods that offer the greatest resolution per unit of time are desired. Therefore, high-resolution chromatographic techniques must be considered in the development of fast LC methods. Monolithic columns, which contain a polymerized porous support structure, provide lower flow resistances than conventional particle-packed columns (1,5,6). These columns can be operated at higher flow rates, although increases in efficiency are moderate. Also, solvent consumption is considerably higher and can be an issue for cost-conscious laboratories. Elevated-temperature chromatography also allows for high flow rates by lowering the viscosity of the mobile phase, which significantly reduces the column backpressure (7). Interesting possibilities arise from the use of high temperatures, such as the use of temperature gradients and purely aqueous mobile phases (8). However, due to the limited availability of packing materials stable at temperatures > 100 °C, method development approaches using elevated temperature chromatography are not yet considered routine.

The most straightforward and accepted way to improve performance and gain speed in HPLC columns has been to reduce the particle diameter of the packing material (9). By proportionally reducing the stationary phase particle diameter (dp) and the column length, separation efficiency is maintained while analysis time is reduced. Thus, shorter columns (150 mm or less) packed with material in the 3-μm size range have now become standard where longer columns (up to 250 mm) with 5-μm materials were once used. However, because the pressure required to pump mobile phase through the column is inversely proportional to the square of the particle diameter, the backpressures required for use of these small-particle columns becomes high. Column manufacturers are now able to reliably produce particles in the sub-2 μm range (10,11), and this presents a challenge to the pressure limitations of conventional HPLC systems. Since chromatographers generally should operate at or above the optimum flow velocity for a given column, even extremely short columns with these particles reach the system pressure limits before their full benefits can be realized. Consequently, there has been much interest in the use of elevated pressures (> 10,000 psi) in LC to take advantage of the separation speed that these particles can provide.

Anton D. Jerkovich, Rosario LoBrutto, and Richard V. Vivilecchia
Pharmaceutical and Analytical Development, Novartis Pharmaceuticals Corporation, East Hanover, New Jersey, e-mail anton.jerkovich@novartis.com
Until recently, research in this area has been restricted to a few academic labs, mostly using non-porous particles packed in fused-silica capillaries (12–19). The use of nonporous particles, however, has been limited in the pharmaceutical industry due to their low sample loading capacity. The need has therefore existed for a commercially feasible system to be used with columns packed with porous particles less than 2 μm in diameter.

The ACQUITY Ultra Performance LC™ (UPLC™) from Waters Corporation (Milford, Massachusetts) is the first commercially available system that addresses the challenge of using elevated pressures and sub-2 μm particles, which makes it a particularly attractive and promising tool for fast LC method development. The ACQUITY system is capable of pumping mobile phase at pressures up to 15,000 psi and utilizes columns that are packed with stationary phase particles 1.7 μm in diameter. The ACQUITY Ultra Performance LC™ (UPLC™) from Waters Corporation (Milford, Massachusetts) is the first commercially available system that addresses the challenge of using elevated pressures and sub-2 μm particles, which makes it a particularly attractive and promising tool for fast LC method development. The ACQUITY system is capable of pumping mobile phase at pressures up to 15,000 psi and utilizes columns that are packed with stationary phase particles 1.7 μm in diameter. The packing material is a porous, silica-based bridged ethyl hybrid (BEH) material. A bonded C18 stationary phase was used for the work presented here, although other column chemistries are available. More than just a new pump, the instrument approaches the use of these small-particle columns from the standpoint of the total HPLC system—both the engineering challenges of operating at high pressures and the high performance expected from such columns necessitates a redesigned injector, reduced system volumes, an increased detector sampling rate, and other improvements.

To be suitable for the analysis of pharmaceutical development samples under good manufacturing practices (GMPs), the UPLC instrument and columns must not only deliver on its promises for fast, high-resolution separations, but do so reproducibly and with the required sensitivity. To this end, a number of fundamental chromatographic characteristics were evaluated. Some parameters that are of concern to analytical chemists involved with pharmaceutical methods development are column performance, system band-broadening, injection and gradient precision, and sensitivity. Results of these investigations are presented here. Also, a Novartis test method was transferred to UPLC to demonstrate the benefits it brings to our existing and future applications.

### Efficiency and Band-Broadening

The performance of a column can be measured in terms of the height equivalent to a theoretical plate ($H$), which can be calculated from the column length $L$, and the column efficiency, or theoretical plates $N$.

$$H = \frac{L}{N} \quad [1]$$

#### Table 1: Column volumes of various ACQUITY column dimensions, calculated using equation 4

<table>
<thead>
<tr>
<th>Column dimensions (mm)</th>
<th>Column volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 × 50</td>
<td>28</td>
</tr>
<tr>
<td>1.0 × 100</td>
<td>55</td>
</tr>
<tr>
<td>2.1 × 50</td>
<td>120</td>
</tr>
<tr>
<td>2.1 × 100</td>
<td>240</td>
</tr>
</tbody>
</table>

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**Figure 1:** Van Deemter curves for a series of parabens on a 2.1 × 100 mm ACQUITY UPLC BEH C18 column. Conditions: 30/70 v/v acetonitrile/water mobile phase, ambient temperature.
The number of theoretical plates is calculated from the equation

$$N = \left( \frac{t_R}{\sigma} \right)^2$$  [2]

where $t_R$ is the analyte’s retention time, and $\sigma$ is the standard deviation of the peak. A lower plate height indicates a more efficient column. The van Deemter equation describes $H$ in terms of its dependence on the linear flow velocity ($u$):

$$H = A + \frac{B}{u} + Cu$$  [3]

where $A$, $B$, and $C$ are the coefficients for eddy diffusion, longitudinal diffusion, and resistance to mass transfer, respectively. The optimum column performance occurs at the minimum of the curve generated by plotting $H$ versus $u$. A minimum plate height of about twice the particle diameter is generally expected for an efficient column.

Figure 1 shows van Deemter curves generated on a $2.1 \times 100$ mm ACQUITY UPLC BEH C18 column with isocratic elution (40/60 v/v acetonitrile/water mobile phase) at flow rates ranging from 0.05–0.45 mL/min (1400–12,000 psi). The sample was a series of parabens plus uracil as a dead time marker, each at a concentration of 50 µg/mL, and the injection volume ranged from 0.5–2 µL, equal to a sample load of 25–100 ng of each compound on the column. Detection was performed at 254 nm. This column yielded a minimum plate height of about 4 m (250,000 plates/m) for butyl-paraben ($k = 8.7$), and about 5.1 m (200,000 plates/m) for methyl-

![Figure 2: The extracolumn contribution to band-broadening for various column dimensions. Conditions: 30/70 v/v acetonitrile/water mobile phase, ambient temperature.](image)

Table II: Injection precision for various injection volumes and partial loop injection techniques on loop sizes of 5, 10, and 20 µL. Values calculated as %RSD of peak area, $n = 6$. Retention factors for phenol and toluene are 1.3 and 12.7, respectively.

<table>
<thead>
<tr>
<th>Injection volume (µL)</th>
<th>20-µL injection loop</th>
<th>10-µL injection loop</th>
<th>5-µL injection loop</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pressure-assisted</td>
<td>Pressure-assisted</td>
<td>Pressure-assisted</td>
</tr>
<tr>
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<td>Phenol</td>
<td>Toluene</td>
<td>Phenol</td>
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<td>0.5</td>
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<td>1.0</td>
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</tr>
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<td>2.0</td>
<td>0.8</td>
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<td>0.9</td>
</tr>
<tr>
<td>5.0</td>
<td>0.4</td>
<td>0.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Full-loop injections
For fastest cycle times, Waters recommends pressure-assisted injection.
For best injector precision, Waters recommends full-loop injection or partial-loop needle overfill with an injection volume of 30–70% of the full loop volume.
paraben ($k = 1.3$). In terms of relative performance, this is equivalent to about 2.3 and 3 times the particle diameter, respectively—slightly worse than what is theoretically expected for particles of this size, although still on par with most typical commercial packing materials. The absolute efficiency of this column (about 25,000 theoretical plates) is only marginally better than what a $3.0 \times 150$ mm, 3-μm particle column typically provides. The speed at which this efficiency can be attained, however, is much greater, showing that speed of analysis is the ACQUITY’s greatest benefit. The higher plate height (lower efficiency) for methylparaben is most likely due to extracolumn sources of band-broadening, which are more pronounced for earlier-eluting components.

This raises an important concern: the small dimensions and high efficiencies of the columns make extra-column band broadening more of a concern than with typical analytical columns (3-5 μm particles, 3-4.6 mm i.d.), where extracolumn broadening is proportionally small enough that it does not significantly add to the overall broadening of the peak. Although system volumes in the ACQUITY have been drastically reduced (total system volume ≲ 100 μL) compared to conventional HPLC systems (up to 1 mL), extra-column broadening is still evident. Indeed, van Deemter analysis of a 1.0 × 50 mm column demonstrated high plate heights of > 6 μm for butyl-paraben and > 12 μm for the earlier-eluting methylparaben.

Our experience has shown us that a good rule of thumb is the post-injection extracolumn volume of the system should not exceed about 10% of the column void volume $V_M$ calculated as

$$V_M = 0.7^* \pi r^2 L \quad [4]$$

where $r$ is the column radius, $L$ is the column length, and 0.7 is the approximate fraction of the column occupied by mobile phase assuming porous particles (20). The column volumes for various ACQUITY column formats are listed in Table I. The post-injection extracolumn volume can be approximated by replacing the column with a “zero dead volume” union, injecting a test analyte at a low flow rate, and calculating the volume from the flow rate and the elution time. This method, while not exact, can give a general order of magnitude of the extracolumn volume. The particular instrument used for this work had a 5-μL injection loop and the low-flow “50/50” detector flow cell, which uses 50-μm capillaries at the inlet and outlet and has a total volume of 500 nL. Nine measurements at various flow rates resulted in values between 8 and 11 μL. This volume, while very small compared to conventional HPLCs, is large enough to cause significant band spreading in the 1.0-mm-i.d. formats.

The extracolumn contribution to the total band spreading can also be determined from these measurements. Any broadening accumulated by the test analyte in this setup will be due solely to non-column sources such as the injector, connection tubing, and detector flow cell. This of course assumes that the analyte zone begins as an infinitely narrow band, which in actuality is not the case. Therefore, the smallest injection volume possible—0.1 μL—was used. Because variances are additive it is the peak variance, rather than peak width, that is representative of individual contributions to band broadening:

$$\sigma^2_{\text{total}} = \sigma^2_{\text{column}} + \sigma^2_{\text{extra-col}} \quad [5]$$

The variance can be calculated from the chromatogram by the equation:

$$\sigma^2 = \left( \frac{W_{4.4\%}}{5} \right)^2 \quad [6]$$

where $W_{4.4\%}$ is the peak width at 4.4% peak height. Variances were measured at flow rates of 10 to 150 μL/min, beyond which elution times were too short to achieve reliable measurements. Since we know from the Golay equation (21) that the variance of a zone in an open tube increases linearly with the flow velocity, a least-squares regression line was fit to the data and extrapolated to higher flow rates to estimate extracolumn variance contributions at those conditions. By comparing variances obtained in this manner with that of peaks retained on a column, the impact of extra-column broadening can be assessed.

The same sample and conditions described above for the van Deemter analyses were used to obtain chromatograms at flow rates of 0.1 and 0.4 mL/min for the 1.0-mm and 2.1-mm diameter columns, respectively. This corresponds to a linear velocity of approximately 3 mm/s for each

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Time</th>
<th>%RSD</th>
<th>Ret. time (n = 200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.50</td>
<td>0.25</td>
<td></td>
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<tr>
<td>2</td>
<td>0.57</td>
<td>0.23</td>
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<tr>
<td>3</td>
<td>0.65</td>
<td>0.20</td>
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<td>4</td>
<td>1.18</td>
<td>0.14</td>
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<tr>
<td>12</td>
<td>2.59</td>
<td>0.06</td>
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column, which is near the optimum velocity. Variances were measured for methyl-, ethyl-, propyl-, and butyl-paraben, whose retention factors (k), measured using uracil as a dead-time marker, ranged from about 1 to 9. The extracolumn variance at the corresponding flow rate is then compared to the peak variance. The contribution of extracolumn broadening to the total broadening of the peak can be expressed as a percentage, and is plotted versus the retention factor for four different column dimensions in Figure 2. Two trends discussed earlier are made evident from this plot: 1) extracolumn broadening has a greater effect on poorly retained components, and in fact becomes the dominant contributor to peak variance at low k values and 2) extracolumn broadening is more pronounced the smaller the column volume is, so that it approaches 100% of peak broadening for 1.0-mm-i.d. columns.

Often, chromatographers will develop isocratic methods for fast assay determination, such as for dissolution or content uniformity testing, or will incorporate an isocratic region at the beginning of a gradient method to retain the more polar constituents of a sample. While the ACQUITY columns offer greater efficiency and speed, the minimum peak width attainable under isocratic conditions will be limited by extra-column broadening, particularly with the 1.0-mm-i.d. columns. Gradient elution presents less of a problem. Typically, sample components will be concentrated onto the head of the column by weak mobile phase starting conditions, reducing extracolumn effects that occur before the sample reaches the column. Early eluters, however, may still be affected. Therefore, it is hard to justify the use of the 1.0-mm columns in the pharmaceutical development environment, where sample availability is rarely an issue. These columns will find their greatest use in labs where sample amount is limited and high sensitivity is required, and for applications using mass spectrometric detection. The 2.1-mm columns, however, exhibit little extra-column broadening for components with k > 5, and are quite suitable for routine methods development.

Injection and Gradient Repeatability

Another performance characteristic essential for a validated HPLC test method is injection precision. The ACQUITY employs a novel injector design, and it was not known how its unique features would affect the injector’s precision. This is not the place to discuss the details of the injector design, but a brief description is warranted. Injections can be performed in a full loop mode or in one of two partial loop modes: pressure-assisted or needle-overfill. For full loop injections, the loop is simply overfilled with sample. The injection valve is then switched, placing the loop in-line with the column. The pressure-assisted partial loop mode uses a pressurized fluid stream to position the sample plug aspirated from the sample vial into the injection loop. In the needle-overfill partial loop mode, the syringe draws an excess of sample into the needle and through the valve while the loop remains in-line with the pump. The valve is switched to bring the loop off-line and the syringe then meters the appropriate volume of sample into the loop. The valve is then switched back again to complete the injection.

To evaluate injection precision, samples of phenol and toluene at concentrations ranging from 0.2 to 9.0 mg/mL, depending on the injection volume, were prepared in 50/50 v/v acetonitrile/water. A set of six injections was performed at each of the four injection volumes: 0.5 μL, 1 μL, 2 μL, and 5 μL; on 5-μL, 10-μL, and 20-μL loops. Partial loop injections were evaluated in the pressure-assisted mode on all three loop sizes; needle-overfill and full loop modes were also evaluated on the 5-μL loop. Isocratic elution (30/70/0.1 v/v/v acetoni-trile/water/TFA) at a flow rate of 0.8 mL/min was used to elute the compounds off a 2.1 × 50 mm, 1.7-μm ACQUITY UPLC BEH C18 column. The column temperature was 35 °C, and detection was performed at 270 nm (phenol) and 261 nm (toluene) with 10 pts/s data collection rate. The peak areas were measured for each injection and the relative standard deviations were determined. The results are displayed in Table II.

The high resolution obtained in extremely short analysis times makes UPLC a very attractive tool for the pharmaceutical development laboratory.

The best precision was obtained with a full loop 5-μL injection, where six injections produced 0.3% RSD for both analytes. This was expected, as overfilling the loop presents less opportunity for variation than transferring and positioning a sample plug in the loop for partial loop injections. The partial loop injections produced varied results. All of the 0.5-μL injections yielded poor precision (> 3% RSD in the pressure-assisted mode). The precision of partial loop injections from 1–5 μL on the 10-μL and 20-μL loops ranged from 0.4-1.6% RSD. Values on the higher end of this range may be unacceptably high for typical system suitability requirements of most validated assay methods. One explanation for the poor precision observed is that in partial loop injections in the pressure-assisted mode the sample plug can potentially experience significant dilution, which may result in some of the sample diffusing out of the sample loop and not being injected onto the column. For this reason, Waters does not recommend injecting more than 50% of the loop volume using the pressure-assisted mode. One would expect the partial loop injection precision to worsen in a smaller loop as accurately positioning the complete sample plug in the loop becomes more difficult. The needle-overfill mode may therefore be a more attractive technique for use with the smaller loops. The sample experiences less dilution in this mode and injections up to 75% of the loop volume may be performed. Moderate improvements in precision were obtained with this mode compared to the pressure-assisted mode on the 5-μL loop.

As one can see, there are multiple factors to consider that make the injection process not a trivial matter. Users will have to keep these in mind and determine for themselves the optimal scenario for the given method, which should include measuring the precision for a particular injection volume, loop size, and mode of injection. For work requiring high precision, it may be more appropriate to exclusively use full loop injections. Waters offers 2, 5, 10, and 20 μL loop sizes; enough choices to make that a viable option.

The gradient reproducibility of the ACQUITY was also assessed. The ability of
the instrument to repeatedly produce an accurate gradient is necessary to ensure reproducible retention times. The sample analyzed was a Novartis development product whose method was successfully transferred to the ACQUITY UPLC system, described in detail below. This sample contained 12 components, each at a level of 1% of the target active concentration. A 10 mM dihydrogen phosphate buffer adjusted to pH 3 with phosphoric acid was used for mobile phase A, and acetonitrile as mobile phase B. The gradient was 5–40% B in 2 min, and 40–60% B in 0.5 min, followed by re-equilibration at starting conditions for 0.5 min, for a total run time of 3.5 min. The flow rate was 0.8 mL/min, which required a peak run pressure of 13,000 psi, and the column used was a 2.1 × 50 mm ACQUITY UPLC BEH C18, held at 35 °C. A total of 200 2-µL injections on a 5-µL loop were performed in sequence over a period of more than 12 h, equal to > 5000 column volumes of mobile phase pumped through the column. Displayed in Table III are the retention time precision results for all 12 peaks. The retention time repeatability for 200 injections was ≈0.25 %RSD for all

12 peaks, indicating that no drift in the gradient occurred throughout the analysis.

Method Transfers

Finally, evaluation of fundamental chromatographic parameters can only go so far in determining the usefulness of the technique. One must eventually test the instrument in the manner in which it is to be used, namely, running test methods with actual samples. Figure 3a shows the analysis of a Novartis development combination product containing three active pharmaceutical ingredients and nine byproducts and degradation products spiked at a level of 1% of the active concentration. This method was originally developed on a Waters® Alliance® Separations Module HPLC using an XTerra™ RP18, 3.5 µm, 4.6 × 100 mm column, and achieves the separation with a run time of 24 min. To transfer this method to the ACQUITY UPLC using a 2.1 × 50 mm ACQUITY column, the flow rate and injection volume were scaled in proportion to the difference in column dimensions in order to obtain the same linear velocity. The original gradient was simplified and adjusted, and the flow rate was increased to obtain the fastest separation possible that still met the resolution requirements of 2.0 between peaks 3 and 4. This utilized a flow rate of 1 mL/min at about 14,000 psi. The chromatogram obtained with this new method is shown in Figure 3b. This sample is a forced degradation of the three active components and is therefore of a different concentration than the sample used to obtain the chromatogram in Figure 3a, which explains the lower signal-to-noise ratio observed. The benefits of the ACQUITY for fast LC method development is strikingly apparent from the run time of the new method developed on the ACQUITY. At 2.8 min, a greater than 8-fold reduction in analysis time is achieved. Most importantly, adequate resolution ($R_s = 2.7$) between the critical peak pair (peaks 3 and 4) is maintained.

Due to potential selectivity differences between columns, an eight-fold reduction in analysis time may be too much to expect for all method transfers. It has been our experience, however, that a five-fold improvement is possible for most reversed-phase LC methods. This will not only enable rapid analysis times, but will speed up the process of developing methods. Provided the selectivity of the various ACQUITY columns can separate the sample components, transferring methods already developed on conventional
instruments and columns to the ACQUITY is straightforward and in most cases could be accomplished within a few hours.

The high efficiency afforded by the ACQUITY columns is also expected to increase sensitivity—an analyte will be more concentrated in a narrower peak and thus give rise to a higher signal. Limit of quantitation solutions of one of the active compounds from the previous method were prepared at 0.05 % and 0.1 % of its target concentration. The injection volume was scaled in proportion to the column dimensions. These gave rise to signal-to-noise ratios of 15 and 22, respectively, using the original 24 min method with a conventional instrument and column. When analyzed with the new ACQUITY method, signal-to-noise ratios of 26 and 38 were obtained—an improvement in sensitivity by a factor of about 1.7. This is not an entirely direct comparison, since the instruments, detectors, and data collection rates used were all different. Regardless of all the variables that can affect peak height and signal-to-noise, a new method was developed that produced greater sensitivity than the former method.

Conclusion
The high resolution obtained in extremely short analysis times makes the UPLC a very attractive tool for the pharmaceutical development laboratory. The increasing number of column chemistries available in the 1.5–2 μm size range make the ACQUITY a flexible method development tool applicable to a wide range of samples. With proper consideration of factors affecting band-broadening, injection and gradient precision, as well as other chromatographic characteristics, the analyst can achieve fast, efficient, and reproducible methods. The results are compelling that 1.5 to 2 μm sized porous stationary phase particles used with high-performance instrumentation such as the ACQUITY UPLC will become the option of choice for the development of fast LC methods in pharmaceutical development in the near future.

Acknowledgments
The authors would like to thank Liz Robertson, Patricia McConville, and Vladimir Binshtock of the Waters Corporation for all of their discussions, assistance, and cooperation during the beta evaluation of the ACQUITY UPLC. They would also like to thank their colleagues at Novartis, specifically Min Yang, Guy Yowell, and Yuri Kazakevich for their support and discussions concerning this work.

References
(2) http://www.acdlabs.com/products/chrom_lab
(3) http://www.rheodyne.com/products/chromatography/drylab
(4) http://www.irstechnologies.net/ChromSword/ChromSwordAuto.htm.
Ultra Performance Liquid Chromatography Coupled to Orthogonal Quadrupole TOF–MS(MS) for Metabolite Identification

In the drug discovery process the detection and identification of the main metabolic routes that a compound will undergo is crucial to identifying a metabolically stable drug candidate which is a requirement for most discovery projects but not all. In the discovery phase it is important that the main metabolites are detected and identified as rapidly as possible to allow the result to feed back into the cycle and influence the synthetic chemistry to either block a potential metabolic route to reduce clearance or avoid a substructure that might result in a potentially reactive or active metabolite.

Liquid chromatography coupled with mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS–MS) is well established as the main analytical technique capable of providing the level of information and throughput required (1) in the pharmaceutical industry. LC–MS for the identification of drug metabolites was first used in the mid 1980s (2,3) with the introduction of the thermospray source which allowed for the relatively easy interfacing of reversed-phase LC solvent systems to mass spectrometers (4). In the late 1980s, the introduction of the atmospheric pressure ionization interface took this approach a step further in terms of performance and ease of use. Through the 1990s, the main advances were instrument performance and capability with triple quadrupoles allowing structural information to be easily obtained to assist in the identification of sites of metabolism. Ion trap mass spectrometers allowed additional information to be generated by being able to carry out MS^n experiments which could give additional information to that obtained from the triple quadrupole (5). The subsequent development of the hybrid quadrupole-orthogonal time-of-flight (TOF) mass spectrometer took the identification of unknown drug metabolites into a new era by providing accurate mass information for both the molecular ion and fragments thereby giving increased confidence in structural assignments. This approach has also been used by Eckers and colleagues for identifying trace impurities in drug formulations (6).

For discovery support where large numbers of compounds need to be screened for their metabolic fate, the initial driving force was high throughput, but this usually compromised both chromatographic resolution and sensitivity. With the metabolic complexity of some samples and the need for more sensitivity as levels being incubated in vitro or dosed in vivo decreased, there has been a trend toward using narrower bore columns to gain more sensitivity and better resolution. However this had the disadvantage of increasing analysis run times. The introduction of the Waters CapLC™ system at the end of the 1990s was ideal for this type of work making the use of 1 mm i.d. columns relatively easy, routine, and reliable. However, average run times were increased from less than 10 min to around 30 min, which could become a potential bottleneck. With the advent of the Waters ACQUITY Ultra Performance LC (UPLC) system, there was the real possibility of using 1 mm i.d. columns at higher flow rates resulting in improved chromatographic resolution, increased sensitivity and a return to run times of around 10 min or less (7,8).

Here, we will compare the UPLC system with the Waters CapLC set up in a column-switching mode which is the LC system currently being used in our laboratory. The results will demonstrate that with UPLC, the analysis time for routine in vitro samples can easily be reduced from 30 min to 10 min, while for more complex matrices...
Improved separation can be achieved relative to the CapLC.

**Experimental**

**Chemicals:** Methanol (HPLC grade) and ammonium acetate were purchased from Fisher Scientific, U.K. Leucine-enkephalin was obtained from Sigma-Aldrich.

**Liquid Chromatography**

HPLC was carried out on a Waters CapLC liquid chromatograph (Waters Corporation, Milford, Massachusetts), which was configured for a column-switching mode. The HPLC trapping cartridge was a $10 \times 1$ mm HyPURITY C18, 3.5 µm (Thermo Electron Corp.) while the analytical column was a $150 \times 1$ mm HyPURITY C18, 3.5 µm.

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**Figure 1:** Ion traces obtained for metabolites on the CapLC.

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**Figure 2:** Ion traces obtained for metabolites on the UPLC.
SEE ALL THAT YOU’VE BEEN MISSING
You’re expected to produce more chromatographic information about your samples in less time. Until now, this seemed impossible due to high throughput performance compromises with conventional HPLC. What if you could simultaneously maximize your data quality and improve your cycle times? You can. Better analyses with more information…all in less time. It’s just the tip of the iceberg with the ACQUITY UPLC System.

MORE DATA = MORE INFORMATION
Introduced in 2004, the Waters ACQUITY Ultra Performance LC™ System is based on a revolutionary holistic instrument design concept, built from the ground up to re-invent liquid chromatography as you know it. Comprising innovative column chemistries, hardware and software, the ACQUITY UPLC is a first-of-its-kind LC system optimized to leverage the potential of patented sub-2-micron particle technology. This concept enables superior chromatographic separations that are up to nine times faster than today’s HPLC systems, with 2-fold better resolution and 3-fold better sensitivity. This means reliable, high quality sample information in less time for truly higher productivity, without compromise.

MORE KNOWLEDGE = BETTER DECISION MAKING
Making more informed decisions in less time — a universal benefit in today’s laboratory. Whether you’re a methods developer, researcher, mass spectrometrist or a chromatographer, you seek a productivity edge. Now you can get more information in a single short run than you’ve ever seen with your current HPLC methods. Smaller particles operate more effectively under high linear velocities, yielding greater efficiency and allowing you to benefit from simultaneous improvements in speed, sensitivity and resolution. The result is unprecedented improvements in the quality of your data with the added benefit of drastically reduced analysis times.

A growing number of scientists around the world are already experiencing the advantages of gaining more quality information from their most challenging separations. See more, know more — the future of liquid chromatography is here today with the Waters ACQUITY UPLC System. To learn more, visit www.waters.com/acquity

“UPLC revealed peaks in the samples that we simply didn’t know were there by conventional LC.”
-Professor Ian Wilson, AstraZeneca Pharmaceuticals
HPLC column (Thermo Electron Corp.). The sample was loaded at 20 µl/min in 0.25% ammonium acetate in water with 2% methanol for 2 min. The valve was then switched and the sample back flushed onto the analytical column with a flow rate of 40 µl/min running a gradient from 5–95% methanol, using 0.025% ammonium acetate in water as the aqueous phase with a total run time of 27 min.

UPLC was carried out on a Waters® ACQUITY UPLC™ system, without column-switching, using a 100 × 1 mm Waters ACQUITY UPLC BEH C18 1.7 µm column with a flow rate of 110 µl/min. The

![Figure 3](image_url)  
*Figure 3:* Mass spectra of oxidized metabolite from UPLC and CapLC.

![Figure 4](image_url)  
*Figure 4:* Selected ion traces for rat urinary metabolites from the UPLC.
same solvents were used for both the UPLC™ and the CapLC. The in vitro samples were analysed using a total run time of 10 min, while for the in vivo samples it was 20 min.

The HPLC column was also run on the UPLC system using the same solvents and gradient as the CapLC but with a higher flow rate of 55 μl/min, but without column-switching.

Radio-flow Detection
The detector was a Lablogic b-RAM (Sheffield, U.K.) fitted with a 50 μl liquid scintillant flow cell. The scintillant flow rate was 0.5 μl/min for both UPLC and CapLC runs.

Figure 5: Selected ion traces for rat urinary metabolites from the CapLC, where metabolite at m/z 515 was not detected.

Figure 6: Nonbackground subtracted spectra obtained from rat urine: spectrum A is the metabolite only found from the UPLC data, spectrum B is a metabolite from the CapLC data with the corresponding spectrum from the UPLC data in C.
Mass Spectrometry
All the MS was carried out on a Waters Micromass® Q-ToF II (Waters, Manchester, U.K.) in either positive or negative ion mode. The analyses were carried out using leucine-enkephalin as the lock-mass via the LockSpray interface. All data were collected in centroid mode. The TOF data were collected between \( m/z \) 100 and 950 with an acquisition rate of 0.4 s/spectrum for most of the data with a rate of 0.9 s/spectrum for the rest.

Samples
The samples used were examples taken from the routine work flow and were either rat liver hepatocyte incubations or rat urine obtained from a bile duct cannulation study.

Results and Discussion
The two main advantages of UPLC over the CapLC is the increased resolution that enables faster analysis times. Figure 1 shows the type of separation from an in vitro sample incubated with rat hepatocytes. This particular compound generated a significant number of metabolites including six oxidised species which were well separated on the CapLC. The UPLC traces in Figure 2 show comparable resolution although the resolution between the six oxidised species on the UPLC could be improved with faster scan rates as the peaks are quite narrow. However the data from the UPLC were generated in 10 min instead of 30 min. In addition to the speed at which the data can be obtained, the quality of the data is also improved. To compare the quality of the spectra from the two systems, Figure 3 shows the spectra for one of the oxidized metabolites taken from the top of the peak. It is clear that the quality of the UPLC spectrum is better than that from the CapLC with much improved signal-to-noise.

Although screening in vitro samples is a significant part of the work process, as projects progress or there are particular issues to address, there is a need to look at in vivo samples to see how the metabolic profile compares with the in vitro results. These matrices are most commonly urine or bile and to ensure that there are no losses of any metabolites they are subjected to no sample work up other than centrifugation for urine and dilution with water followed by centrifugation for bile. However, the level of the background endogenous material found in those samples is much higher than that found in in vitro preparations. This is where the increased resolution and peak capacity of the UPLC system should have an impact by providing greater separation of compound related material from endogenous with less likelihood of interference or possible suppression. For this type of sample good sepa-
ration and sensitivity is more important than speed of analysis.

The selected ion traces in Figure 4 are for a number of metabolites found in rat urine from the UPLC, while the corresponding results obtained from the CapLC are shown in Figure 5. From these traces there are two observations. The first is that the metabolite with $m/z$ 515 was not detected at all in the CapLC data, possibly as a result of ion suppression. The second is that there is an endogenous component with $m/z$ 419 eluting at 15.32 min close to the metabolite at 14.95 min. This endogenous component is present in the UPLC trace but elutes around at 12.73 min, well separated from the peak of interest at 10.48 min. One issue with in vivo samples is the level of the background that the endogenous components generate making it more difficult to pick out compound related peaks. Figure 6 demonstrates the quality of the spectra that can be obtained from the UPLC compared to the CapLC. The spectra are single spectra with no background subtraction with spectrum B from the CapLC and spectrum C comparing the same metabolite from the UPLC. From this, it is quite clear that the UPLC gives a much higher quality spectrum with the base peak being the molecular ion and less endogenous interference while in the CapLC spectrum the metabolite is less than 30% of the base peak which is an endogenous interference. Spectrum A is the metabolite that could only be detected from the UPLC data and again shows the quality of the spectrum with the isotope pattern confirming it is compound related.

To determine the fate of drug candidates in vivo, it is best to work with radio-labelled materials. This approach can provide a more definitive quantitative measure but also indicate where to look in the mass spectral data for metabolites. To do this requires running the sample through a radio-flow detector and matching the radio traces obtained with the mass spectral data. The radio detector can contribute to some peak broadening as the peaks are being mixed with scintillant so increased resolution and sharper peaks can improve the quality of the radio trace. Figure 7 shows the summed selected ion traces for a rat urine sample from the CapLC and the UPLC both with a 20 min run time. Typical peak widths from the CapLC are 12–15 s while for the UPLC this was reduced to 6–10 s. By comparing the radio traces in Figure 8 the peak widths are 30 s for the CapLC and 20 s for the UPLC data. In addition, peak 4 in the MS traces did not produce a peak in the radio trace from the CapLC data but does in the UPLC trace.

As a final comparison, a rat hepatocyte incubation was run on the CapLC, the ACQUITY UPLC system using the ACQUITY column and also on the UPLC using the HPLC C18 column to determine how an ordinary column would perform on the UPLC system. The resultant traces are shown in Figures 9 (CapLC), 10 (UPLC with ACQUITY column), and 11 (UPLC with HPLC C18). The peak widths for the various traces were around 6–7 s for the UPLC with ACQUITY column and 12–15 s for the HPLC column on both the CapLC and UPLC. The best results were obtained on the ACQUITY column with all peaks being resolved and the best spectral quality, as shown in Figure 12, for the weakest metabolite with $m/z$ 502. Increased sensitivity when using the ACQUITY system
UPLC–MS does offer significant gains over the CapLC–MS for metabolite identification. For screening in vitro samples it is possible to reduce analysis times by two thirds while still retaining chromatographic resolution and sensitivity with an improvement in spectral quality. For the analysis of more complex in vivo samples it does offer big improvements in separation and again the gain in spectral quality is even more crucial making it much easier to detect compound related peaks in the first place. From the data shown it also allowed the identification of a metabolite that was not even detected when using the CapLC. The sharper peaks also gave added benefit when generating radio chromatograms to compare with the mass spectral data. As a final point, it is also a very effective and capable HPLC and should allow for the replacement of other systems with this giving the choice of using conventional HPLC columns up to their recommended pressure limits, typically 4000 psi, as well as the high pressure capable columns. The very low dead volume in the system makes this an excellent HPLC system with the significant advantage over conventional HPLC systems of having the high pressure capability.

To push the UPLC capability further it would be necessary to have a mass spectrometer that is capable of scanning fast enough to be sure of getting good quality data. However we have clearly demonstrated that using the Q-Tof II it does give significant advantages in chromatographic resolution, sensitivity and a big reduction in analysis time.

**References**

Assay Transfer from HPLC to UPLC for Higher Analysis Throughput

A typical HPLC assay was transferred and optimized for a Waters ACQUITY UPLC™ system to achieve both higher sample analysis throughput and better assay sensitivity. Strategies to expedite future method transfers were compiled. Analysis of operation costs and sample throughput found UPLC cost advantageous over HPLC.

Increasing demand for greater pharmaceutical analysis throughput prompted the testing of the Waters ACQUITY Ultra Performance LC (UPLC™). This system claims to provide faster analyses through the use of a novel separation material of very fine particle size (1.7 μm) and unique core chemistry (1–5).

To effect fast separations on this material, the column hardware and instrument have significant design modifications from typical HPLC. The UPLC operates at higher pressures (up to 15,000 psi.), injects samples into a smaller system dwell volume, and captures detector signals at high data rates for fast eluting peaks. A new needle design has been claimed to substantially reduce carryover which can aid in the lowering of limits of quantitation (LOQ).

In this work, an HPLC method for quality control (QC) was optimized for UPLC. Strategies to reduce total runtime, lower cost per assay, and promote instrument uptime were considered.

Method Development
The original 10-min HPLC QC assay was developed to quantify the content of a heterocyclic drug (Cpd A) in organic solvent extracts. An internal standard (IS) was used to compensate for sample preparation losses and a terminal washing gradient was necessary to remove late eluting interferences.

Initial transfer of the HPLC assay to UPLC was accomplished by simply applying a scaling factor to the mobile phase flow rate and the sample injection volume. This scaling factor was derived from the ratio of the column cross sectional areas in order to retain the mobile phase linear velocity.

Chromatograms from this UPLC method had very narrow peaks, and the excessive resolution indicated opportunity for method improvement. The mobile phase flow rate was increased until limited by column backpressure. However, subsequent column lifetime studies indicated that reducing total run time by increasing organic solvent content was more economical. A dramatic decrease in solvent consumption was also obtained. Chromatograms in Figure 1 compare the original HPLC method to those of the initial scaling and the final UPLC conditions. Parameters of the HPLC and final UPLC methods are listed in Table I.

Method Optimization Guidelines and Observations
During the course of optimizing the UPLC method, considerations to expedite future method transfers were developed, and the following recommendations were made:

● Increase elution solvent strength to reduce run times taking advantage of the high resolution potential of UPLC columns (see Table II).

● Increase mobile phase flow rate secondarily to solvent strength in order to promote longer column lifetimes. While high mobile phase linear velocities with good resolution are possible (Figure 2), as with any column, routine operation at 80% maximum rated pressure led to shortened lifetimes. In our experience, UPLC operation around 8000 psi or less provided comparable or lower column cost per assay than HPLC. Maintaining low flows as much as possible also reduces solvent and waste disposal costs, although these are already an order of

Ying Yang and Craig C. Hodges
Alexza Molecular Delivery Corporation, Palo Alto, California 94303, e-mail: yyang@alexza.com.
magnitude less than HPLC.

- Reduce column re-equilibration times by taking advantage of the low system dwell volume. Programmed changes in the mobile phase take time to reach the column. The small UPLC dwell volume (measured as 110 μL, 15% of that of the HPLC) allowed in part the abbreviation of the original assay. Column re-equilibration was accomplished during next sample loading in the UPLC, further increasing throughput.

- Reduce injection volumes appropriately for the column diameter to achieve good peak shapes. Peak splitting can occur when too large of a strong sample solvent bolus overwhelms the packing at the column head. While this assay method tolerated 5 μL injections, volumes of 1–3 μL are more typical starting points in our experience. Note that smaller injection volumes may be compensated by enhanced peak height from use of the high resolution columns and by the low carryover from the UPLC injector (measured as 10% of the HPLC carryover for this analyte) to achieve an equivalent or even lower LOQ. An alternative to smaller injection volumes might be to lower sample solvent strength to accomplish sample focusing on the head of the column.

- Utilize partial loop-fill injections in preference to full loop-fill. Partial loop-fill precision was good even at volumes up to 80% of the loop total volume (Figure 3). Typical laboratory practice is to limit sample volume injections to roughly 50% of the total loop volume. The UPLC injection system, which utilizes air-gap sandwiching of the sample, allows better utilization of the sample loop and

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**Table I: Original HPLC versus optimized UPLC assay parameters**

<table>
<thead>
<tr>
<th></th>
<th>HPLC Assay</th>
<th>UPLC Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>XTerra C18, 50 x 4.6 mm, 4 μm particles</td>
<td>ACQUITY UPLC BEH C18, 50 x 2.1mm, 1.7 μm particles</td>
</tr>
<tr>
<td><strong>Flow Rate</strong></td>
<td>3.0 mL/min</td>
<td>0.6 mL/min</td>
</tr>
<tr>
<td><strong>Needle Wash</strong></td>
<td>Methanol</td>
<td>Strong Needle Wash: 200 μL Methanol; Weak Needle Wash: 600 μL ACN:H₂O 10:90</td>
</tr>
<tr>
<td><strong>Injection Volume</strong></td>
<td>20 μL</td>
<td>3 μL partial loop fill or 5 μL full loop fill with automatic overfill</td>
</tr>
<tr>
<td><strong>Gradient (time in min) (ACN:H₂O)</strong></td>
<td>T0 (25:75), T6.5 (25:75), T7.5(95:5), T9 (25:75), T10 (25:75)</td>
<td>T0 (36:64), T1.1 (95:05), T1.3 (36:64)</td>
</tr>
<tr>
<td><strong>Flow Rate</strong></td>
<td>3.0 mL/min</td>
<td>0.6 mL/min</td>
</tr>
<tr>
<td><strong>Total Run Time</strong></td>
<td>10 min</td>
<td>1.5 min</td>
</tr>
<tr>
<td><strong>Total Solvent Consumption (including 0.5 min of delay time in between injections)</strong></td>
<td>Acetonitrile:10.5 mL Water: 21.0 mL</td>
<td>Acetonitrile: 0.53 mL Water: 0.66 mL</td>
</tr>
<tr>
<td><strong>Plate Count for Cpd A</strong></td>
<td>2000</td>
<td>7500</td>
</tr>
<tr>
<td><strong>USP Resolution</strong></td>
<td>3.2</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>LOQ</strong></td>
<td>−0.2 μg/mL</td>
<td>0.054 μg/mL</td>
</tr>
<tr>
<td><strong>Carry-over</strong></td>
<td>&lt; 0.05% with needle wash</td>
<td>0.01%</td>
</tr>
<tr>
<td><strong>Delay Volume</strong></td>
<td>−720 μL</td>
<td>−110 μL</td>
</tr>
</tbody>
</table>

---

**Figure 1**: Chromatograms (from top to bottom): original HPLC, initial scaling to UPLC showing peak shape improvement and possibility for further method optimization, and final UPLC method. Order of peak elution: internal standard (IS) then Cpd A.
Figure 2: A van Deemter plot derived from UPLC experimental data indicates that use of high flow rates is a plausible strategy to decreasing overall runtime. This should be balanced with backpressure effects on overall column lifetime (see text).

Table II: Adjusting mobile phase parameters utilizing resolution potential of UPLC

<table>
<thead>
<tr>
<th></th>
<th>HPLC Original</th>
<th>UPLC Initial Scaling</th>
<th>UPLC Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate (mL/min)</td>
<td>3.0</td>
<td>0.63</td>
<td>0.60</td>
</tr>
<tr>
<td>% ACN in Mobile Phase</td>
<td>25</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Plate Count for Cpd A</td>
<td>2000</td>
<td>9100</td>
<td>7500</td>
</tr>
<tr>
<td>USP Resolution Between A and IS</td>
<td>3.2</td>
<td>6.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Figure 3: Peak area data generated by partial loop-fill mode of a 5 μL nominal (4.8 μL actual) sample loop in the UPLC injector. For standard loop injectors, the deviation from linear injection volume, as seen above in the 5-μL injection, occurs at much lower loop utilization so the general rule is to only load 40–50% of the loop capacity.

higher injection precision, reducing the need for use of the full loop-fill mode. From a practical point of view, full loop fill requires substantially greater sample movement considering overfill functions. This likely increases subsequent needle washing, which may impact sample throughput and increase wear of the washing hardware. Larger sample volume transfers also increases exposure to sample particulates, lowering long-term instrument reliability.

- If full loop-fill mode is utilized, perhaps for very high precision requirements, ensure adequate loop overfilling. A significant laminar flow velocity differential in the loading sample between its wall interface and center is created in the very narrow bore tubing of the UPLC injector. Overfilling the sample loop by at least four loop volumes was found necessary to fully displace wash solvent from the 5 μL injector loop. For this instrument, the manufacturer has determined and set as the default the optimum overfill volume with typical sample solvents for each sample loop size. Operators can specify other overfill volumes for unusual sample compositions.

- Choose the proper composition and volume of weak sample wash to obtain good peak shape. A portion of the weak sample wash solvent will be co-injected with partial-loop filled samples. The weak solvent wash should therefore mimic the initial conditions mobile phase in solvent.
strength. Utilizing the weak wash solvent as a sample diluent in the sample loop may enhance sample focusing onto the column. The volume of the weak wash must be sufficient to purge the former strong wash solvent from the loop.

Preliminary Method Validation

Preliminary assessment was made of the new assay and the instrument for linearity and linear range, precision, accuracy, system suitability, and sample carry over.

Linearity and Lower Limit of Quantification (LLOQ)

With the potential greater sensitivity of UPLC, the scope of the assay application was broadened to address samples which could differ in concentration by 500-fold. The same UPLC separation method was calibrated and found acceptably linear for two assay ranges (Figures 4 and 5). With an LLOQ of 54 ng/ml, the low range UPLC assay allowed analyses more typically addressed by liquid chromatography–mass spectrometry. Notably, this particular UPLC system is configured with a photodiode array detector. Use of a wavelength-specific detector could provide an even lower limit of quantification.

Precision and Accuracy

Triplicate injections were made at specified concentrations to assess precision (repeatability) and accuracy. Precision was evaluated

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**Figure 4:** Linear correlation between concentration and peak area at lower concentrations from 0.054 to 1.30 μg/mL (R² = 0.996 with 1/X² weighting).

**Table III: Evaluation of precision and accuracy for low range calibration**

<table>
<thead>
<tr>
<th>Theoretical Conc. (μg/mL)</th>
<th>Peak Area</th>
<th>Precision¹ (% RSD)/Result</th>
<th>Calculated Conc. (μg/mL)</th>
<th>Accuracy² (% Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.054</td>
<td>11834</td>
<td>4.5</td>
<td>0.0484</td>
<td>−10.3</td>
</tr>
<tr>
<td></td>
<td>12127</td>
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<td>0.0519</td>
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<tr>
<td></td>
<td>12897</td>
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<td>0.0611</td>
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<tr>
<td>0.325</td>
<td>35647</td>
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<td>0.332</td>
<td>2.3</td>
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<tr>
<td></td>
<td>34344</td>
<td></td>
<td>0.316</td>
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<tr>
<td></td>
<td>36703</td>
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<td>0.345</td>
<td>6.2</td>
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<td>0.645</td>
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<td></td>
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<td>0.6</td>
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<tr>
<td></td>
<td>62264</td>
<td></td>
<td>0.649</td>
<td>0.8</td>
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<tr>
<td>1.3</td>
<td>115988</td>
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<td>1.290</td>
<td>−0.7</td>
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<tr>
<td></td>
<td>114863</td>
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<td>1.277</td>
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</tr>
<tr>
<td></td>
<td>115826</td>
<td></td>
<td>1.288</td>
<td>−0.9</td>
</tr>
</tbody>
</table>

¹ Acceptance criterion: < 5.0%, all passed.
² Acceptance criteria: ±5.0%, except lowest concentration ±15.0%, all passed.

---

**Table IV: Evaluation of precision and accuracy for low range calibration**

<table>
<thead>
<tr>
<th>Theoretical Conc. (μg/mL)</th>
<th>Peak Area</th>
<th>Precision¹ (% RSD)/Result</th>
<th>Calculated Conc. (μg/mL)</th>
<th>Accuracy² (% Deviation)</th>
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</thead>
<tbody>
<tr>
<td>0.645</td>
<td>62518</td>
<td>0.3</td>
<td>0.646</td>
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<td></td>
<td>62173</td>
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<td>0.642</td>
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<td></td>
<td>62264</td>
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<td>0.643</td>
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<td>1.3</td>
<td>115988</td>
<td>0.1</td>
<td>1.308</td>
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<tr>
<td></td>
<td>114863</td>
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<td>1.294</td>
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<tr>
<td></td>
<td>115826</td>
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<td>1.306</td>
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<tr>
<td>5.18</td>
<td>428428</td>
<td>0.1</td>
<td>5.174</td>
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<td></td>
<td>428756</td>
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<td>5.178</td>
<td>−0.03</td>
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<td></td>
<td>429553</td>
<td></td>
<td>5.188</td>
<td>0.2</td>
</tr>
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<td>2094015</td>
<td>0.2</td>
<td>25.78</td>
<td>−0.07</td>
</tr>
<tr>
<td></td>
<td>2088395</td>
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<td>25.71</td>
<td>−0.3</td>
</tr>
<tr>
<td></td>
<td>2097868</td>
<td></td>
<td>25.83</td>
<td>0.1</td>
</tr>
</tbody>
</table>

¹ Acceptance criterion: < 5.0%, all passed.
² Acceptance criteria: ±5.0%, all passed.
by the peak area relative standard deviation (RSD). Accuracy was assessed by back-calculation of the injection peak areas using the calibration curve to give the calculated concentration for each injection. These values were compared to the theoretical value and reported in terms of % deviation from the theoretical value. The results for both low and high range assays passed acceptance criteria (Tables III and IV).

**System Suitability**

Five replicate injections were made to evaluate system suitability. The results passed all the common USP acceptance criteria (Table V).

**Injection-to-Injection Sample Carry-Over**

Contamination of a sample injection by residues of the previous sample in the instrument (carry-over) can set the boundary for an assay’s LLOQ. Carry-over frequently leads to failure of tests for precision, accuracy, and system suitability. However, depending on the protocol details of these studies, significant carry-over effects may not be revealed. Direct measurement of carry-over was performed here to anticipate inaccuracies arising in potentially mixed sets of concentrated and dilute samples.

The UPLC instrument had design features to reduce sample carry-over: a novel needle-in-needle injector design as well as two separate injector wash solvents. In this assay, 200 µL methanol were used as the first wash to remove the bulk of organic residues, followed by 600 µL water:ACN (90:10) to displace the strong solvent and bring the remaining sample loop, needle, and valve solutions to a composition compatible with initial method conditions.

Carry-over was evaluated here by analyzing a solvent blank sample after each of the calibration standards and measuring the area of any peak appearing at the analyte retention times. No interference peak was detected in the blanks run after the five lower concentration standards. For blanks run after injections of the highest concentration standard, faint peaks slightly above noise were measured at 0.01% of the analyte peak in the previous injection. This was acceptable for this assay, although carry-over may have been reduced further by optimizing the wash solvent parameters. In comparison, carry-over on the HPLC system was 5 to 10 fold higher.

**Summary**

A QC HPLC assay to quantitate a heterocyclic pharmaceutical in organic solvent extracts has been successfully transferred and optimized for UPLC. Preliminary assessment indicates that the assay can be validated. Guidelines to expedite the development of future UPLC assays were compiled. The application of UPLC will be cost advantageous. While UPLC column expense per analysis will be comparable to or slightly less than HPLC, solvent consumption and waste disposal charges should decrease better than an order of magnitude. Reduction of assay time by five-fold dramatically improves instrument return on investment and reduces the total number of instruments needed if only HPLC were employed.

**References**

(5) M. E. Swartz and B. Murphy, *Pharm. Formulation Quality* 6(5).
The Evaluation and Application of UPLC for the Rapid Analysis of Dose Formulations

The use of Ultra Performance Liquid Chromatography (UPLC™) with UV and MS detection was evaluated in several pharmaceutical applications. Initial studies characterized the commercial instrumentation performance parameters for isocratic and gradient separations as a function of flow rate using several commercially available drugs as model probe compounds. Parameters examined included reproducibility of retention time and peak area, as well as theoretical plates, resolution, and column ruggedness. The use of high-speed separations for dose formulation strength analysis was evaluated using two model drug compounds: mefenamic acid and chloramphenicol, in a dimethylacetamide/polyethylene glycol-200 vehicle. Accuracy, precision, and resolution were examined under high-speed gradient conditions for the dose formulation analysis.

The pharmaceutical industry is under intense pressure to increase productivity and put new drugs onto the market in a shorter time period (1). Various approaches including high-throughput screening, combinatorial chemistry, proteomic/genomic target identification techniques, high-throughput in vitro screening to determine physiochemical and absorption/distribution/metabolism/elimination properties of compounds, rapid in vivo pharmacokinetic screening, and the use of biomarkers and pharmacogenomics are being employed in drug discovery and development activities. A common theme in these approaches is the need to provide high-quality data at a faster rate to drive decision making processes.

Analytical chemists are challenged to find faster ways of delivering quality data across a range of project-driven needs. A number of approaches are being employed to increase separation throughput including dual high performance liquid chromatography (HPLC) column switching techniques (2,3), direct injection approaches for biological samples (4,5), high-speed supercritical fluid chromatography (6), parallel (96-capillary) capillary electrophoresis (7,8), parallel (24-lane) HPLC (9,10), monolithic HPLC columns (11,12), and ultrahigh performance chromatography (UPLC) (13–17). UPLC was pioneered in the late 1990s by the Jorgenson (13–15) and Lee (16,17) groups and typically involves the use of 1–2 mm particles at much higher pressures (15,000–100,000 psi) than conventional HPLC instruments (4000–6000 psi). The use of columns packed with 1-µm particles in combination with high pressures allows dramatic decreases in analysis time with little compromise in column performance. The relatively flat nature of the van Deemter plot for these small particles at higher linear velocities accounts for the ability to operate at high flow rates without severe effects on column efficiency (13,15). Recently, commercial instrumentation capable of operating up to 15,000 psi combined with columns packed with 1.7 µm particles has become available in the form of the Waters® ACQUITY UPLC™ system. This report describes the basic performance of this commercial instrumentation, as well as pharmaceutical applications for the rapid analysis of dose formulations.

**Experimental**

**ACQUITY UPLC Performance Characterization: Isocratic Conditions:** An ACQUITY UPLC system (Waters, Milford, Massachusetts) equipped with a UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) was evaluated under isocratic conditions using a water/acetonitrile/formic acid (65/35/0.1; v/v/v) mobile phase pumped at flow rates from 0.1 to 1.2 mL/min. The column temperature, data sampling rate, filter constant, injection volume, and detection wavelength were 55 °C, 20 ps/s, fast, 5 µL and 233 nm, respectively. A mixture of NAP (10 mg/mL) and KR (100 mg/mL) in...
Table I: Gradient conditions

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.00</td>
<td>0.50</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.50</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
<td>2.10</td>
<td>0.50</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>Fast</td>
<td>0.00</td>
<td>1.00</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>1.00</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.51</td>
<td>1.00</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>

Table II*: Ketorolac — Average (%RSD). Retention Time, Peak Area, Peak Height, and Theoretical Plates (N) as a Function of Flow Rate (n = 3 injections at each flow rate)

<table>
<thead>
<tr>
<th>Flow Rate (mL/min)</th>
<th>t&lt;sub&gt;r&lt;/sub&gt; min</th>
<th>Avg (%RSD)</th>
<th>Peak Area × 10&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Avg (%RSD)</th>
<th>Peak Ht × 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Avg (%RSD)</th>
<th>N</th>
<th>Avg (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>4.159 (0.06)</td>
<td>315 (0.28)</td>
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<td>8878 (1.00)</td>
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<tr>
<td>0.20</td>
<td>2.188 (0.03)</td>
<td>157 (0.23)</td>
<td>485 (0.71)</td>
<td>13257 (1.12)</td>
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<tr>
<td>0.30</td>
<td>1.513 (0.04)</td>
<td>105 (0.04)</td>
<td>450 (0.23)</td>
<td>14136 (0.44)</td>
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<td>0.40</td>
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<td>0.50</td>
<td>0.965 (0.00)</td>
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<td>13261 (0.41)</td>
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<tr>
<td>0.60</td>
<td>0.824 (0.07)</td>
<td>52.1 (0.17)</td>
<td>326 (0.28)</td>
<td>12186 (0.78)</td>
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<td>0.70</td>
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<tr>
<td>0.80</td>
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<td>270 (0.34)</td>
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<tr>
<td>0.90</td>
<td>0.585 (0.20)</td>
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<tr>
<td>1.2</td>
<td>0.461 (0.13)</td>
<td>25.1 (0.54)</td>
<td>195 (0.57)</td>
<td>8374 (0.67)</td>
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</tr>
</tbody>
</table>

* t<sub>r</sub> = Retention Time, Peak Ht = peak height, and N = Theoretical Plates

Results and Discussion

UPLC System Performance: Isocratic analysis: A standard solution containing KR and NAP as model drug compounds was examined by UPLC under isocratic conditions to determine the instrument performance as a function of flow rates from 0.1 to 1.2 mL/min. Representative chromatograms for low, mid, and high flow rate conditions are shown in Figure 1. Symmetrical peak shapes were obtained under all flow conditions and KR and NAP were well resolved under all conditions. The average values and reproducibility (%RSD) for the retention time, peak area, peak height, theoretical plates (N), and resolution obtained for three replicate injections under each flow condition are shown in Tables II and III for KR and NAP, respectively. The RSD values obtained for retention time reproducibility were, in general, less than 0.1%. The peak area and peak height reproducibility were, in general, less than 1% RSD and most values ranged between 0.1 and 0.4% RSD. Theor-
Theoretical plates decreased by 41 and 26% for KR and NAP, respectively, when operating at the highest flow rate, 1.2 mL/min, relative to the optimal values found at 0.3–0.4 mL/min. Similarly, there was a 17% decrease in resolution when operating at the highest flow rate relative to the optimal flow rate. This small decrease in resolution is contrasted with the four-fold decrease in analysis time.

The long-term performance of the UPLC BEH C18 column was determined by performing 4000 isocratic injections of the KR/NAP standard at high pressure (~13,500 psi, 1.25 mL/min). The column performance was periodically monitored during the injections by analyzing the KR/NAP standard at a lower flow rate (0.5 mL/min). The retention times, peak shapes, theoretical plates, and the resolution were found to remain constant throughout the 4000 injections (data not shown).

**Gradient analysis:** The performance of the UPLC instrument under standard (0.5 mL/min flow rate with 2 min linear ramp from initial to final mobile phase condition) and fast (1.0 mL/min flow rate with 0.5 min linear ramp from initial to final mobile phase condition) gradient conditions were examined using a probe sample containing CA (50 mg/mL), NAP (75 mg/mL), and MFA (50 mg/mL). Representative chromatograms for the standard and fast gradient conditions are shown in Figure 2. All three peaks are well resolved under both gradient conditions with the last peak, MFA, eluting at 1.54 and 0.56 min for the standard and fast gradient, respectively. Using the fast gradient conditions, an injection-to-injection time of 1 min could be achieved.

The average value and %RSD obtained for the retention time, peak area, and resolution for each compound are shown in Table IV. The RSD values for the retention time of all three compounds for the standard and fast gradient methods were less than 0.07% and 0.30%, respectively. The peak area RSD values for all compounds under standard and fast gradient conditions were less than 0.70%. Resolution decreased from a value of 13.3 under the standard conditions to 4.10 under the fast gradient conditions but as seen in Fig 2 all peaks were well resolved under the fast gradient conditions.

### Table III*: Naproxen — Average (%RSD), Retention Time, Peak Area, Peak Height, Theoretical Plates (N), and Resolution as function of Flow Rate (n = 3 injections at each flow rate)

<table>
<thead>
<tr>
<th>Flow Rate (mL/min)</th>
<th>( t_r ) min Avg (%RSD)</th>
<th>Peak Area ( \times 10^4 ) Avg (%RSD)</th>
<th>Peak Ht ( \times 10^3 ) Avg (%RSD)</th>
<th>N Avg (%RSD)</th>
<th>Resolution Avg (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>7.202 (0.08)</td>
<td>533 (0.30)</td>
<td>386 (0.99)</td>
<td>6320 (0.10)</td>
<td>11.2 (0.10)</td>
</tr>
<tr>
<td>0.20</td>
<td>3.806 (0.03)</td>
<td>267 (0.21)</td>
<td>449 (0.21)</td>
<td>10287 (0.95)</td>
<td>14.3 (0.37)</td>
</tr>
<tr>
<td>0.30</td>
<td>2.639 (0.04)</td>
<td>178 (0.13)</td>
<td>434 (0.11)</td>
<td>11650 (0.51)</td>
<td>15.2 (0.19)</td>
</tr>
<tr>
<td>0.40</td>
<td>2.043 (0.05)</td>
<td>133 (0.17)</td>
<td>413 (0.38)</td>
<td>12185 (0.75)</td>
<td>15.6 (0.31)</td>
</tr>
<tr>
<td>0.50</td>
<td>1.686 (0.03)</td>
<td>1064 (0.11)</td>
<td>400 (0.20)</td>
<td>11869 (1.13)</td>
<td>15.3 (0.44)</td>
</tr>
<tr>
<td>0.60</td>
<td>1.440 (0.07)</td>
<td>87.9 (0.12)</td>
<td>366 (0.23)</td>
<td>11438 (0.78)</td>
<td>15.0 (0.33)</td>
</tr>
<tr>
<td>0.70</td>
<td>1.262 (0.05)</td>
<td>75.3 (0.11)</td>
<td>346 (0.13)</td>
<td>11047 (0.38)</td>
<td>14.7 (0.35)</td>
</tr>
<tr>
<td>0.80</td>
<td>1.124 (0.00)</td>
<td>65.9 (0.15)</td>
<td>325 (0.27)</td>
<td>10689 (0.42)</td>
<td>14.4 (0.34)</td>
</tr>
<tr>
<td>0.90</td>
<td>1.015 (0.10)</td>
<td>58.1 (0.22)</td>
<td>305 (0.24)</td>
<td>10173 (0.49)</td>
<td>14.0 (0.16)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.928 (0.00)</td>
<td>51.1 (0.83)</td>
<td>283 (0.73)</td>
<td>9762 (0.27)</td>
<td>13.7 (0.08)</td>
</tr>
<tr>
<td>1.1</td>
<td>0.853 (0.07)</td>
<td>46.2 (0.76)</td>
<td>267 (0.75)</td>
<td>9361 (0.46)</td>
<td>13.3 (0.10)</td>
</tr>
<tr>
<td>1.2</td>
<td>0.791 (0.07)</td>
<td>42.3 (1.00)</td>
<td>254 (1.11)</td>
<td>8981 (0.47)</td>
<td>12.9 (0.13)</td>
</tr>
</tbody>
</table>

* \( t_r \) — Retention Time, Peak Ht — Peak Height and N — Theoretical Plates.

**Figure 2:** Representative chromatograms for a combined chloramphenicol/mefenamic acid obtained under: (a) fast and (b) standard gradient UPLC conditions.

### Rapid Dose Formulation Analysis

The analysis of CA and MFA in the
DMAC/PEG200 dose formulation was done under the standard and fast gradient conditions for three replicate dilutions of the dose formulation using replicate \( n/H11005 \) injections for each dilution. The concentrations of CA and MFA in the diluted dose formulations were determined by interpolation from a linear regression standard curve (Table V). Very similar results were obtained by the standard and fast gradient approach in terms of the found concentration, although the data for the fast analysis was slightly more variable than the standard conditions but was still very acceptable for drug discovery efforts.

Conclusions

The ACQUITY UPLC System was shown to provide accurate and reproducible results for rapid isocratic and gradient analysis of drug molecules in dose formulations. In the rapid isocratic mode, retention times, peak shapes, theoretical plates, and resolution for a multicomponent drug standard were found to remain constant over 4000 injections. Using fast gradient conditions, injection-to-injection cycle times of 1 min could be obtained, while still maintaining acceptable within-specification results for repeatability for both peak retention time and area. The utility of the UPLC system for the rapid, accurate and precise analysis of drugs in dosage formulations was demonstrated using a model drug system. The UPLC system provided the same accuracy, precision, and ruggedness as a standard HPLC system but allowed decreased analysis times resulting in higher sample throughput.

References


Table IV*: Performance of UPLC versus gradient conditions (n = 5 injections)

<table>
<thead>
<tr>
<th>Standard Condition</th>
<th>Chloramphenicol (50 mg/mL)</th>
<th>Naproxen (75 mg/mL)</th>
<th>Mefenamic Acid (50 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>MFA</td>
<td>CA</td>
<td>MFA</td>
</tr>
<tr>
<td>t_r, min</td>
<td>Peak Area</td>
<td>t_r, min</td>
<td>Peak Area</td>
</tr>
<tr>
<td>(Avg (%RSD))</td>
<td>(Avg (%RSD))</td>
<td>(Avg (%RSD))</td>
<td>(Avg (%RSD))</td>
</tr>
<tr>
<td>0.682 (0.066)</td>
<td>535530 (0.37)</td>
<td>1.07 (0.076)</td>
<td>499236 (0.44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fast Condition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>MFA</td>
<td>CA</td>
<td>MFA</td>
</tr>
<tr>
<td>0.323 (0.28)</td>
<td>252980 (0.63)</td>
<td>0.441 (0.28)</td>
<td>232183 (0.51)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>MFA</td>
<td>CA</td>
<td>MFA</td>
</tr>
<tr>
<td>Avg [Found], mg/mL (%RSD)</td>
<td>Avg [Found], mg/mL (%RSD)</td>
<td>Avg [Found], mg/mL (%RSD)</td>
<td>Avg [Found], mg/mL (%RSD)</td>
</tr>
<tr>
<td>1 1.403 (0.12)</td>
<td>1.400 (0.35)</td>
<td>0.850 (0.96)</td>
<td>0.845 (1.22)</td>
</tr>
<tr>
<td>2 1.404 (0.10)</td>
<td>1.414 (0.26)</td>
<td>0.858 (0.38)</td>
<td>0.862 (0.38)</td>
</tr>
<tr>
<td>3 1.399 (0.16)</td>
<td>1.422 (0.14)</td>
<td>0.845 (0.63)</td>
<td>0.853 (1.03)</td>
</tr>
<tr>
<td>Average 1.402 (0.16)</td>
<td>1.412 (0.79)</td>
<td>0.851 (0.75)</td>
<td>0.853 (1.03)</td>
</tr>
</tbody>
</table>

*Res = Resolution for adjacent pairs: Chloramphenicol/Naproxen and Naproxen/Mefenamic Acid.

Table V: Dose formulation analysis

<table>
<thead>
<tr>
<th>Dilution</th>
<th>CA Avg [Found], mg/mL (%RSD)</th>
<th>Standard</th>
<th>Fast</th>
<th>MFA Avg [Found], mg/mL (%RSD)</th>
<th>Standard</th>
<th>Fast</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>1.403 (0.12)</td>
<td>1.400 (0.35)</td>
<td>0.850 (0.96)</td>
<td>0.845 (1.22)</td>
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<tr>
<td>2</td>
<td>1.404 (0.10)</td>
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<td>3</td>
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<td>Average</td>
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<td>1.412 (0.79)</td>
<td>0.851 (0.75)</td>
<td>0.853 (1.03)</td>
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Developing Columns for UPLC: Design Considerations and Recent Developments

Chromatographers are faced with the challenge of developing separations that completely characterize the constituents of their sample. A new tool for meeting these challenges became generally available in 2004. This new class of separation science, Ultra Performance Liquid Chromatography or UPLC, provides improved resolution, speed, and sensitivity. This is achieved through the use of columns with very small particle packings and a matching family of instruments developed simultaneously to provide full compatibility between chemistry and instrumentation. In describing this new separation power, it is essential to consider the key parameters that influence peak resolution and ultimately lead to successful chromatographic methods.

Retention ($k$ or $k’$) and selectivity ($\alpha$) are chemical factors describing the interaction among the analyte molecules, the mobile phase, and the stationary phase. In contrast, efficiency ($N$ or plate count) describes the physical process of band-broadening during the separation. Developing a chromatographic method is based upon the systematic manipulation of these three parameters.

Most method development strategies focus on retention and selectivity because they are easy and economical to manipulate. Resolution is improved by increasing the retention ($k$) of all of the peaks. Increasing retention, however, increases peak width, resulting in lower sensitivity, and reduces sample throughput. Selectivity describes the elution sequence of the peaks relative to one another, that is, relative retention. It can be manipulated by several parameters including mobile phase pH, organic modifier, and bonded phase.

Efficiency is less often used to improve a separation because it is difficult to change experimentally and because any improvements only contribute to resolution as the square root. Efficiency, however, can be significantly improved by reducing the diameter of the particle. A column packed with 1.7 µm particles would offer a 1.7 fold improvement in resolution compared to a column packed with 5 µm material. This resolution increase is defined by narrower, lower volume peaks so sensitivity is also increased.

This paper will focus on the challenges of improving resolution and efficiency by utilizing highly efficient 1.7 µm particle packed columns. The requirements include the design and development of the new chromatographic particle for UPLC™ separations are described. Columns packed with this new UPLC particle must meet or exceed reproducibility and longevity expected for other modern HPLC columns under conditions that are more mechanically and chemically demanding. Additionally, several new bonded phases provide flexibility for methods development, enabling the introduction of new products to be brought to market faster.
base chromatographic particle, the preparation of reproducible columns with maximized life using these particles, and the provision of modern reversed-phase selectivity with different stationary phase ligands.

New Particle Technology

The use of smaller particle packing materials increases resistance to flow so that the columns operate at higher backpressure. In addition, the optimal linear velocity for maximum separation efficiency of a 1.7 µm particle necessitates operation at higher flow rates, generating even higher pressures, sometimes as high as 15,000 psi. Silica-based materials do not possess the mechanical strength or efficiency necessary to meet the demands of UPLC separations. The definition of a new particle to meet these requirements must, therefore, include improved physical stability. This strength must be achieved without compromising the mass loading capacity of the material that is related to the large surface area produced with fully porous packing material. The new material must also be stable to a wide range of chemical operating conditions while minimizing any secondary or mixed mode interactions with a wide range of analytes.

A new bridged ethylsiloxane/silica (BEH) hybrid particle was synthesized to meet these demands (Figure 1). It provides improved mechanical and chemical stability for UPLC separations.

![Figure 1: Bridged ethylsiloxane/silica hybrid particle provides improved mechanical and chemical stability for UPLC separations.](image1)

![Figure 2: Mechanical and chemical aging study over 1000 injections at pH 11.3. Column: ACQUITY UPLC BEH C18 2.1 × 50 mm, 1.7 µm. Conditions: acetonitrile-methyl pyrrolidine buffer, 45:55 (v/v) at 0.9 mL/min. Test probes: butyrophenone, protriptyline and amitriptyline.](image2)

### Table I: BEH particle

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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<tr>
<td>Pore Diameter*</td>
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<tr>
<td>Pore Volume*</td>
<td>0.7 mL/g</td>
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<tr>
<td>Surface Area*</td>
<td>185 m²/g</td>
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<tr>
<td>90/10 Ratio*</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Expected or approximate values.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>ACQUITY UPLC</th>
<th>C18</th>
<th>C8</th>
<th>Shield RP18</th>
<th>Phenyl</th>
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</thead>
<tbody>
<tr>
<td>BEH Chemistry</td>
<td></td>
<td>Trifunctional C18</td>
<td>Trifunctional C8</td>
<td>Monofunctional Embedded Polar Group</td>
<td>Trifunctional C6 Phenyl</td>
</tr>
<tr>
<td>Ligand Type</td>
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<td>3.1 µmol/m²</td>
<td>3.2 µmol/m²</td>
<td>3.3 µmol/m²</td>
<td>3.0 µmol/m²</td>
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<td>Ligand Density</td>
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<td>18%</td>
<td>13%</td>
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<td>15%</td>
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<td>pH Range</td>
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</tbody>
</table>
inorganic hybrid, with ethylsiloxane bridges both on the surface and throughout the body of the material, provides a broader range of chemical stability, especially the pH operating range (pH 1–12), while minimizing interactions of the matrix with any analyte functionalities. The properties of this packing material are summarized in Table I. These characteristics are typical of modern reversed-phase HPLC packings. The slightly larger pore size improves the accessibility for larger analytes, while the surface area is about the same as first generation methyl hybrid packings. The particle size distribution is among the narrowest of modern packings.

**Column Lifetime**
Columns packed with this new UPLC particle must meet or exceed the reproducibility and longevity expected for other modern HPLC columns. Column lifetime is a broad term that reflects both physical and chemical changes to the packing as well as the adsorption of sample components. Chemical stability depends primarily on the effect of mobile phase pH and solvent selection. The higher operating pressures associated with sub-2 µm particle packed column could also

---

**Figure 3:** Column stability maintained over 2200 analyses at pH 2.0 with protein precipitated rat plasma samples. Column: ACQUITY UPLC BEH C18 2.1 × 50 mm, 1.7 µm. Mobile phase A: 0.1% trifluoroacetic acid in water, mobile phase B: 0.08% trifluoroacetic acid in acetonitrile. Gradient from 10–40% B over 1 minute curve 7, 40–90% B from 1.0 to 1.1 minutes, hold for 0.4 min, 95–10% B from 1.5–1.6 minutes, hold for 0.5 min. Flow rate 0.7 mL/min, 5.0 µL injection; temperature 30 °C; detection UV at 272 nm.

**Figure 4:** Selectivity difference between C18 and C8 alkyl chain columns. Column: ACQUITY UPLC BEH C18 and C8 2.1 × 100 mm, 1.7 µm. Isocratic 28% methanol; at flow rate 0.5 mL/min; temperature 50 °C; 5.0 µL injection; detection UV at 254 nm. Analytes: 1 HMX, 2 RDX, 3 1,3,5-TNB, 4 1,3-DNB, 5 NB, 6 Tetryl, 7 TNT, 8 2-Am-4,6-DNT, 9 4-Am-2,6-DNT, 10 2,4-DNT, 11 2,6-DNT, 12 2-NT, 13 4-NT, 14 3-NT.
compromise bed stability. All of these phenomena are accelerated at elevated temperatures. The physical and chemical effects of mobile phase extremes were examined as shown in Figure 2. Both the mechanical (pressure tolerance) and chemical (pH and temperature) stability were measured as retention and efficiency, with no losses over 2000 injections. The same long term stability and performance is observed when using complex sample matrices shown in Figure 3. In this case, protein precipitated rat plasma was injected after evaporation and reconstitution in the initial mobile phase conditions. The column maintained initial peak capacity and selectivity for over 2200 injections. While it is never possible to predict column life absolutely for all combinations of sample and operating conditions, these experiments are consistent with this new UPLC particle meeting or exceeding the number of injections expected for traditional HPLC columns.

Column Selectivity

A 1.7 μm particle packed column provides significant improvements in resolution because efficiency is better. Separation of the

Figure 5: Selectivity difference between C18 and embedded polar group columns for phenolic compounds. Column: ACQUITY UPLC BEH C18 and Shield RP18 2.1 × 50 mm, 1.7 μm. Isocratic 45% methanol with 0.1% formic acid; at flow rate 0.6 mL/min; temperature 40 °C; 5.0 μL injection; detection UV at 270 nm. Analytes: 1 quercetin, 2 kaempferol, 3isorhamnetin.

Figure 6: Similar selectivity is often observed between C18 and embedded polar group. Isoelutropic conditions are used to distinguish changes in retentivity and selectivity. Column: ACQUITY UPLC BEH C18 and Shield RP18 2.1 × 100 mm, 1.7 μm Mobile phase A: 20 mM ammonium bicarbonate pH 10.0, mobile phase B: acetonitrile; at flow rate 0.5 mL/min; temperature 30 °C; 10.0 μL injection; detection UV at 210 nm. Analytes: forced degradation of terbinafine HCl by 8.0 N hydrochloric acid.
components of a sample, however, still requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations: ACQUITY UPLC™ BEH C18 and C8 (straight chain alkyl columns), ACQUITY UPLC BEH Shield RP18 (embedded polar group column), and ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl). The characteristics of these stationary phases are summarized in Table II. Each provides a different combination of hydrophobicity, silanol activity, hydrolytic stability, and chemical interaction with the analytes. The effect of these properties on separations can be described briefly.

The C18 and C8 UPLC columns have alkyl chain bonded phases trifunctionally bonded to the particle surface to ensure the best hydrolytic stability. Compared to the C18 column, the shorter chain length C8 bonded phase is less hydrophobic, and, therefore, less retentive in general. Although selectivity differences seldom result from chain length differences, changes in peak elution order can occur. As shown in Figure 4, a set of 14 nitroaromatic compounds were analyzed on both C18 and C8 stationary phases. Baseline resolution was achieved on both stationary phases. However, less retention is observed on the C8 column. There is also a change in the elution order for the peaks.

An embedded polar group column can exhibit significantly different selectivity compared to linear alkanes (1,2). The ACQUITY UPLC BEH Shield RP18 column includes an embedded carbamate group that shows preferential retention of hydrogen-bond donors. Figure 5 demonstrates the selectivity differences between the straight chain alkyl C18 and the embedded polar group column for a set of flavanoids.

For analytes that do not specifically interact with the embedded polar group, the column behaves as a shorter chain length alkyl column, as shown in Figure 6. The embedded polar functionality also suppresses surface silanol activity, reducing peak tailing, especially for basic analytes. Finally, the embedded polar group provides compatibility with highly aqueous mobile phases. The embedded carbamate group allows the stationary phase to resist pore dewetting by increasing the water concentration at the surface layer of the pores. The combination of the characteristics of ligands with embedded polar groups provides unique features, most importantly, an alternative selectivity to alkyl ligands.

Columns with phenyl ligands provide another alternate selectivity. Due to the bonding orbital interactions, phenyl columns provide unique and specific selectivity with aromatic compounds and other analytes with similar \( \pi \) electrons. In Figure 7, the separation of nonsteroidal anti-inflammatory drugs on the phenyl column is compared to that on a C18 column. The selectivity differences can be magnified by changing the organic modifier from acetonitrile to methanol, increasing the retention of \( \pi \)-acids (2).

**Conclusion**

The performance barriers of traditional chromatographic packings have been removed with the development of a new, highly efficient, mechanically strong, 1.7 \( \mu \)m bridged ethylsiloxane/silica hybrid particles developed specifically to meet the challenge of routinely applying UPLC in the modern chromatographic laboratory. These particles can be packed in columns that meet or exceed the lifetimes expected for modern HPLC columns. Stability over a broad pH operating range combined with the several available bonded phases provide flexibility for methods development. This flexibility enables methods development to be more efficient, allowing products to be brought to market faster. The power of these ultra-efficient columns is combined with a low dispersion Ultra Performance LC™ system to successfully transfer existing HPLC methods or to develop new, fast chromatographic methods that offer substantial improvements in resolution, sensitivity and sample throughput.
Ultra Performance Liquid Chromatography: New Boundaries for the Chromatography Frontier

By M. E. Swartz

Waters ACQUITY UPLC System

BIBLIOGRAPHY

Waters ACQUITY UPLC System Literature and Presentation

References

Peer-Reviewed Publications


Articles


BIBLIOGRAPHY

ACQUITY UPLC System, Brochure (2004). 720000820EN.


Ultra Performance LC by Design, Brochure (2004). 720000880EN.

Waters ACQUITY UPLC FlexCart, Product Bulletin (2005). 720001089EN.

Waters ACQUITY UPLC Sample Organizer, Product Bulletin (2005). 720001088EN.

Connections INSIGHT Service for the ACQUITY UPLC System, Brochure (2005). 720001019EN.

Posters


“A Rapid Automated Approach to the Generation and Visualization of In Vivo Metabolism, Solubility, and Log D Using LC/MS/MS and UPLC/MS/MS,” W. Potts, R. Plumb, and K. Yu, (Montreux 2004). 720001008EN.


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CZECH REPUBLIC 420 2 617 11384
DENMARK 45 46 59 8080
FINLAND 358 09 506 4140
FRANCE 33 1 3048 7200
GERMANY 49 6196 400600
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HUNGARY 36 1 350 5086
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IRELAND 353 1 448 1500
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PEOPLE’S REPUBLIC OF CHINA 86 10 8451 8918
POLAND 48 22 833 4400
PUERTO RICO 787 747 8445
RUSSIA/CIS 7 095 931 9193
SINGAPORE 65 6278 7997
SPAIN 34 93 600 9300
SWEDEN 46 8 555 11 500
SWITZERLAND 41 62 889 2030
TAIWAN 886 2 2543 1898
UK 44 208 238 6100
USA 508 478 2000 • 800 252 4752