[ACQUITY UPLC SYSTEM APPLICATION NOTEBOOK]

- UPLC® BASICS
- METHOD TRANSFER, DEVELOPMENT, AND VALIDATION
- UPLC/MS ADVANTAGES

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[JEREMY DESAI, PH.D.]
KNOWS THAT WHEN TIME ISN'T ON HIS SIDE, WATERS IS.
Dr. Jeremy Desai is the Executive Vice President of Research and Development at Apotex, a leading generic pharmaceutical company. Apotex is in an industry known for tight deadlines. For example, recently Desai's team had three months to meet a regulatory deadline. With the ACQUITY UPLC® System from Waters®, his scientists completed 88 days of chemistry work in just 30 days. They used Waters NuGenesis® Scientific Data Management System to improve their collaboration, create required documentation, and meet the target filing date. This will hopefully give Apotex the opportunity to be one of the first to launch the generic drug version. And put time back on their side.
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UPLC Technology: Having a Significant Impact on Scientific Discovery and Business Performance

With the introduction of the ACQUITY UltraPerformance LC® system four years ago, Waters sparked a new wave of innovation in LC; something the industry hadn't seen in over 30 years. Using particles half the size and four times the pressure of standard HPLC, UPLC® technology translates into nine times the speed, twice the resolution, and three times the sensitivity.

From the beginning, with UPLC technology we set out to create a technology platform that would solve problems that couldn't be solved before and a platform with "legs." That has certainly proven to be the case.

Since 2004, we have announced a series of new applications and industry-specific solutions built on the ACQUITY UPLC® platform. In 2005, it was the nanoACQUITY UPLC® System, for those working with extremely small amounts of sample and requiring maximum peak capacity. In 2006, it was ACQUITY® TQD, a benchtop, ultra-compact, tandem-quadrupole, API mass detector-based system. In 2007, we introduced the ACQUITY UPLC Fluorescence (FLR) Detector, VanGuard Pre-Columns for UPLC separations, and ACQUITY UPLC HSS T3 columns. Pittcon 2008 saw the introduction of UPLC-based systems for food safety, physiologic amino acids, and process control. With the recent introduction of the Waters PATROL™ UPLC Process Analyzer, we've made it possible for manufacturers to take advantage of UPLC technology's analytical speed to improve manufacturing efficiency. And our newest innovation, the TRIZAIC™ UPLC System, delivers nano-scale separations that substantially surpass chip-based LC systems on the market today.

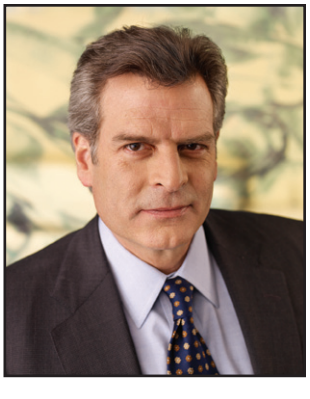
By any measure ACQUITY UPLC is likely to be the most successful and prolific new laboratory instrument technology over the past two decades. At last count, more than 200 UPLC-related articles have appeared in the world's most respected peer-reviewed scientific journals. Unit installations now number in the thousands, and a large percentage of them are on the front-end of mass spectrometers. It's been proven time and again that an ACQUITY UPLC System produces sharper peaks, increased peak heights, and better signal-to-noise performance, leading to greater all-around MS sensitivity.

For our customers operating in highly competitive industries our breakthrough technologies are also having a measurable, significant impact on their business performance.

Thanks to ACQUITY UPLC technology, one major generic pharmaceutical company succeeded in completing its analytical work for a major regulatory filing in 25 man-days versus the 88 days it would have taken with HPLC. AIT Labs switched from GC/MS and immunoassay techniques to UPLC/MS to get three times faster analyses, save $15,000/month in labor and supply costs, and certify results in half the time it used to take. And Dominion Diagnostics is able to offer its clients real-time differential drug displays with a "tremendous degree of specificity, selectivity, and accuracy."

If you would like to see your investment in LC and MS pay off in greater quality of results, speed to market, and efficiency, then help is no further away than typing www.waters.com/uplc into your internet browser.

No doubt about it, these are exciting times for us in the industry. May they continue for a long time to come.



ACQUITY UPLC® System

Application Notebook – June 2008

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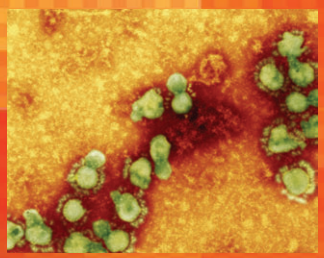
[TAKE A CLOSER LOOK]

SEE WHY TOP COMPANIES USE ACQUITY UPLC TO GET TO MARKET FASTER.



ACQUITY UPLC® doesn't just get better results, it enables laboratories to get those results to market faster. That's why it's used by every major organization interested in improving laboratory performance. What will it do for you? Visit www.waters.com/ACQUITY

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Chromatographic System Requirements for Exploiting the Full Potential of Sub-2 µm Porous Particle LC

Kenneth J. Fountain, Robert S. Plumb, Diane M. Diehl, and Eric S. Grumbach, Waters Corporation, Milford, Massachusetts

High Performance Liquid Chromatography (HPLC) has become the workhorse of many analytical chemistry laboratories. HPLC is used in diverse applications, including pharmaceutical R&D, pharmaceutical product quality testing, clinical diagnostic testing, environmental analysis, and food and beverage safety analysis. This is mainly due to HPLC's analytical versatility, ease of use, sample compatibility, and the selectivity and resolving power of the chromatographic process.

The chromatographic separating power of an HPLC system depends upon the selectivity of the mobile phase/stationary phase system and the efficiency of its column. Column efficiency is dependent upon two factors: column length and packing particle size (d_p). Column efficiency increases with the square root of the column length and is inversely proportional to the particle size.

As chromatography has developed over the last 30 years, we have seen reductions in column particle size, from irregular 20 µm particles in the 1970s, to spherical 10 µm particles in the early 1980s, to 5 and 3 µm materials in the 1990s, to the variety of sub-2 µm column chemistries that have been commercially available since 2004.

Modern chromatographers today face challenging pressures on their laboratory: to increase productivity while also dealing with more and more complicated separation problems. They need to meet increasingly rigorous scientific demands while aligning with business drivers: from product innovations, to cost reductions, to faster sample processing times, to increased profitability. These novel sub-2 µm particle columns offer the most promise for LC to meet such disparate challenges without sacrificing performance.

The use of sub-2 µm particle columns has seen increased popularity since their introduction, however these materials produce higher backpressures than conventional 5 and 3 µm columns. This application note discusses the effects of chromatographic system operating pressures on the performance of these sub-2 µm particle columns, and how a holistically-designed system maximizes their separation power.

Experimental

The chromatographic performance of columns with particle sizes ranging from 1.7 to 5 µm was investigated in both gradient and isocratic modes using a variety of test probes and conditions.

LC conditions

Conditions shown below are for a didanosine mixture only. All other conditions appear in their respective figure captions.

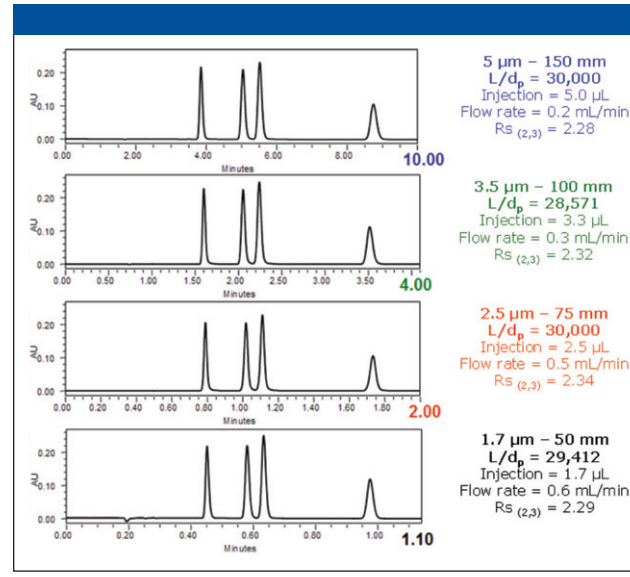


Figure 1: Isocratic separation of 1-methylxanthene, 1,3-dimethyluric acid, theobromine, and 1,7-dimethylxanthene (all 25 µg/mL) while maintaining a constant L/d_p ratio. Peaks are listed in order of elution.
- 5, 3.5, and 2.5 µm columns: Waters XBridge™ C_{18}.
- 1.7 µm column: ACQUITY UPLC BEH C_{18}.
- All columns were 2.1 mm I.D.
- The isocratic mobile phase was 95% H_2O, 5% ACN with 0.1% formic acid.
- The separation was performed at 38 °C; UV @ 280 nm.

LC Systems:	Waters Alliance® HPLC 2695 System (HPLC) Waters ACQUITY UPLC® System (UPLC®)
Column Temp.:	30 °C
Mobile Phase A:	10 mM CH_3COONH_4 with 0.02% acetic acid
Mobile Phase B:	Acetonitrile
Gradient:	Hold at 2% B for 0.54 min, then to 15% B in 6.7 min, reset to 2% B at 7.94 min, equilibrate until 10.76 min (gradient for 4.6 × 150 mm, 5 µm)
Injection Volume:	28.8 µL for HPLC; 2 µL for UPLC
Detection:	Photodiode Array (PDA) UV @ 254 nm
Sampling Rate:	5 Hz for HPLC; 20 Hz for UPLC
Time Constant:	0.1

Columns and flow rates are listed on their respective chromatograms. All flow rates and gradient conditions were scaled to d_p.

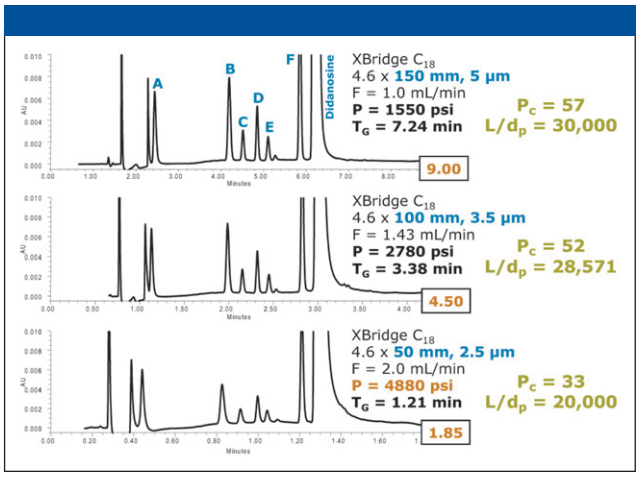


Figure 2: Separation of didanosine and its impurities under gradient conditions. Flow rate was scaled based on d_p.

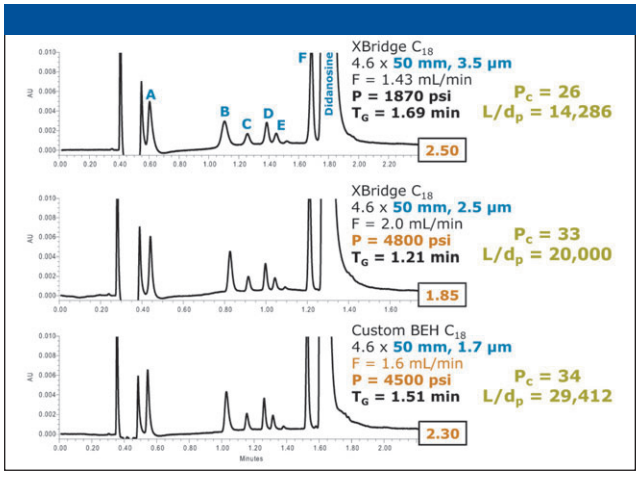


Figure 3: Effect of particle size on peak capacity for didanosine and its impurities. Flow rate was scaled based on d_p.

Results

Isocratic separations

The resolving power of different LC columns can be compared directly using the L/d_p ratio of each (column length/particle size). For example, a 5 µm, 150 mm length column (L/d_p = 30,000) has approximately the same resolving power as a 1.7 µm, 50 mm length column (L/d_p = 29,412).

The data shown in Figure 1 illustrates the separation of a simple isocratic test mix using 5, 3.5, 2.5, and 1.7 µm columns. It is clear that the resolving power of each column is identical.

Also note that analysis time decreases as the particle size decreases. This is because shorter columns and higher linear velocities can be used with smaller particle columns without compromising chromatographic efficiency. The result is an analysis time that is 9× faster on the 1.7 µm column when compared to the 5 µm column.

Gradient separations

Comparing the resolving power of different columns under gradient conditions is somewhat more complicated. Besides maintaining a constant L/d_p ratio, it is also important to properly scale the gradient conditions based on column dimensions. For example, if the column length is decreased by a factor of two and the flow rate is increased by a factor of two, then the gradient duration should be decreased by a factor of four.

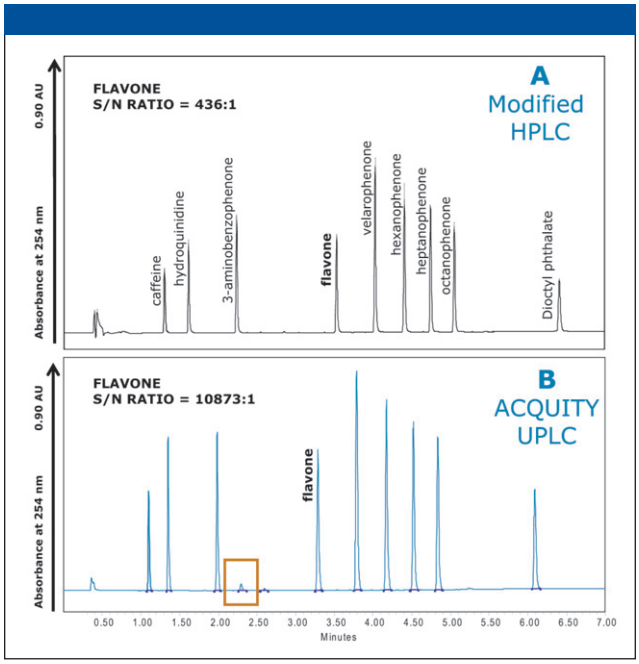


Figure 4: Comparison of baseline noise and instrument sensitivity using a modified HPLC system (A) versus the ACQUITY UPLC System (B).
- Mobile phase A was 0.05% TFA in H_2O, mobile phase B was 0.05% TFA in ACN.
- Gradient from 5 to 95% in 5.17 min, hold for 1.7 min, reset.
- Flow rate 0.6 mL/min, 50 °C, UV @ 254 nm.

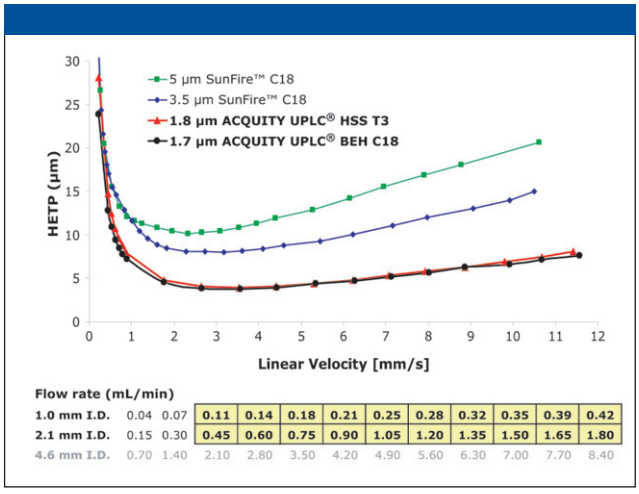
The data in Figure 2 show the separation of didanosine and its impurities on 5, 3.5, and 2.5 µm columns using conventional HPLC instrumentation. When the separation is scaled from the 5 µm column to the 3.5 µm column, the L/d_p ratio is kept constant, and peak capacities are nearly the same.

When the separation is transferred to the 2.5 µm column the peak capacity decreases significantly. This is because the conventional HPLC system is not capable of operating at the conditions needed to preserve the separation. A 75 mm length column is needed to maintain the same L/d_p ratio; however, this length would cause the system to exceed the maximum system pressure (5000 psi). In order to use these smaller particle columns effectively and take advantage of their performance, a system with higher operating pressure is required.

Increasing performance of small-particle LC columns

Besides reducing analysis time, smaller-particle LC columns can be used to increase overall resolving power in a chromatographic system. An incremental gain in performance should be seen by decreasing particle size and keeping column length constant.

Figure 3 shows that as the particle size is reduced from 3.5 to 2.5 µm, there is an 18% increase in peak capacity, which correlates well with the theoretical expectations. This is not the case with the 1.7 µm column. Pressure limitations of the conven-



Flow rate (mL/min)											
1.0 mm I.D.	0.04	0.07	0.11	0.14	0.18	0.21	0.25	0.28	0.32	0.35	0.39	0.42
2.1 mm I.D.	0.15	0.30	0.45	0.60	0.75	0.90	1.05	1.20	1.35	1.50	1.65	1.80
4.6 mm I.D.	0.70	1.40	2.10	2.80	3.50	4.20	4.90	5.60	6.30	7.00	7.70	8.40

Figure 5: van Deemter plots for 5, 3.5, 1.8, and 1.7 µm particle columns.
- Test probe is heptanophenone.
- Mobile phase is 70:30 ACN/H_2O.
- Temperature is 30 °C.
- All columns in the 2.1 × 50 mm format.

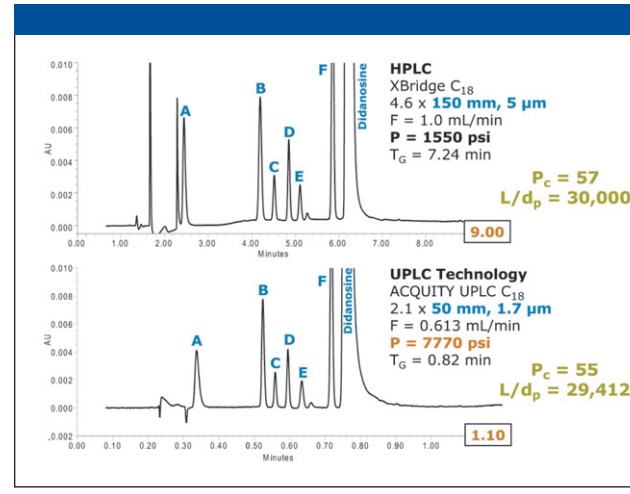


Figure 6: Ultra-fast separation of didanosine and its impurities using UPLC technology.

tional HPLC system do not allow its operation at the properly-scaled flow rate of 2.94 mL/min.

These data, combined with those in Figure 2, illustrate that in order to exploit the chromatographic potential of these sub-2 µm particle columns, it is necessary to run at pressures above the operating range of conventional HPLC systems.

Extended pressure capability is not the sole requirement when designing a chromatographic system to work with sub-2 µm particles. These materials are packed into narrow-bore columns (< 2.1 mm I.D.), and the chromatographic peaks produced are extremely narrow.

Therefore, the volume of the LC system itself must be carefully controlled in order to minimize peak dispersion and prevent loss of performance. This includes minimizing pre-column dispersion of the injected sample and post-column peak broadening prior to detection.

For gradient separations, a large system volume can result in a more significant gradient delay, which can decrease separation power and add unnecessary time to the analysis. Conventional HPLC systems have delay volumes in the range of 700 to 1450 µL. These systems can be altered and configured to reduce this volume somewhat by removing or bypassing some of the fluid paths (mixer, pulse damper, etc.). However, this can result in a loss of signal-to-noise for critical peaks present at low levels (Figure 4A). This is due to an increase in baseline noise caused by removal of critical components in the mobile phase pump.

The ACQUITY UPLC chromatography system has been purposely designed to operate with sub-2 µm particle columns. It has the capability of operating at pressures up to 15,000 psi (~1,000 bar) and has a system volume that is an order of magnitude lower than conventional HPLC systems. Thus no system modification is needed to realize the LC benefit of small column particles, and baseline noise is minimized.

The result is that the native UPLC system shows a 25× improvement in signal-to-noise over a modified HPLC system, which is especially important when detecting and quantifying low-level impurity peaks in a mixture (Figure 4B).

Maximizing the performance benefits of small-particles with UPLC technology

Sub-2 µm particle columns provide significant benefits in terms of productivity and chromatographic performance simply because shorter columns and faster flow rates can be used without adversely effecting performance. However, these columns can be used in such a way as to maximize the full potential of UPLC technology.

From the van Deemter equation we understand that the minimum plate height decreases as particle size decreases (Figure 5). In addition, the optimal linear velocity range increases as particle size decreases, which allows us to use sub-2 µm particle columns at even higher linear velocities without loss in chromatographic performance.

As mentioned previously, this necessitates an LC system that is capable of operating at higher backpressures. Once this limitation has been overcome, as it has been with the design of the ACQUITY UPLC System, small particle columns can be used at elevated flow rates to generate either ultra-fast or ultra-high resolution separations.

Ultra-fast separations can be performed by increasing the flow rate and proportionally decreasing the gradient time to keep a constant column volume in all steps of the gradient. Ultra-high resolution separations can be achieved by increasing flow rate and keeping analysis time constant.[1]

Examples of these two approaches can be seen in the following figures. Figure 6 shows the directly-scaled separation of didanosine and its impurities on a 5 µm and 1.7 µm column. In this example, the L/d_p ratio remained constant, the flow rate was scaled according to d_p, and the gradient times were adjusted based on

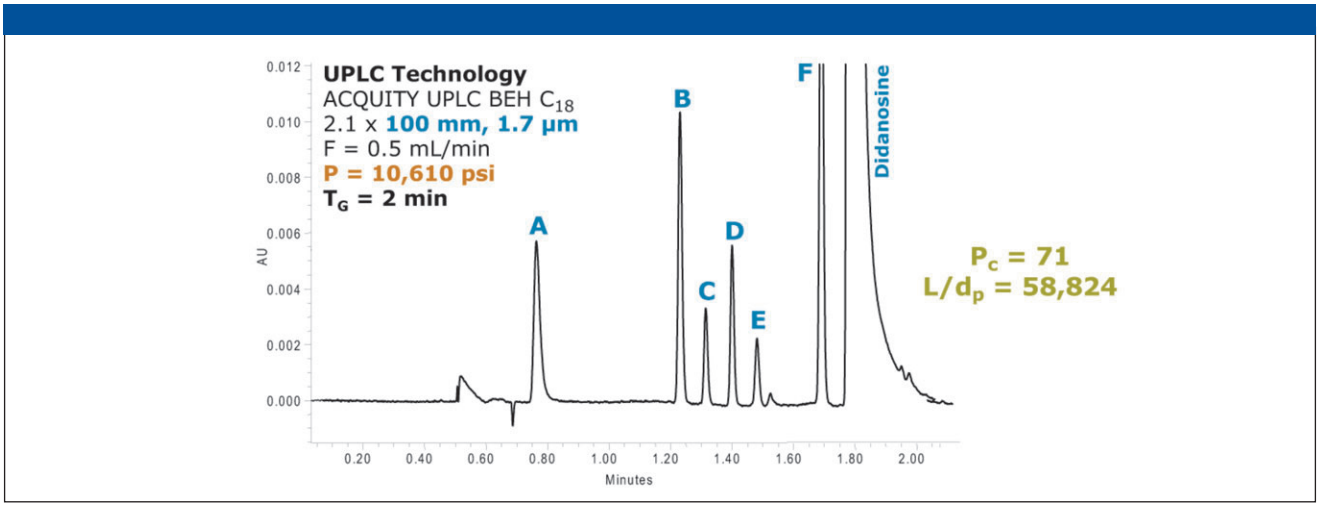


Figure 7: Ultra-high resolution separation of didanosine and its impurities, performed on a 100 mm length column in 2 min.

column volume. Note that the peak capacities achieved are the same in both cases, only the analysis time on the 1.7 µm column is 49 s instead of more than 7 min on the 5 µm column.

By increasing the column length to 100 mm (doubling L/d_p), peak capacity was increased by 30% while maintaining a relatively short gradient time of 2 min (Figure 7), which is still more than 3.5× less than the original HPLC method shown in Figure 6.

Conclusion

Chromatographers today are challenged with producing more information, processing more samples, and returning higher quality information in a shorter time frame. The development and commercialization of sub-2 µm porous particle LC columns has yielded the promised levels of performance with the added benefits of faster analysis time.

However, in order to take advantage of this increased performance, it is necessary to run sub-2 µm columns on a system specifically designed to maximize their full separation power.

This means that the system must be capable of operating at the backpressures generated by small particle columns operated at their optimal flow rate. It also means that the system volume must be minimized in order to prevent a loss in performance caused by band-spreading.

It is clear from the data presented here that a conventional HPLC system cannot be successfully modified to meet these criteria, and therefore cannot be used to realize the full potential of sub-2 µm particles.

The use of sub-2 µm particles in combination with the ACQUITY UPLC System provides superior performance for developing new separation methods or transferring existing HPLC methods to UPLC technology. The flexibility of this technology allows the chromatographer to optimize analyses for speed and productivity (with no loss in resolution), or for maximum resolution in the same analysis time.

References

(1) Neue, UD. 1997. *HPLC Columns: Theory, Technology, and Practice.* New York, NY: Wiley.

HPLC to UPLC Migration: Taking the Guesswork out of Transfer and Development

Waters Corporation, Milford, Massachusetts

High performance liquid chromatography (HPLC) has proven to be the dominant technology used in laboratories worldwide during the past 30-plus years.

One of the primary drivers for the growth and continued use of HPLC has been the evolution of the packing materials used to effect the separation. One of the underlying principles of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2 µm, not only is there a significant gain in efficiency; the efficiency doesn't diminish at increased linear velocities or flow rates as dictated by the van Deemter equation.

The use of smaller particles provides enhanced chromatographic speed, resolution, and sensitivity; this advancement represents the evolution of a new technique: UltraPerformance LC®, or UPLC®.

With UPLC, it is now possible to take full advantage of chromatographic principles to run separations on shorter columns and/or with higher flow rates for increased speed, with superior resolution and sensitivity.

So, the word is out. More and more laboratories are migrating to UPLC as the advantages of speed, resolution and sensitivity are born out in application after application.

But are the theoretical increases translating to real increases in laboratory efficiency and productivity? For methods in development, the answer is a resounding, "Yes!" Lab after lab has adopted this exciting new technology, and has notified their QC lab managers to get ready.

In addition, companies are evaluating the possible return on investment derived from migrating existing HPLC methods to UPLC.

The following testimonials are just a small composite sampling of customer experiences with implementing this exciting new technology, UPLC.

Faster Method Development

How long does it take to develop an HPLC method? A few days? A few weeks?

HPLC method development scouting runs can typically take 30 to 60 min or more. So when tasked with the evaluation of several column chemistries, different solvents, or pHs, the hours pile up.

With a typical analysis time of a few minutes or less, UPLC methods can be developed in a fraction of the time of HPLC methods-laboratory throughput can be improved dramatically, perhaps by an order of magnitude. This marked enhancement allows for additional scouting runs, ultimately resulting in more robust methods and greater productivity.

One Waters field application laboratory worked with a customer to develop a single QC method for several over-the-counter (OTC) cough/cold actives currently analyzed with multiple HPLC methods with long (>20 min) analysis times.

Using UPLC, a single, repeatable, quantitative 3 min method was developed in under 3 hours. Throughput was increased by over 300 percent, with a 75 percent reduction in analysis time and 80 percent reduction in solvent waste.

Column History Revealed with ACQUITY UPLC eCord Technology

You've witnessed it before; the method development chemist pulls a column out of the drawer, develops a method and it can't be reproduced on another column.

For columns in routine use, you may wonder what was the last solvent used? How many injections were performed, and under what conditions? Is the column still operating to specifications? For that matter, what are the specifications?

There is an easy way to review and record column specifications and track column use with ACQUITY UPLC® eCord™ Technology, which provides an easily traceable and paperless column history.

The column's eCord connects to a memory chip that plugs into a reader in the ACQUITY UPLC Column Manager (a thermostated module capable of holding up to four columns and includes column switching capabilities for automating method development). This chip contains all column manufacturing information, including packing batch number, serial number, part number, and the column dimensions as well as the information currently reported in a certificate of analysis.

All this information is transferred to the data station where it is recorded and stored with results. The eCord keeps a running history of column use, recording and sharing information such as number of injections, highest temperature and pressure experienced and the last methods run.

This information allows a user to have prior knowledge of a column's history before putting it to use. It also allows all of this column information to be stored and retrieved with every result. Bottom line: less down time for method transfer.

Migrating to UPLC: Scaling the Way to Cutting Costs

Migrating from HPLC to UPLC can at first appear challenging. But some easy steps using a few equations that geometrically scale the original method to the new column dimensions can make the process quite easy. These equations take into account changes in gradient time, flow rate and injection volume.

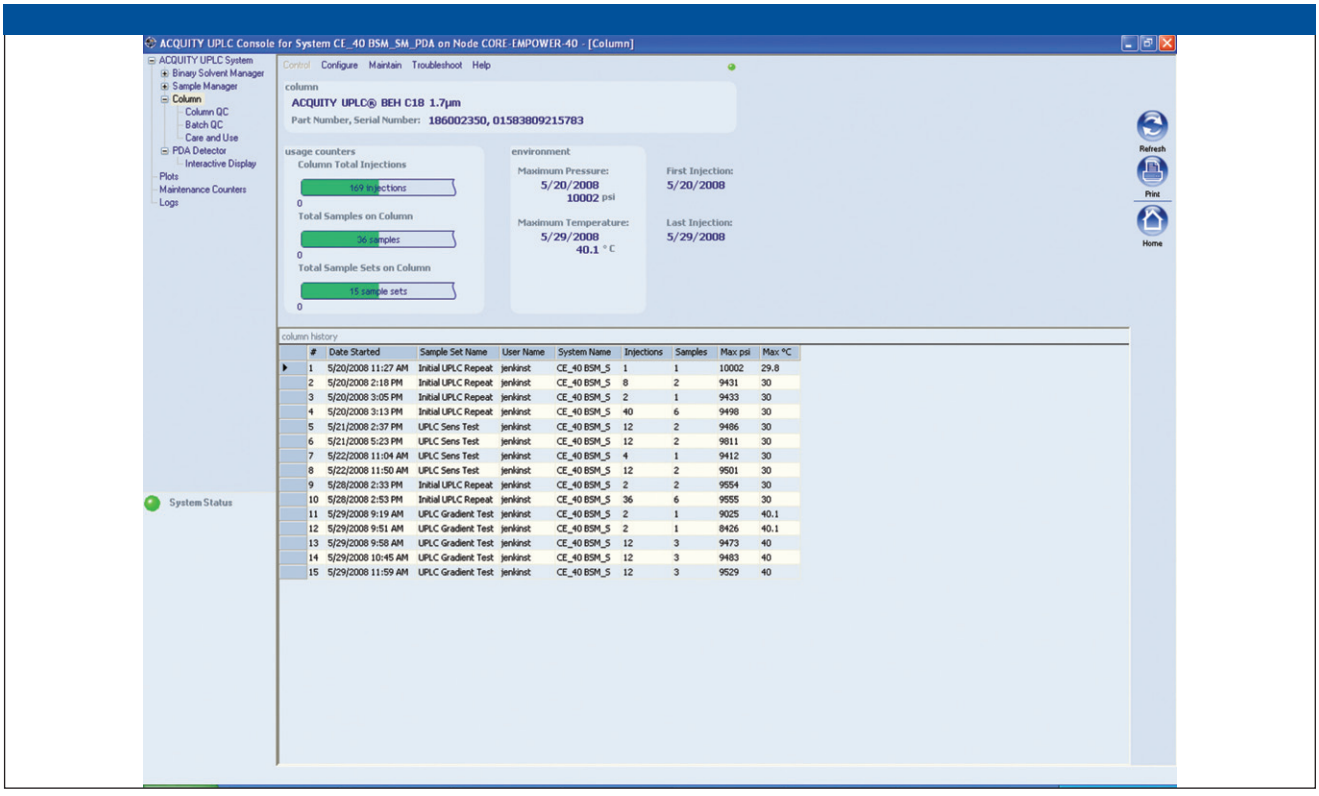


Figure 1: ACQUTIY UPLC eCord Technology and user history file.

The gradient is scaled from HPLC to UPLC using: $L_2/L_1 \times t_{g1} = t_{g2}$ where $L_1$and L_2 are the lengths of the HPLC and UPLC columns, and t_{g1} and t_{g2} are the times of each gradient step respectively.

Flow rate is scaled taking into account the difference in the diameter of the two columns: $(d_2)^2/(d_1)^2 \times F_1 = F_2$ where d_2 and d_1 are the column diameters and F_1 and F_2 the flow rates.

To keep the column volumes proportional, the gradient steps should be readjusted for the new flow rate: $(F_2 \times t_{g2})/F_3 = t_{g3}$ where F_2 and t_{g2} are the flow rate and gradient time of the geometrically scaled values (typically 650 µL/min for small molecules on a 2.1 mm I.D. column) and F_3 and t_{g3} are the optimized values.

The injection volume is scaled taking into account the volumes of the two columns: $V_1 \times [(r_2^2 \times L_2)/(r_1^2 \times L_1)] = V_2$ where r_2^2 and r_1^2 are the radii of the columns, L_1 and L_2 are the lengths of the columns, and V_1 and V_2 are the injection volumes.

As shown in the chromatogram using the above approach, a <10 min UPLC chromatogram was obtained from what was previously a 65 min HPLC method. Total analysis time to release the batch in QC decreased from 13 h to 2 h.

Savings in labor, reduced solvent usage and disposal, and the ability to release more batches more quickly expediting delivery to patients and revenue recognition made a compelling case for this customer to migrate to UPLC.

The customer is now prioritizing their product list to evaluate

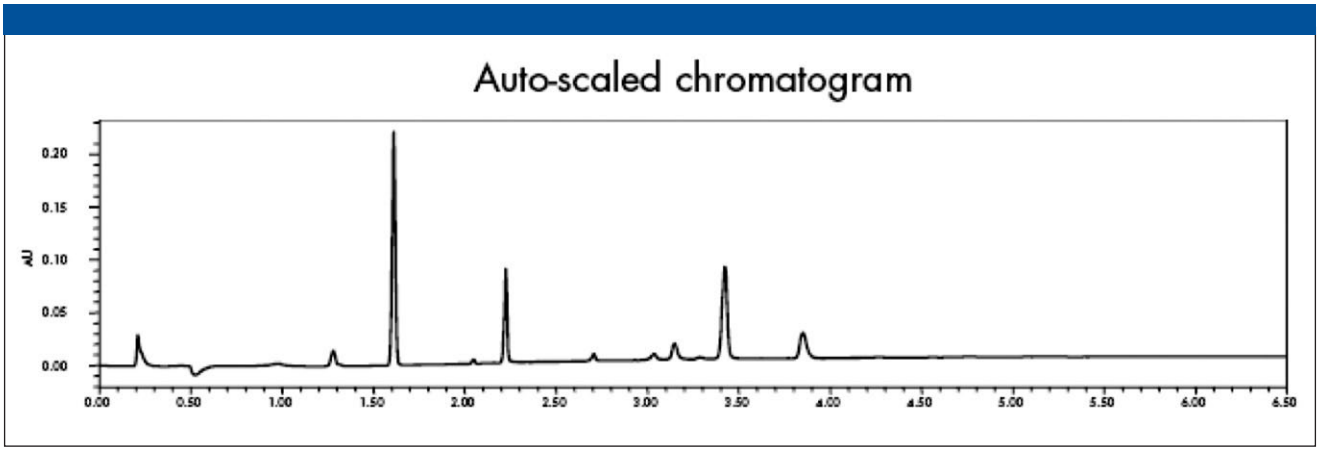


Figure 2: UPLC related substances assay.

migrating all of their methods to UPLC.

ACQUITY UPLC BEH Column Chemistries

Combined with the increased efficiencies of UPLC technology, the ACQUITY UPLC BEH C_{18}, C_8, Shield RP18, and Phenyl column chemistries enable the rapid development of faster and more robust separations.

ACQUITY UPLC BEH C_{18} and C_8 columns were designed to be the universal columns of choice for most UPLC separations by providing the widest pH range. ACQUITY UPLC BEH C_{18} and C_8 columns incorporate trifunctional ligand bonding chemistries, which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7 µm BEH particle to deliver the widest usable pH operating range.

ACQUITY UPLC BEH Shield RP18 columns are designed to provide selectivities that complement the ACQUITY UPLC BEH C_{18} and C_8 phases. ACQUITY UPLC BEH Shield RP18 columns combine Waters' patented Shield Technology with BEH Technology™ by incorporating an embedded carbamate group into the bonded phase ligand.

The alternate selectivity and excellent peak shape from the embedded polar group ligand, when combined with the wide pH range and ultra-efficiency of the 1.7 µm BEH particle, provide a necessary and powerful tool for UPLC method development. ACQUITY UPLC BEH Phenyl columns utilize a trifunctional C_6 alkyl tether between the phenyl ring and the silyl functionality.

This ligand, combined with the same proprietary endcapping processes as the ACQUITY UPLC BEH C_{18} and C_8 columns, provides long column lifetimes and excellent peak shape. This unique combination of ligand and endcap on the 1.7 µm BEH particle creates a new dimension in selectivity and efficiency for challenging UPLC separations.

ACQUITY UPLC HSS Column Chemistries

The ACQUITY UPLC High Strength Silica (HSS) particle is a 100% silica particle designed specifically for 15,000 psi (1000 bar) applications. ACQUITY UPLC HSS column chemistries include C_{18}, C_{18} SB and T3.

The ACQUITY UPLC HSS C_{18} column is an ultra-performance general purpose C_{18} bonded phase that provides superior peak shape for base, increased retention (vs. ACQUITY UPLC BEH C_{18} columns), and extremely long column lifetimes at low pH. The selectivities and retention observed with HSS C_{18} columns will resemble that of most modern, fully endcapped silica-based C_{18} HPLC columns.

The ACQUITY UPLC HSS C_{18} SB (Selectivity for Bases) column is an unendcapped C_{18} bonded phase optimized for low pH method development applications and provides alternative selectivities, especially for basic compounds, as compared to most fully endcapped C_{18} chemistries.

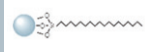
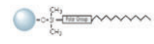
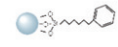
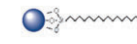
The ACQUITY UPLC HSS T3 column is aqueous mobile phase-compatible and designed to retain and separate polar organic molecules.

Column Scouting Approaches for Method Development

There are many approaches to method development. One common approach calls for evaluating different columns, pH's and solvents, commonly called column scouting. Once the various conditions are scouted, additional optimization runs are made.

Because of the number of runs, in the HPLC world method development time is measured by the calendar. With UPLC, the combination of increased separation speed and uncompromising resolution yields runs in minutes or less. Feedback is instantaneous, meaning development is often done on the fly. Faster

Table I: UPLC Column Chemistries. ACQUITY UPLC Columns are available in more than 50 combinations of configurations, chemistries and offerings including 1.7 µm BEH UPLC particles, 1.8 µm HSS UPLC particles, seven bonded-phases, and reversed-phase and HILIC.

	BEH Particle					HSS Particle		
	C_{18}	C_8	Shield RP18	Phenyl	HILIC	C_{18}	C_{18}SB	T3
Chemistry								
Ligand Type	Trifunctional C_{18}	Trifunctional C_8	Monofunctional Embedded Polar Group	Trifunctional C_6 Phenyl	—	Trifunctional C_{18}	Trifunctional C_{18}	Trifunctional C_{18}
Ligand Density*	3.1 µmol/m²	3.2 µmol/m²	3.3 pmol/m²	3.0 pmol/m²	—	3.2 µmol/m²	1.6 pmol/m²	1.6 µmol/m²
Carbon Load*	18%	13%	17%	15%	—	15%	8%	11%
Endcap Style	Proprietory	Proprietory	TMS	Proprietory	—	Proprietory	None	Proprietory
pH Range	1–12	1–12	2–11	1–12	1–8	1–8	2–8	2–8

*Expected or approximate values.

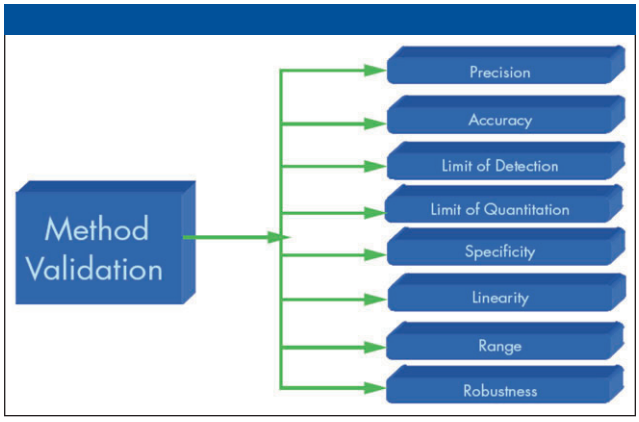


Figure 3: Common USP method validation parameters.

method development also means more time to evaluate a greater number of conditions, resulting in more robust methods.

And part of the beauty of UPLC is that it is governed by the same rules and theory as HPLC; there is nothing new to learn. Conventional chemometric software modeling approaches can also be used for even greater efficiency and throughput.

The result: more robust, accurate methods developed in a fraction of the time required for HPLC.

Migrating to UPLC: Method Validation

For a method in development, a compelling argument can be made for significant cost savings by migrating to UPLC. But what about methods for released products already in QC? Is revalidation necessary?

ACQUITY UPLC column chemistries fall into the broad USP designations of L1 (C_{18}), L7 (C_8) and L11 (phenyl). So in reality, migrating from HPLC to UPLC is really no different than making the switch from one column to another since both techniques share the same liquid chromatographic theory, rules, and mechanism of separation. The amount of revalidation is dependent on a company's SOPs and interpretation of the regulations.

Therefore migrating to 1.7 µm ACQUITY UPLC BEH columns is no different than what has been done historically in moving from the original 10 µm, L1 columns, to the 5 µm or 3.5 µm columns commonly in use today. Migration to UPLC is really quite simple, because it equates merely to a change in the method.

However, in a regulated environment, when a change is made to an existing analytical method, both of the following must also be considered:

1. A full validation of the new UPLC method should be conducted showing that it is suitable for its intended use.

2. A method equivalency study should also be conducted to show that the new UPLC method is better than or the same as the prior method.

A proper equivalency study demonstrates the sameness of two analytical methods.

If the new UPLC method is shown to be better or equivalent then no prior notification needs to be given to the FDA to proceed with the change. Prior notification is needed if the new procedure has lower quality attributes, such as being less precise or less specific. In this situation, an update needs to be included in the annual report of the NDA. For early stage compounds, an update to the IND should be provided to inform the regulatory agency of the new methodology.

An equivalency study (option 2) is also recommended if trending information is being gathered, e.g. stability samples where data is collected over time. It is prudent to demonstrate method equivalency of the HPLC method and the UPLC method so that out of trend results are not obtained in error.

So yes, revalidation is necessary; but like method development runs, method validation runs are completed in a fraction of the time by UPLC compared to HPLC, placing much less strain on resources.

Even with typical validation protocols often exceeding 250 injections, the speed of the ACQUITY UPLC System can reduce validation time to hours instead of weeks.

Automated Method Validation

Empower™ 2 Method Validation Manager (MVM) software not only automates the chromatographic method validation workflow, it also addresses many of the process limitations, challenges, and bottlenecks that analysts face with the typical chromatographic method validation process.

MVM assists you in adhering to your companies' corporate validation requirements while performing the validation workflow in use today. MVM allows the entire chromatographic method validation process, from protocol planning, through data acquisition, data processing, data review and approval, as well as final reporting to be performed within the compliant-ready environment of Empower 2. So, many of the manual and error prone steps such as exporting data to other software applications, checking for transcription errors, verifying data placement and calculation syntax, data review and approval, and final reporting are either streamlined or eliminated entirely.

Streamlining the chromatographic method validation workflow offers up to an 80% reduction in the time and cost associated with the method validation process.

ACQUITY UPLC Assay of Amoxicillin and Potassium Clavulanate from an Oral Suspension

Daniel Root and Andrew Aubin, Waters Corporation, Milford, Massachusetts

The migration and consolidation of legacy methods to the Waters® ACQUITY UPLC® System is a major focus for companies adopting UltraPerformance LC® (UPLC®) technology. Those companies that invest in the process are rewarded with significant savings in analysis times and operational costs while conserving or improving overall chromatographic performance.

Many laboratories are choosing to redevelop legacy methods during this migration in order to take full advantage of the strengths of the ACQUITY UPLC System, creating even more robust methods in the process. However, some legacy methods may not require full redevelopment; users can still realize significant method improvement by simply transferring their method from HPLC to UPLC.

For example, the assay of amoxicillin and potassium clavulanate from an oral suspension[1] is an established HPLC method that can be simply migrated to the ACQUITY UPLC with minimum time and effort. Amoxicillin (Figure 1A) is a ß-lactam antibiotic with primary activity against gram-positive bacteria. To broaden the effect of this drug on select gram-negative bacteria and to combat resistant strains, potassium clavulanate (Figure 1B) is added to formulations of amoxicillin to inhibit amoxicillin degradation by the enzyme ß-lactamase.

This application note summarizes the results of a simple migration of a USP-based HPLC assay to a UPLC-based method.

Experimental

Materials

Amoxicillin and potassium clavulanate standards, as well as sodium phosphate, were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). Methanol was purchased from Fisher (Fair Lawn, New Jersey). Water used in the study was purified with a Milli-Q Gradient A10 system (Millipore, Billerica, Massachusetts). The standard and assay preparations were made according to the USP method[1].

Instruments and conditions

The USP HPLC verification was performed on an Alliance® 2695 Separations Module equipped with a 2996 Photodiode Array (PDA) Detector. A Waters XBridge™ C_{18} 5 µm 4.6 × 250 mm column was selected to satisfy the USP L1 phase requirement.

The UPLC development was performed on an ACQUITY UPLC System consisting of a Binary Solvent Manager (BSM), Sample Manager (SM) and Tunable UV detector (TUV). A Waters ACQUITY UPLC BEH C_{18} 1.7 µm 2.1 × 100 mm column was selected for the separtion. All instruments were controlled and data collected and analyzed using Waters Empower™ 2 Software.

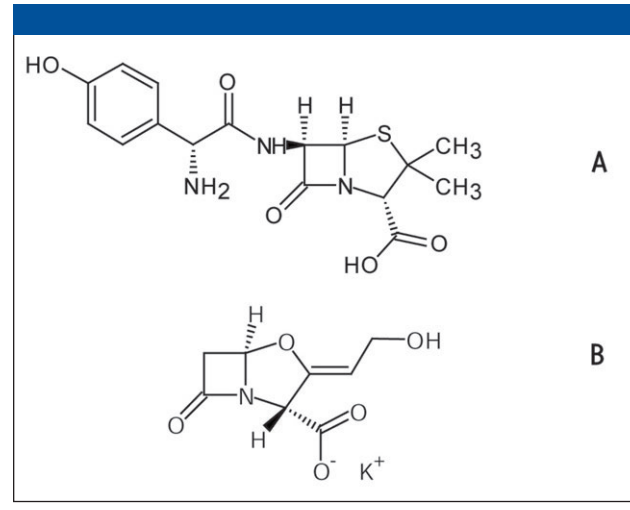


Figure 1: (A) amoxicillin and (B) potassium clavulanate.

Verification of the HPLC method

The isocratic HPLC method, from the USP, was verified using a sodium phosphate buffer, pH 4.4 in the proportion 95:5 with HPLC grade methanol. A 20 µL injection volume was analyzed on the Alliance HPLC System and detected at 220 nm. No sample or column temperatures were stated in the USP method, so a 15 °C sample temperature and 30 °C column temperature were selected.

The run time was about 7 min, and the flow rate was 1.5 mL/minute. The USP acceptance criteria for this assay were: a resolution of 3.5 or greater for the two components, a USP tailing value of not more than 1.5, a USP plate count of 550 or more and a percent RSD for area of 2% or less for replicate injections. The HPLC assay met the acceptance criteria and was then transferred to the ACQUITY UPLC System.

Transfer to the ACQUITY UPLC

The verified HPLC method was transferred to UPLC using the Waters ACQUITY UPLC Calculator. As suggested by the calculator, a 1.7 µm, 2.1 × 100 mm ACQUITY UPLC BEH C_{18} column was selected for the assay (in order to conserve the

Table I: Comparison of the mean ACQUITY UPLC assay results to the USP acceptance criteria.

USP Acceptance Criterion	UPLC Potassium Clavulanate	UPLC Amoxicillin
R ≥3.5	8.0	
Tailing≤1.5	1.0	1.1
>550 plates	4232	7676
%RSD area <2%	1.0	1.1

L/d_p ratio of the HPLC column used in the HPLC assay) with a 1.7 µL injection at a flow rate of 0.40 mL/minute. Detection wavelength (220 nm), mobile phase composition, standard and assay preparations remained unchanged. The sample compartment and column temperatures were maintained at 15 °C and 30 °C, as in the verification. The run time under these new conditions was 3.5 min with UPLC compared to 7 min for the HPLC method. Figure 2 shows the result of the ACQUITY UPLC assay.

Table I compares the assay results to the USP acceptance criteria. This calculator-transferred method easily exceeded the assay acceptance criteria as stated in the USP. The unknown peaks, B and C, were observed in both the original HPLC and transferred UPLC assays.

Results and Conclusions

This transfer was an example of how smoothly an established legacy method can be migrated to the ACQUITY UPLC System. In comparison to the original HPLC assay, the run time was shortened by more than 50 percent (HPLC at 7 min; UPLC at 3.5 min) and the flow rate reduced by 3.75 times, delivering a savings in time (resulting in an increased sample throughput) as well as overall cost of analysis by reducing solvent usage and waste disposal costs.

Streamlining the migration of this legacy method by using the ACQUITY UPLC Calculator made the transfer quick and simple. In a laboratory already performing the assay, verification would be unnecessary and the transfer process would call for only a single step.

The subsequent validation experiments required following any method transfer could be further facilitated by the application of the Empower 2 Method Validation Manager.

References

(1) The United States Pharmacopeia USP 29, The National Formulary NF24, United States Pharmacopeial Convention, Inc. 2006, pg. 161.

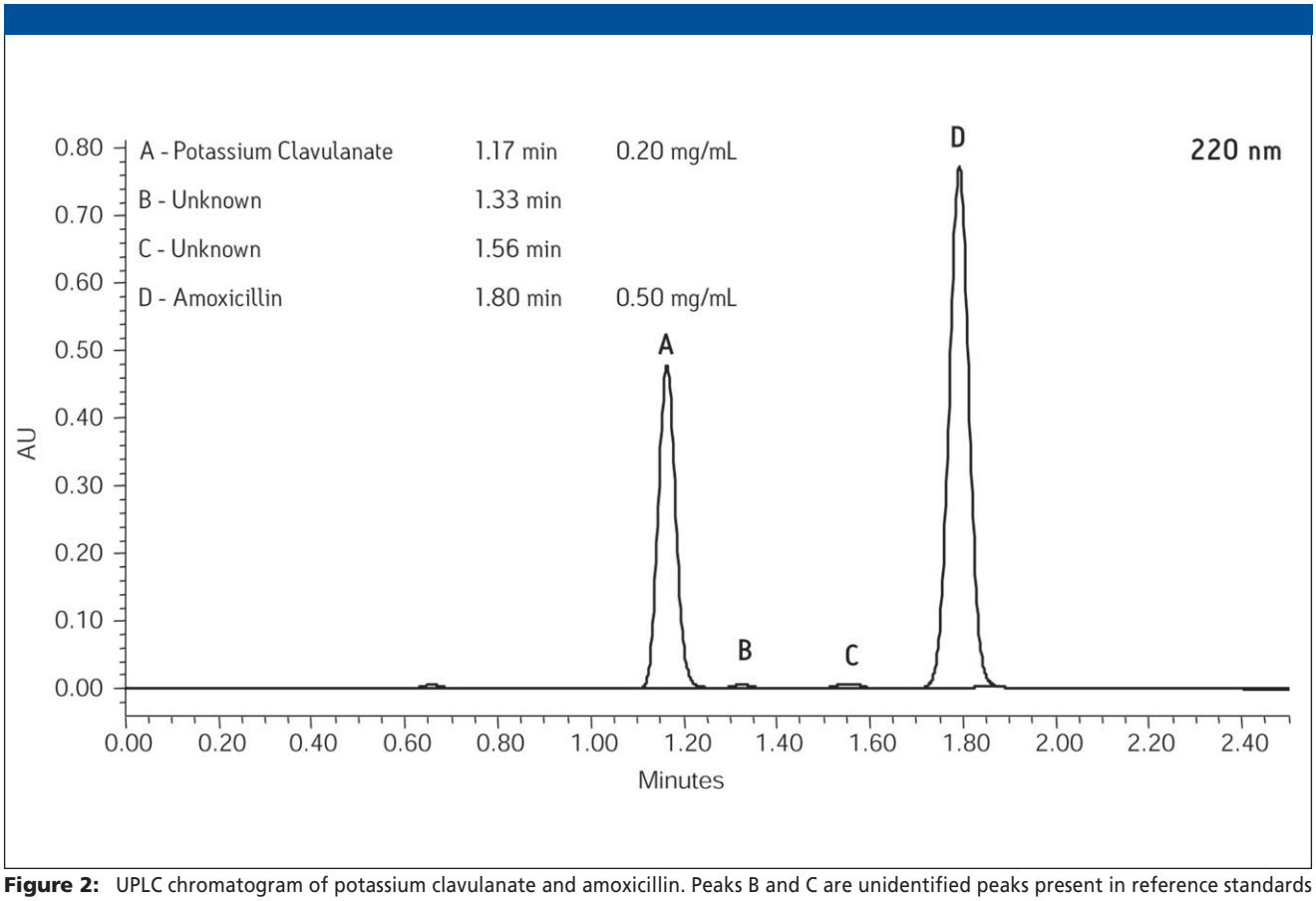


Figure 2: UPLC chromatogram of potassium clavulanate and amoxicillin. Peaks B and C are unidentified peaks present in reference standards and the oral suspension drug product.

Utilization of UPLC and Empower 2 CDS for Efficient Method Development of an Impurity Profile of Simvastatin and Related Impurities

Michael D. Jones, Paul Lefebvre, and Rob Plumb, Waters Corporation, Milford, Massachusetts

Many pharmaceutical analytical applications are focused on the identification and quantification of the active pharmaceutical ingredient (API) and related impurities. This activity requires a high resolution validated methodology, which is often time consuming to develop. The method development bottleneck results from the requirement to generate a quantitative and qualitative profile of impurities, enabling the reporting of the identity and quantity of each chemical moiety.[1]

The impurities that are frequently present are a small fraction of the main component, with identification and reporting requirements of impurity peaks at 0.05% area relative to the API. Due to the low concentration of these impurities, high instrument sensitivity and selectivity become a necessity in order to demonstrate process compliance to regulatory agencies without compromising the quality throughput needed to meet the fiscal demands of the business.

When taken orally, simvastatin, a well-known prescribed class of statin for lowering cholesteral, hydrolyzes to the b-hydroxy acid form, which acts as an inhibitor of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase enzyme involved in the *in vivo* synthesis of cholesterol.[2] Recently the patent rights for production of simvastatin have expired, allowing it to be manufactured by generic pharmaceutical companies.

There are several methods for analyzing simvastatin and its related impurities. Two official methods utilizing HPLC gradient methodology are reported in the European Pharmacopoeia (EP) and the United States Pharmacopoeia (USP).[3,4] These methodologies are typically time consuming, with analysis times in excess of 30 min. To meet the business needs of a generic pharmaceutical company, a faster methodology is required that does not compromise analytical quality.

This study demonstrates the utility of UltraPerformance LC® (UPLC®) technology and Empower™ 2 Software to aid in the efficient method development process of impurity profiles for pharmaceutical drug entities.

In this application, we show how the HPLC method for simvastatin has been redeveloped on UPLC and is compatible for MS. The analytical goals were to meet the requirements stated in the USP 30 - NF 25 monograph for simvastatin drug substance for chromatographic purity and possibly be used for the assay. Empower 2 custom reporting, custom fields, and spectral analysis were used in streamlining the decision making process during method development.

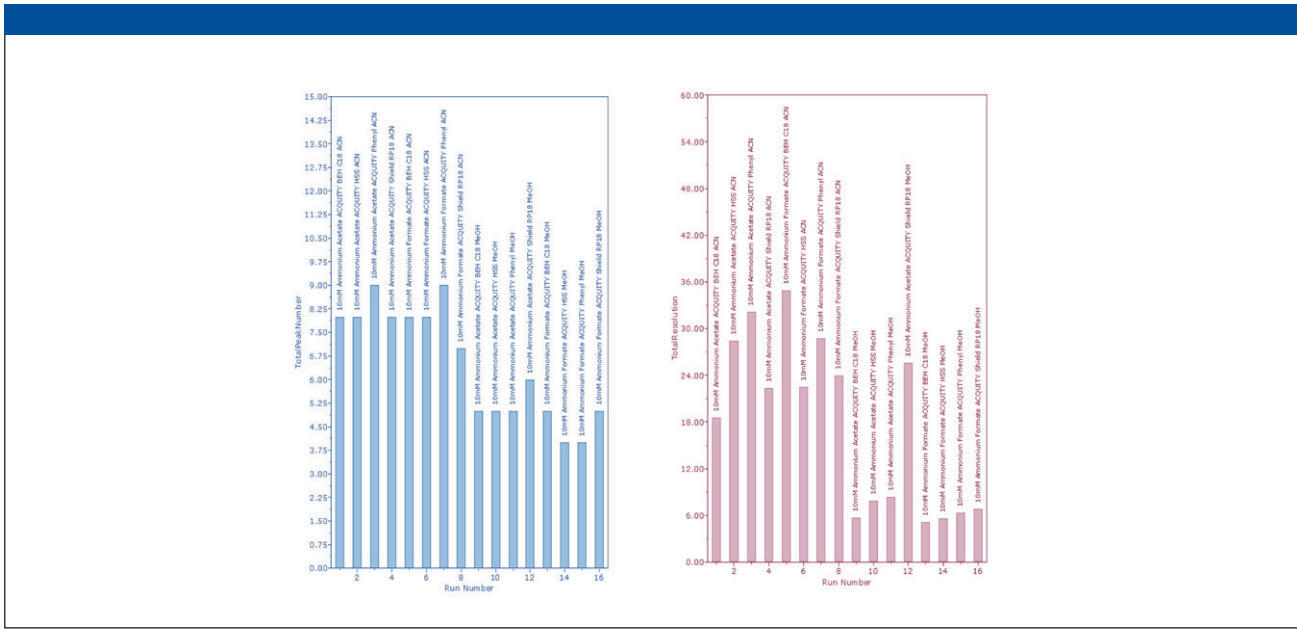


Figure 1: The Empower 2 software summary plots, reporting values for total peak number and total resolution for each injection of the method screening process that was performed for the simvastatin impurity profile.

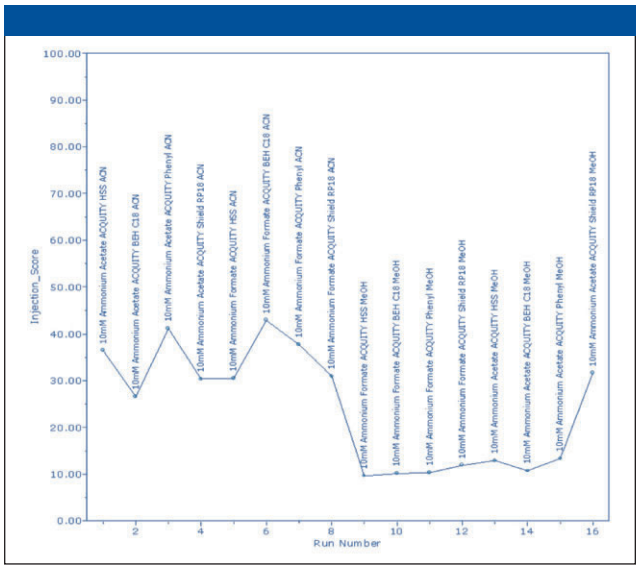


Figure 2: Custom summary plot of calculated "Best set of injections" based on the summary bar charts from Figure 1.

Method development and UPLC conditions

LC System:	Waters® ACQUITY UPLC® System
Column:	2.1 × 50 mm, 1.7 µm (1.8 µm for HSS)
Column 1:	ACQUITY UPLC HSS C_{18} column
Column 2:	ACQUITY UPLC BEH C_{18} column
Column 3:	ACQUITY UPLC Phenyl column
Column 4:	ACQUITY UPLC Shield RP_{18} column
Column Temp.:	30 °C
Flow Rate:	800 µL/min

Mobile Phase A1: 15 mM ammonium formate, pH 4.0
Mobile Phase A2: 15 mM ammonium acetate, pH 4.0
Mobile Phase B1: Acetonitrile
Mobile Phase B2: Methanol
Gradient: Linear 2 to 100% B1 / 3 min (ACN)
Linear 2 to 100% B2 / 5 min (MeOH)

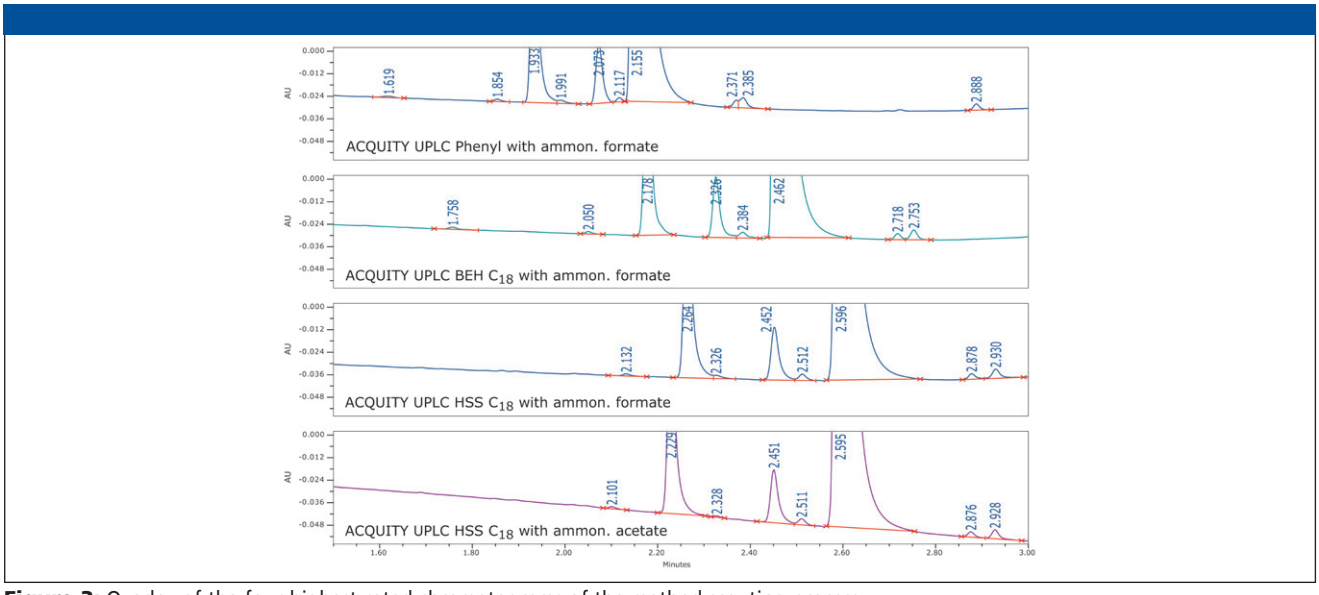


Figure 3: Overlay of the four highest-rated chromatograms of the method scouting process.

Experimental

Traditionally, method scouting involves an experimental process of screening columns, mobile phase composition, and pH. In this particular application test case, some parameters can be eliminated immediately before the scouting process to better speed the analysis time and limit collection of unwanted data.

Columns with 50 mm lengths decrease analysis time while evaluating which chemistry and solvent conditions will work best. Further research revealed that the pH range for optimum simvastatin analysis is best within pH 4 to 6, due to the rapid hydrolytic degradation of simvastatin above pH 6 and spontaneous degradation at pH 9.[5] In substitution of the screening at alkaline pH, two different types of buffers at pH 4.0 were used during the scouting injections.

Instrumentation

The UPLC method development was performed on a Waters ACQUITY UPLC system consisting of a Binary Solvent Manager (BSM), Sample Manager (SM) and Photodiode Array detector (PDA). Various 1.7 µm ACQUITY UPLC columns were selected for the separation as described in the method conditions. All instruments were controlled and data collected and analyzed using Waters Empower 2 software. The ACQUITY UPLC Column Manager was employed to allow for the simple automated selection of four different columns.

Results

Mining the data

Empower 2 CDS was employed to mine data without the need

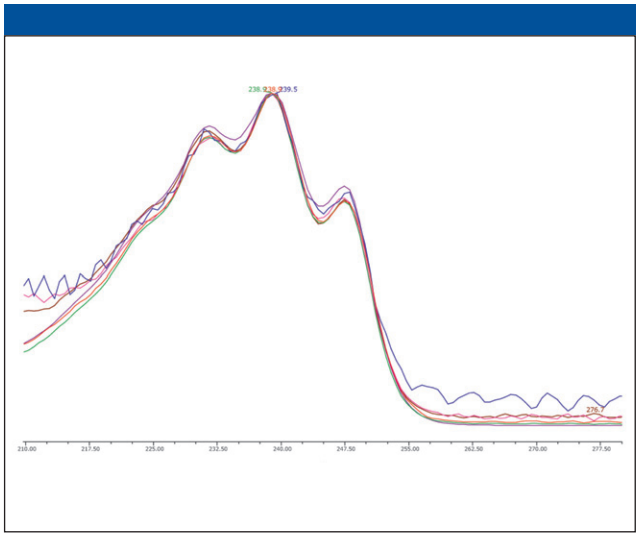


Figure 4: Normalized UV PDA spectral data for the simvastatin and impurity peaks. The data illustrates spectral similarity between the API and identified peaks, suggesting a related structural origin.

for manual review of the numerous injections in whole data sets. Simple drop-down menus within the CDS allow for the rapid review of the effects of buffer type, solvent, pH, and column type. Interpretation of the method scouting data of simvastatin and related impurities, in conjunction with these custom reporting features, resulted in an easy-to-read summary report.

The report describes per each injection of varied condition. The total number of detected peaks and total resolution of these values were automatically calculated to determine an injection score. Injection scores can be configured to account for any chromatographic criteria that the method development group uses to make decisions (Figure 1).

Empower custom reporting

Customized summary plots can either be bar charts or line plots (Figure 2). The summary plots indicated that the phenyl column with the ammonium formate buffer would yield the best average results. However, the phenyl column had difficulty resolving peaks RT=2.371 min and RT=2.385 min. A review of the four chromatograms, giving the greatest number of peaks and highest total resolution number (Figure 3), confirmed that the conditions for the ACQUITY UPLC HSS C_{18} column with ammonium acetate resulted in the best resolution between the critical pairs of peaks RT=2.229 min and RT=2.328 min.

Utilizing Empower for spectral analysis

It was determined from the screening experiments that all of the peaks present in the ultraviolet trace were spectrally similar to simvastatin. The optical characteristics of the ACQUITY UPLC PDA detector allowed for the generation of data with high spectral quality even at the low levels of detection allowing such determinations. Each peak was integrated and the UV spectral analysis (when normalized) clearly showed which impurity peaks were simvastatin-related (Figure 4).

The mass spectra data generated by the ACQUITY® SQ detector facilitated peak tracking during the method optimization process. Furthermore, the MS data allowed the confirmation of known impurities and identified the presence of an unknown impurity peak of [M+H] ion of m/z 421.2 that was not present on the UV chromatographic trace (Figure 5). The mass spectra also indicated NH_3 adduct formations. The adduct formation was present in the spectra of all of the impurities and the API. The integrated peaks were assessed during the method optimization to obtain the best possible resolution from the API.

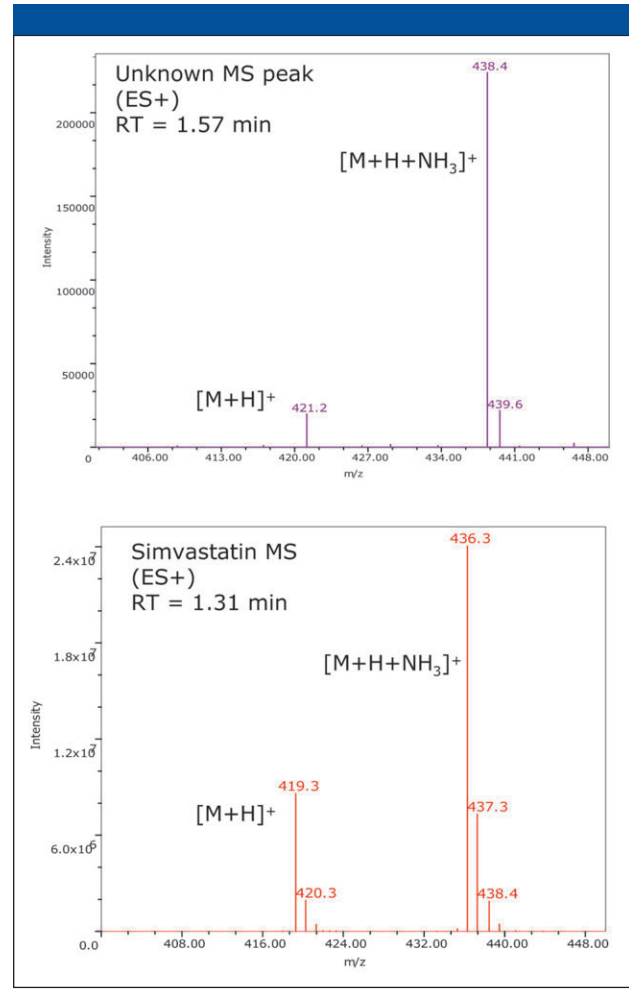


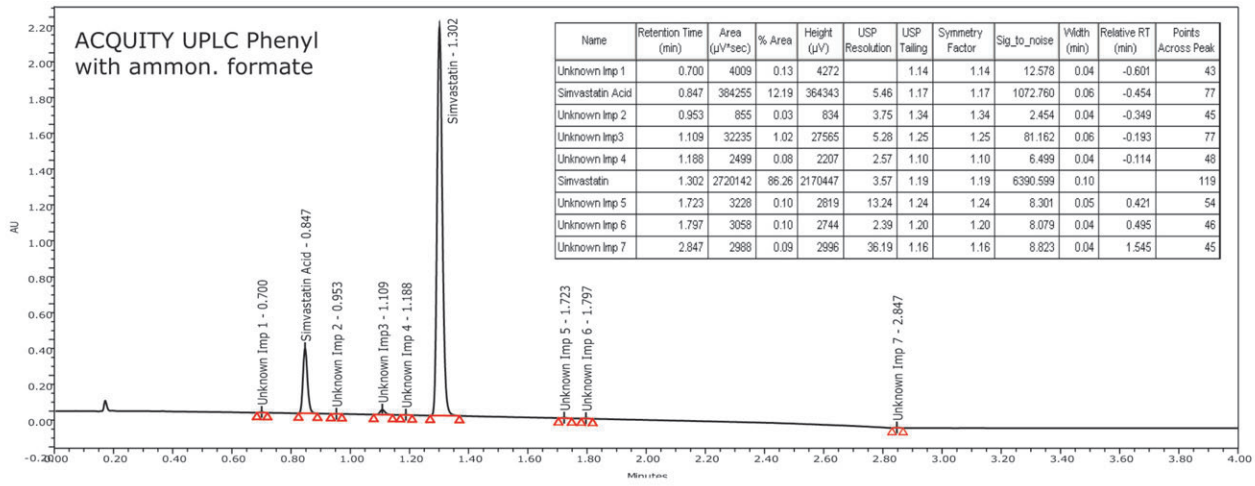
Figure 5: MS spectra of the unknown peak at RT=1.566 min. The adduct formation was consistent of observations of the simvastatin MS spectra (RT=1.31 min).

Method optimization

The UPLC method was optimized for the 1.8 µm ACQUITY UPLC HSS T3 2.1 × 50 mm column with ammonium acetate pH 4.0 and acetonitrile as the mobile phase. MS confirmation of many specified impurities and unspecified impurities facilitated peak tracking during the method optimization.

An experimental design of four injections were performed, including two different linear gradient slopes (five minutes and ten minutes) and two different temperatures (30 and 50 °C) to optimize the LC separation. The resulting data was collected and entered into chromatographic modeling software.

Optimal conditions for the 1.8 µm ACQUITY UPLC HSS C_{18} 2.1 × 50 mm column to maximize chromatographic speed



Name	Retention Time (min)	Area (µV*sec)	% Area	Height (µV)	USP Resolution	USP Tailing	Symmetry Factor	Sig_to_noise	Width (min)	Relative RT (min)	Points Across Peak
Unknown Imp 1	0.700	4009	0.13	4272		1.14	1.14	12.578	0.04	-0.601	43
Simvastatin Acid	0.847	384255	12.19	364343	5.46	1.17	1.17	1072.760	0.06	-0.454	77
Unknown Imp 2	0.953	855	0.03	834	3.75	1.34	1.34	2.454	0.04	-0.349	45
Unknown Imp3	1.109	32235	1.02	27565	5.28	1.25	1.25	81.162	0.06	-0.193	77
Unknown Imp 4	1.188	2499	0.08	2207	2.57	1.10	1.10	6.499	0.04	-0.114	48
Simvastatin	1.302	2720142	86.26	2170447	3.57	1.19	1.19	6390.599	0.10		119
Unknown Imp 5	1.723	3228	0.10	2819	13.24	1.24	1.24	8.301	0.05	0.421	54
Unknown Imp 6	1.797	3058	0.10	2744	2.39	1.20	1.20	8.079	0.04	0.495	46
Unknown Imp 7	2.847	2988	0.09	2996	36.19	1.16	1.16	8.823	0.04	1.545	45

Figure 6: Final method. 1.8 µm ACQUITY UPLC HSS C_{18} 2.1 × 50 mm column. A: 15 mM ammonium acetate, B: acetonitrile, 0.8 mL/min, 40 °C. Peak table for the final simvastatin methodology. Resolution of Rs >2.0 was achieved and peaks can be identified as low as 0.02% area.

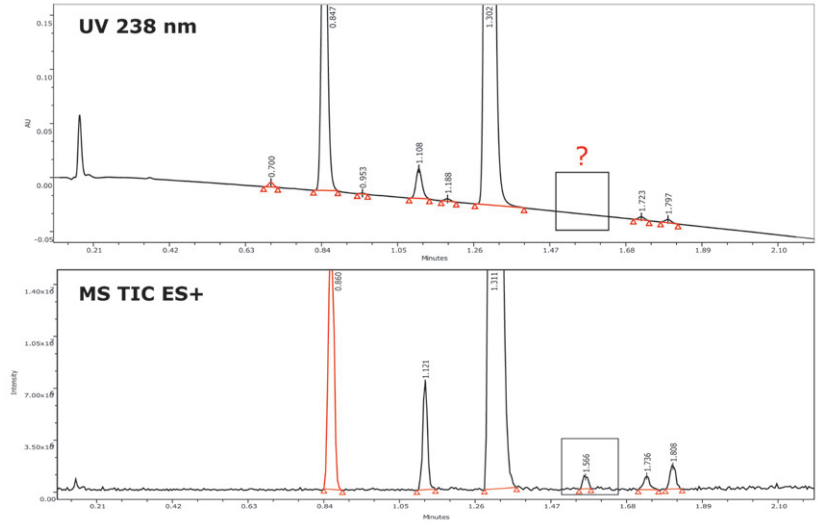


Figure 7: Zoomed baseline of the UV-MS final method. The presence of the unknown peak at 1.566 in the MS TIC was not present in the UV range.

and resolution yielded a flow rate of 800 µL/min with a gradient from 52% B to 100% B over 2.5 min, with a 1.5 min hold at 100% B to elute the dimer at 2.85 min at 40 °C. The final method conditions resulted in the chromatogram displayed in Figures 6 and 7.

Final method, UPLC conditions

LC System: Waters ACQUITY UPLC system
Column Dimensions: 2.1 × 50 mm, 1.8 µm
Column 1: ACQUITY UPLC HSS C_{18} column
Column Temp.: 40 °C
Flow Rate: 800 µL/min
Mobile Phase A: 15 mM ammonium acetate, pH 4.0
Mobile Phase B: Acetonitrile
Gradient: 52% B to 100% B over 2.5 min with a 1.5 min hold at 100% B
MS System: Waters ACQUITY SQ detector
Scan Range: 100 to 1000
Scan Rate: 10,000 amu/sec
Cone Voltage: 20 V
Source Temp.: 150 °C
Desolvation Temp.: 450 °C
Desolvation Flow: 800 L/Hr
Total Run Time: 4.0 min
Inj.-to-Inj. Run Time: 5.0 min

Conclusion

An efficient method development screening process was

employed utilizing short UPLC columns and a generic gradient to fast-track the method analysis screening time. The process takes advantage of UPLC technology, delivering rapid method scouting.

The use of short UPLC columns allowed many column chemistries to be screened quickly in an automated manner using the ACQUITY UPLC Column Manager. Further optimization for resolution was achieved by varying gradient slope and temperature.

Data collected on the ACQUITY UPLC PDA and ACQUITY SQ detectors allowed for UV-MS spectral analysis within Empower 2 software, which facilitated peak tracking (MS data), simvastatin relation (UV-MS data), and preliminary peak confirmation of identification. The use of specific labeling custom fields in Empower 2 allowed for the creation of custom reports to help expedite the mining of the resulting data which would normally take a considerable amount of manual review.

The utilization of the ACQUITY UPLC system and Empower 2 software provided a timely solution to the method development challenges associated with impurity profiling.

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(2) Vickers S, Duncan CA, Chen IW, Rosemary A, Duggan DE. *Drug Metab. Dispos.* 1990; 18: 138—145.
(3) European Pharmacopoeia. Council of Europe, Strasburg Cedex. 2002; 4th Edition.
(4) (2002) United States Pharmacopoeia, USP30-NF25, p. 3179. Pharmacopeial Forum: Vol. 32 (1) 141.
(5) Alvarez-Lueje et al, *J. AOAC Inter.* 2005; 88 (6): 1631–1636.

A Systematic Approach Towards UPLC Methods Development

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Waters Corporation, Milford, Massachusetts

Reversed-phase HPLC methods development can take anywhere from weeks to months, incurring large operational cost. By utilizing UltraPerformance LC® (UPLC®) Technology for methods development, a 6-fold improvement in throughput can be realized. This, in turn, reduces cost per sample and time of analysis considerably while maintaining or improving separation integrity. By developing rapid, high resolution analytical methods, products can be brought to market faster, therefore, improving the overall profitability of the assay.

A new method can be developed efficiently if experimental design is well thought out. Common methods development approaches include: conducting a literature search, trial and error, a step-wise iterative approach or a systematic screening protocol. A systematic screening protocol that explores selectivity factors such as pH, organic modifier and column chemistry, will be the premise of this strategy. This approach allows chromatographers to quickly determine which experimental parameters are most effective in manipulating the selectivity of a separation. By employing this strategy, the total number of steps necessary to develop a method are reduced, therefore, providing an efficient and cost effective approach.

In this application note, combinations of selectivity factors (pH, column chemistry and organic modifier) in UPLC separations were examined to develop high resolution chromatographic methods. Once the best combination of factors was selected, gradient slope and temperature were optimized. This methods development approach is demonstrated by developing a separation for paroxetine hydrochloride and its related compounds.

Experimental Conditions

LC conditions

Columns:	ACQUITY UPLC® BEH C_{18} 1.7 µm ACQUITY UPLC BEH Shield RP18 1.7 µm ACQUITY UPLC BEH Phenyl 1.7 µm ACQUITY UPLC HSS T3 1.8 µm		
Dimensions:	2.1 × 50 mm		
Mobile Phase:			
A1	20 mM Ammonium Formate, pH 3.0		
A2	20 mM Ammonium Bicarbonate, pH 10.0		
B1	Acetonitrile		
B2	Methanol		
Flow Rate:	0.5 mL/min		
Gradient:	Time (min)	%A	%B
	0.0	95	5
	5.0	10	90
	5.01	95	5
	5.5	95	5

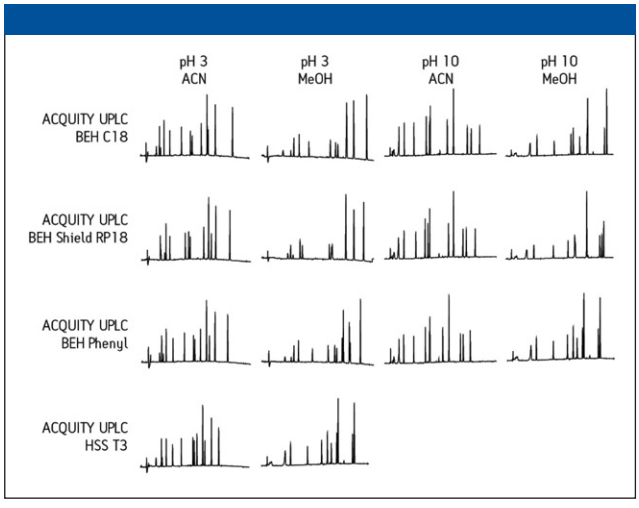

Figure 1: UPLC methods development experimental matrix.

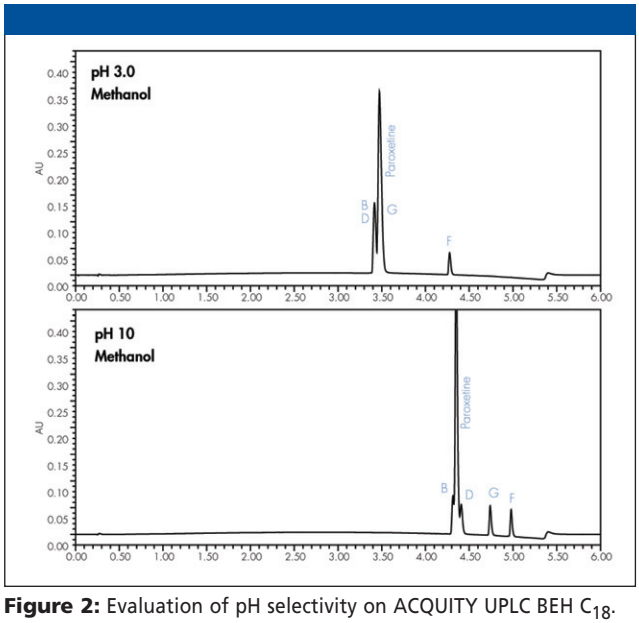

Figure 2: Evaluation of pH selectivity on ACQUITY UPLC BEH C_{18}.

Inject Volume:	4.0 µL
Temperature:	30 °C
Detection:	UV Scan 200–350 nm
Sampling Rate:	20 pts/sec
Time Constant:	0.1
Instrument:	Waters ACQUITY UPLC with ACQUITY UPLC Column Manager and ACQUITY UPLC PDA detector

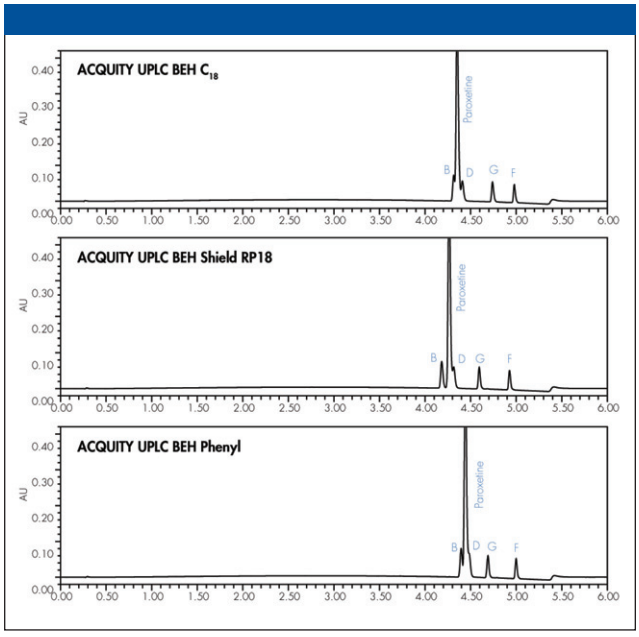

Figure 3: Comparison of column selectivity in methanol at alkaline pH.

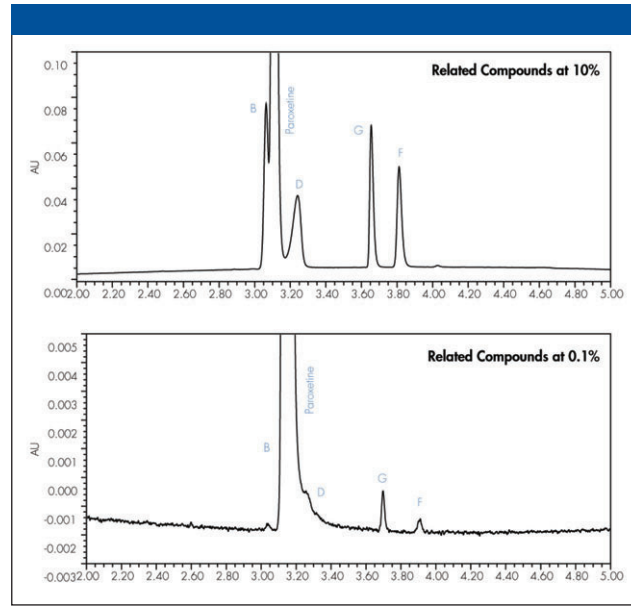

Figure 5: Related compounds at 10% versus 0.1% of paroxetine.

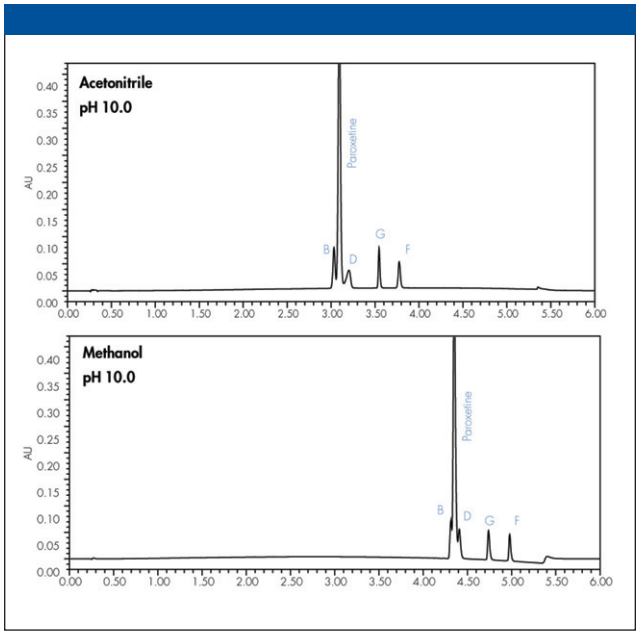

Figure 4: Evaluation of solvent selectivity on ACQUITY UPLC BEH C_{18}.

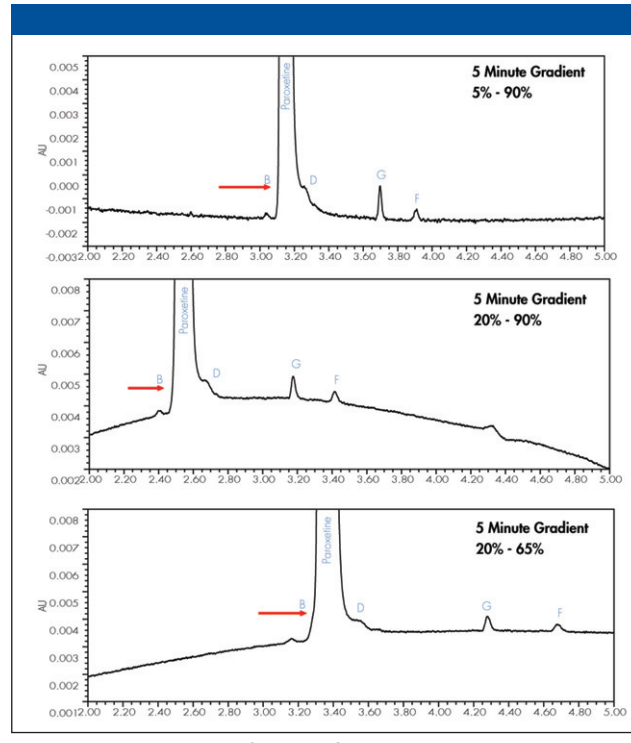

Figure 6: Monitoring influence of gradient slope reduction.

Results and Discussion

As depicted in Figure 1, a result matrix of fourteen chromatograms is generated by evaluating 3 hybrid (BEH) columns at low and high pH and a silica (HSS) column at low pH, with two different organic modifiers. Each experimental result was evaluated for retentivity, peak shape and resolution.

Step 1: Select the pH

By first evaluating the data acquired at low and high pH, the retention characteristics, loadability and overall resolution of the mixture of analytes can quickly be determined. Paroxetine is an alkaline species with a pKa of 9.8. It is, therefore, in its neutral charge state when the mobile phase is increased to pH 10. As seen in Figure 2, acidic mobile phase pH results in poor resolution of paroxetine and related compounds. Alkaline pH provides better retention and resolution of all components due to the neutral charged states of the analytes.

Step 2: Select column chemistry

Once pH is selected, a comparison of different stationary phases is made. As shown in Figure 3, all three BEH columns show

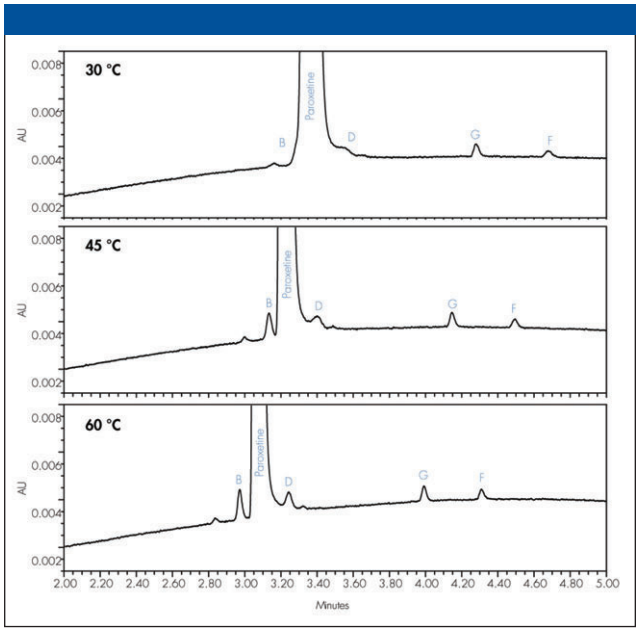

Figure 7: Influence of temperature on separation.

potential for resolving all components. The ACQUITY UPLC BEH C_{18} was selected to carry out the separation.

Step 3: Select organic modifier

Lastly, the organic modifier is selected. Methanol offers a different selectivity than acetonitrile, and is a weaker elution solvent at equivalent concentration. This results in greater retention of the analytes. For this set of components, acetonitrile offers a better separation, as depicted in Figure 4.

Optimization

During our initial method screening, the related compounds were spiked into the solution at a 10% concentration level relative to paroxetine for ease of identification. For method optimization, the concentration of the related compounds was reduced from 10% of paroxetine to the target concentration of 0.1%, as shown in Figure 5. However, at the 0.1% concentration level, inadequate resolution among paroxetine and related compounds B and D resulted due to disparate levels of concentration making for a more challenging separation.

In efforts to improve the separation, gradient slope and temperature were manipulated.

Optimization: Gradient slope

Changing gradient slope is often a balance between resolution and sensitivity. Although selectivity change can occur, most often a steeper gradient slope will result in a reduction in resolution and an increase in sensitivity, while a shallower gradient slope will result in an increase in resolution and a decrease in sensitivity.

In efforts to improve resolution, the gradient slope was flattened by changing the % organic at the start and then endpoint of the gradient. In this case, marginal improvement was made by altering the gradient slope as depicted in Figure 6. Using the 20 – 65% acetonitrile gradient, the influence of column temperature was then explored.

Optimization: Temperature

Temperature affects every chemical process that occurs. Analyte diffusivity, sample loadability and peak shape dramatically

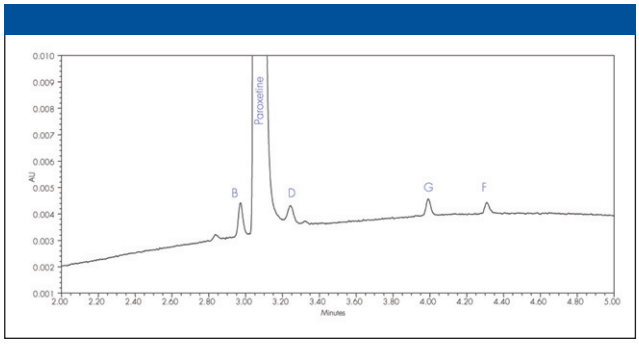

Figure 8: Final separation of Paroxetine and related compounds B, D, G, and F at the 0.1% level.

improved with increasing temperature. At 60 °C, adequate separation of related compounds from paroxetine was achieved; therefore, no further optimization was necessary.

Final conditions

Separation was performed on a ACQUITY UPLC BEH C_{18} 2.1 × 50 mm, 1.7 µm column at 60 °C. Mobile Phase A contained 20.0 mM ammonium bicarbonate with 1.2% ammonium hydroxide. Mobile Phase B was acetonitrile. A 5 minute gradient from 20 to 65% acetonitrile was performed. Flow rate was 0.5 mL/min.

Business impact

Productivity improvements associated with employing UPLC technology for methods development are depicted below in Table I. By comparing the UPLC methods development strategy outlined previously to one directly scaled to conventional HPLC, a 6-fold improvement in time is observed. This significantly reduces the overall instrument time required to develop chromatographic methods to 1 work day opposed to 1 work week with conventional HPLC.

Conclusion

A systematic approach towards chromatographic methods development that monitors selectivity change in a separation by manipulating pH, column chemistry and organic modifier was described. By utilizing UPLC Technology for methods development, a 6-fold improvement in throughput can be realized. This, in turn, reduces cost per sample and time of analysis considerably while maintaining or improving separation integrity. By developing rapid, high resolution analytical methods, products can be brought to market faster, therefore, improving the overall profitability of the assay.

Table I: Comparison of productivity between UPLC Technology and HPLC for methods development.

Methods Development Time					
UPLC Technology 2.1 × 50 mm, 1.7 µm, 0.5 mL/min			**Conventional HPLC 4.6 × 150 mm, 5 µm, 1.0 mL/min**		
pH 3	**acetonitrile**	**Time**	**pH 3**	**acetonitrile**	**Time**
Flow Ramp		5 min	Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min	Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min	Sample Injection (2 replicates)		79.2 min
pH 3	**methanol**	**Time**	**pH 3**	**methanol**	**Time**
Flow Ramp		5 min	Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min	Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min	Sample Injection (2 replicates)		79.2 min
Column Purge		6 min	Column Purge		43.2 min
pH 10	**acetonitrile**	**Time**	**pH 10**	**acetonitrile**	**Time**
Flow Ramp		5 min	Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min	Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min	Sample Injection (2 replicates)		79.2 min
pH 10	**methanol**	**Time**	**pH 10**	**methanol**	**Time**
Flow Ramp		5 min	Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min	Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min	Sample Injection (2 replicates)		79.2 min
Column Purge		6 min	Column Purge		43.2 min
Time		120 min	Time		740 min
Screening Time					
3 Hybrid (BEH) Columns		6 Hours	**3** Hybrid (BEH) Columns		36.9 Hours
1 Silica (HSS) Column		1 Hour	**1** Silica (HSS) Column		6.1 Hours
Total Screening Time		**7 Hours**	**Total Screening Time**		**43 Hours**

Empower 2 Method Validation Manager Software: Tool for Rapid Method Validation

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Within the compliant laboratory, the validation of analytical methods is a fact of life. Regulatory agencies must have documented evidence that the analytical methods employed by a laboratory yield accurate and reliable results. The laboratories, utilizing advanced planning and good scientific judgment, rely on validation as a means of assuring confidence in the results generated from their analytical methods. From both perspectives, there is no argument that analytical method validation is an important process and a permanent aspect of compliant laboratory operation.

Method validation is a demanding activity. It requires a large investment in personnel, materials, instruments, supervision, and, most of all, time. Some of the more time-consuming aspects of validation involve the creation of validation protocols and sample lists, tracking of the workflow from protocol to final reporting, the performance of calculations, and the intense need to organize and manage raw and processed data. The potential for errors in the many steps of the validation process is large and the time delay when errors occur can be costly.

Waters® Empower™ 2 Method Validation Manager (MVM) Software, coupled with the Waters ACQUITY UPLC® System, can dramatically address these time-consuming elements of analytical method validation. The advantages of using the ACQUITY UPLC System have been reported previously.

MVM Software is designed to streamline the set-up, execution, calculation, and reporting of a method validation. It provides easy data tracking and complete organization of validation data and results monitored by the built-in oversight of automated error checking.

MVM is a business-critical software that reduces the time and costs required to perform chromatographic method validation by as much as 80%. Because MVM allows the entire chromatographic method validation process to be efficiently performed within Empower 2, fewer software applications need be deployed, validated, and maintained. Software training and support is also minimized.

When less software is required, the software that is business-essential can be deployed more quickly and efficiently.

In addition, Method Validation Manager allows you to be fully compliant with governmental regulations by providing data security, a full set of user privileges, audit trails, and automatic data documentation; providing you with the necessary information and complete data traceability required for final reports and to pass audits and data reviews.

To illustrate the straightforward operation and comprehensive functionality of MVM Software, this application note summarizes a basic assay validation of acetaminophen. Screenshots from MVM are presented with the validation results to help demonstrate the application of this software to the validation process.

Experimental

Materials

Acetaminophen RS was purchased from Sigma-Aldrich Co. (St. Louis, MO). Methanol was acquired from Fisher (Fair Lawn, NJ). Water was purified with a MilliQ Gradient A10 System from Millipore (Billerica, MA).

UPLC conditions

The assay was performed on a Waters ACQUITY UPLC System consisting of a Binary Solvent Manager (BSM), Sample Manager (SM), and Tunable UV Detector (TUV). A Waters ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 × 50 mm Column was selected for the separation. All instruments were controlled, and data were collected and analyzed, using Empower 2 Method Validation Manager Software.

Assay conditions

Mobile Phase:	90:10 water/methanol, mixed by pump
Flow:	0.65 mL/min
Temperature:	40 °C
Injection Volume:	1.0 µL
Wavelength:	243 nm
Run Time:	2 min
Retention Time:	0.7 min

Method

Solution preparation

The acetaminophen working standard was made from a 1:9 dilution of a 0.1 mg/mL acetaminophen stock standard. 10 mg of acetaminophen RS was weighed accurately into a 100 mL volumetric flask, diluted to mark, and mixed with mobile phase. A 1.0 mL aliquot was then transferred to a 10 mL volumetric flask, diluted to mark, and mixed with mobile phase. The final concentration of the working standard was 0.01 mg/mL acetaminophen RS.

Table I: Method suitability criteria

Parameter	Acceptance Criterion
%RSD RT min	≤1.0%
%RSD area acetaminophen in std	≤2.0%
USP tailing	≤1.5%
USP plates	≥1000

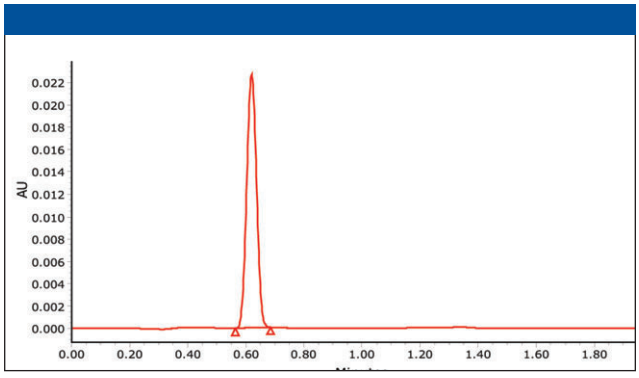


Figure 1: Analysis of acetaminophen.

Acetaminophen sample preparation

Sample preparations for this method were made using the following procedure:

Weigh and finely powder at least 20 tablets. Transfer an accurately weighed portion, equivalent to about 100 mg of acetaminophen, to a 200 mL volumetric flask. Add approximately 100 mL of mobile phase and shake the solution for 10 minutes, then sonicate for 5 minutes. Fill the flask to mark with mobile phase.

Transfer a 5.0 mL aliquot of the above solution to a 250 mL volumetric flask, dilute to mark with mobile phase, and mix. The final concentration of this preparation should be approximately 0.01 mg/mL acetaminophen.

Method system suitability criteria

The method system suitability criteria are listed in Table I.

Acetaminophen analysis with the ACQUITY UPLC System is shown in Figure 1.

Validation protocol and execution

The elements of the written validation protocol for this method were easily transferred into the validation protocol method template of MVM (Figure 2).

The following validation tests were performed in this study:

- Robustness (for three factors)
- Repeatability
- Intermediate precision (different analyst)
- Linearity
- Accuracy
- Solution stability (24 hours)

Individual tests and their associated acceptance criteria were configured.

Complete sample set methods were constructed and then saved as templates within the validation protocol method (Figure 3).

Since the validation protocol called for method suitability parameters to be met by each analysis, system precision requirements were also configured.

During the process of test configuration and sample set method construction, errors were automatically caught by MVM.

Using the update status button and responding to error messages from the message center effectively guides all troubleshooting activity.

The validation protocol method was saved within a validation

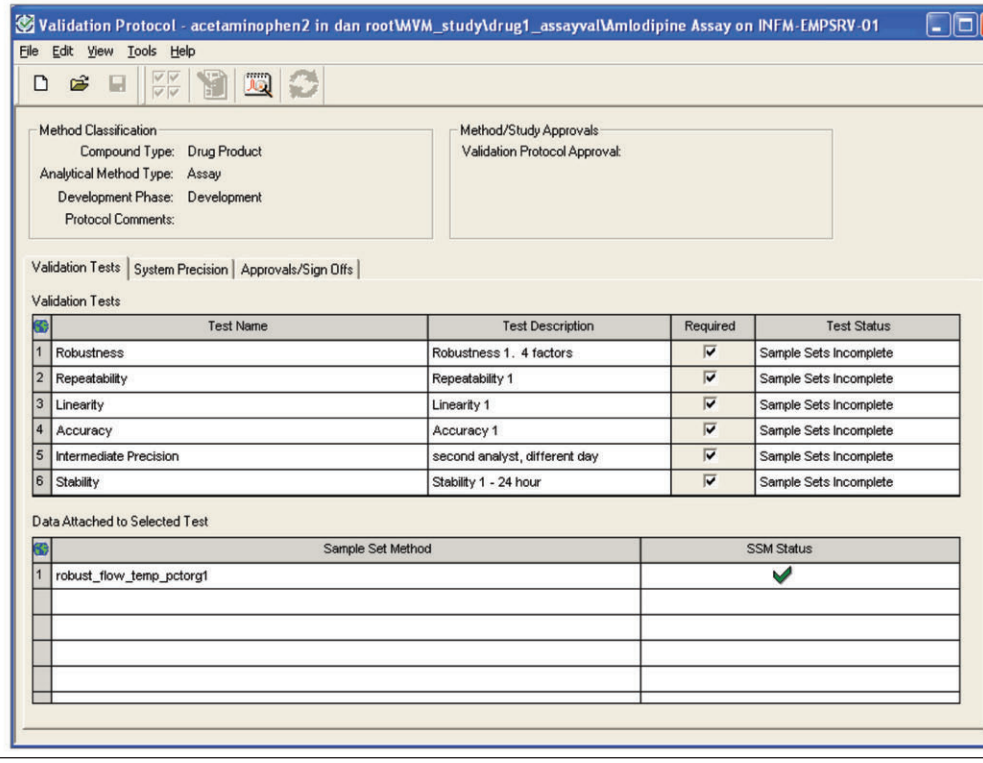
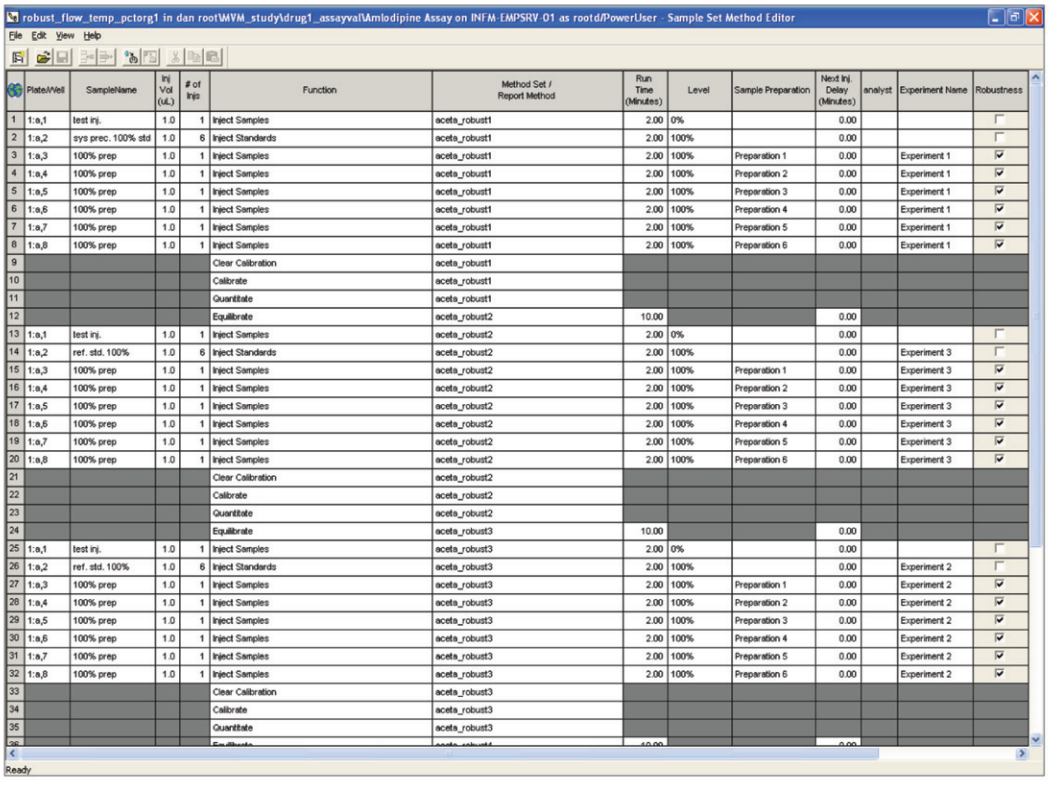
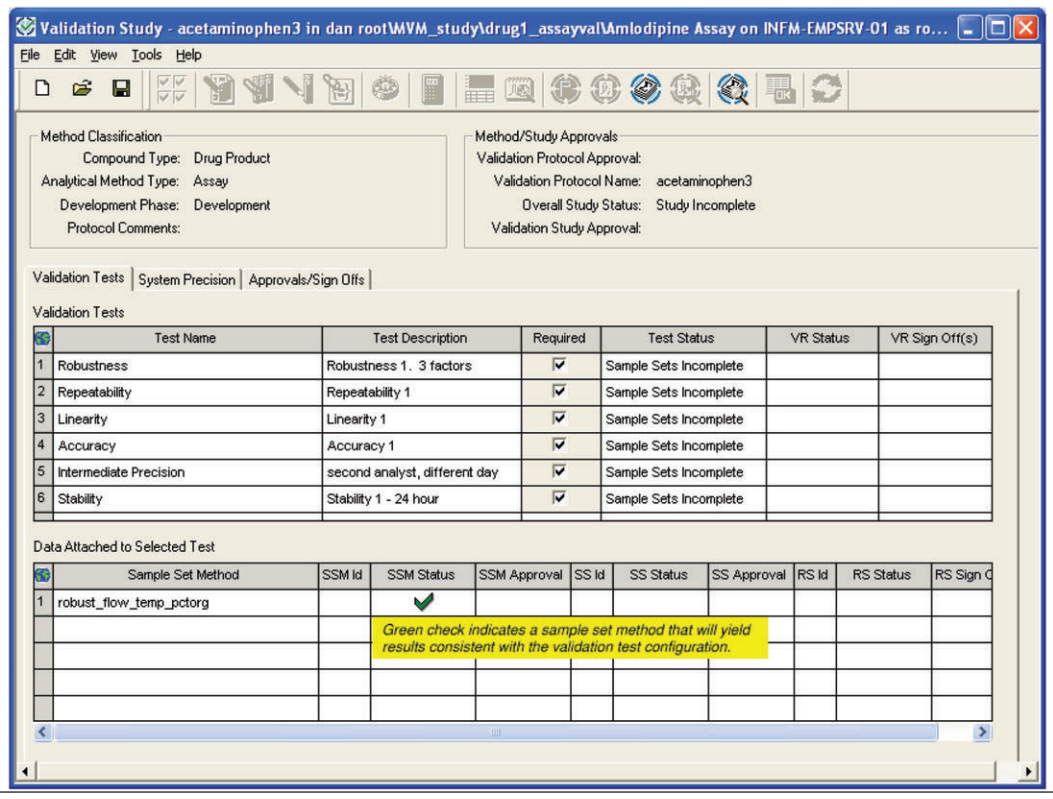


Figure 2: The written protocol can be easily transferred to Empower 2 Method Validation Manager.



	Plate/Well	SampleName	Inj Vol (uL)	# of Injs	Function	Method Set / Report Method	Run Time (Minutes)	Level	Sample Preparation	Next Inj. Delay (Minutes)	analyst	Experiment Name	Robustness
1	1:a,1	test inj.	1.0	1	Inject Samples	aceta_robust1	2.00	0%		0.00			☐
2	1:a,2	sys prec. 100% std	1.0	6	Inject Standards	aceta_robust1	2.00	100%		0.00			☐
3	1:a,3	100% prep	1.0	1	Inject Samples	aceta_robust1	2.00	100%	Preparation 1	0.00		Experiment 1	☑
4	1:a,4	100% prep	1.0	1	Inject Samples	aceta_robust1	2.00	100%	Preparation 2	0.00		Experiment 1	☑
5	1:a,5	100% prep	1.0	1	Inject Samples	aceta_robust1	2.00	100%	Preparation 3	0.00		Experiment 1	☑
6	1:a,6	100% prep	1.0	1	Inject Samples	aceta_robust1	2.00	100%	Preparation 4	0.00		Experiment 1	☑
7	1:a,7	100% prep	1.0	1	Inject Samples	aceta_robust1	2.00	100%	Preparation 5	0.00		Experiment 1	☑
8	1:a,8	100% prep	1.0	1	Inject Samples	aceta_robust1	2.00	100%	Preparation 6	0.00		Experiment 1	☑
9					Clear Calibration	aceta_robust1							
10					Calibrate	aceta_robust1							
11					Quantitate	aceta_robust1							
12					Equilibrate	aceta_robust2	10.00			0.00			
13	1:a,1	test inj.	1.0	1	Inject Samples	aceta_robust2	2.00	0%		0.00			☐
14	1:a,2	ref. std. 100%	1.0	6	Inject Standards	aceta_robust2	2.00	100%		0.00		Experiment 3	☐
15	1:a,3	100% prep	1.0	1	Inject Samples	aceta_robust2	2.00	100%	Preparation 1	0.00		Experiment 3	☑
16	1:a,4	100% prep	1.0	1	Inject Samples	aceta_robust2	2.00	100%	Preparation 2	0.00		Experiment 3	☑
17	1:a,5	100% prep	1.0	1	Inject Samples	aceta_robust2	2.00	100%	Preparation 3	0.00		Experiment 3	☑
18	1:a,6	100% prep	1.0	1	Inject Samples	aceta_robust2	2.00	100%	Preparation 4	0.00		Experiment 3	☑
19	1:a,7	100% prep	1.0	1	Inject Samples	aceta_robust2	2.00	100%	Preparation 5	0.00		Experiment 3	☑
20	1:a,8	100% prep	1.0	1	Inject Samples	aceta_robust2	2.00	100%	Preparation 6	0.00		Experiment 3	☑
21					Clear Calibration	aceta_robust2							
22					Calibrate	aceta_robust2							
23					Quantitate	aceta_robust2							
24					Equilibrate	aceta_robust3	10.00			0.00			
25	1:a,1	test inj.	1.0	1	Inject Samples	aceta_robust3	2.00	0%		0.00			☐
26	1:a,2	ref. std. 100%	1.0	6	Inject Standards	aceta_robust3	2.00	100%		0.00		Experiment 2	☐
27	1:a,3	100% prep	1.0	1	Inject Samples	aceta_robust3	2.00	100%	Preparation 1	0.00		Experiment 2	☑
28	1:a,4	100% prep	1.0	1	Inject Samples	aceta_robust3	2.00	100%	Preparation 2	0.00		Experiment 2	☑
29	1:a,5	100% prep	1.0	1	Inject Samples	aceta_robust3	2.00	100%	Preparation 3	0.00		Experiment 2	☑
30	1:a,6	100% prep	1.0	1	Inject Samples	aceta_robust3	2.00	100%	Preparation 4	0.00		Experiment 2	☑
31	1:a,7	100% prep	1.0	1	Inject Samples	aceta_robust3	2.00	100%	Preparation 5	0.00		Experiment 2	☑
32	1:a,8	100% prep	1.0	1	Inject Samples	aceta_robust3	2.00	100%	Preparation 6	0.00		Experiment 2	☑
33					Clear Calibration	aceta_robust3							
34					Calibrate	aceta_robust3							
35					Quantitate	aceta_robust3							

Figure 3: Sample set method.



	Test Name	Test Description	Required	Test Status	VR Status	VR Sign Off(s)
1	Robustness	Robustness 1. 3 factors	☑	Sample Sets Incomplete		
2	Repeatability	Repeatability 1	☑	Sample Sets Incomplete		
3	Linearity	Linearity 1	☑	Sample Sets Incomplete		
4	Accuracy	Accuracy 1	☑	Sample Sets Incomplete		
5	Intermediate Precision	second analyst, different day	☑	Sample Sets Incomplete		
6	Stability	Stability 1 - 24 hour	☑	Sample Sets Incomplete		

	Sample Set Method	SSM Id	SSM Status	SSM Approval	SS Id	SS Status	SS Approval	RS Id	RS Status	RS Sign O
1	robust_flow_temp_pctorg		✔							

Figure 4: The green check mark in this validation manager window indicates that the sample set method is consistent with the user-configured test criteria.

Table II: Experimental design of robustness from MVM

Experiment #	Column Temperature °C	Percent Organic	Flow rate mL/min
1	37	8	0.750
2	43	8	0.550
3	37	12	0.550
4	43	12	0.750

template project. Next, a validation working project was started and a new study was initiated based on the validation protocol method template.

The validation manager window lists the test configurations and acceptance criteria for the validation study. Additional functionality includes indicators that show test status and required approval (Figure 4). Since complete sample set methods are contained in the validation protocol method, the study can now be executed. Standards and samples were prepared then analyzed on the ACQUITY UPLC System as the previously established sample set methods.

Results

Robustness

Robustness was evaluated using a 1/2 fractional factorial experimental design. The parameters assessed were flow rate, percent organic in the mobile phase, and column temperature (Table II). Because the sample preparation procedure of this method is direct from the United States Pharmacopeia, only selected instrumental parameters were evaluated.

The acceptance criteria for the test were:

1) The amount of acetaminophen determined must fall within 5% of the target value.

2) The %RSD of the amount must be no more than 3%.

A parameter that fails these criteria will need to be tightly controlled when performing the assay.

The results of the robustness testing indicate that all three factors – percent organic, flow rate, and column temperature – had statistically significant effects on the determination of acetaminophen by this method.

Referring to the effects plot in the validation result review window, varying the percent organic by ± 2%, the temperature by ± 3 °C, and the flow rate by ± 0.1 mL/min produced a 1%, 5%, and 10% effect on the assayed acetaminophen amount respectively.

In this case, robustness was evaluated for only the primary effects of the three factors with no consideration given to interaction. However, additional factors and the assessment of possible interactions between them, can all be performed easily and the results analyzed with MVM Software's powerful statistical techniques with a minimum of effort on the part of the validation analyst.

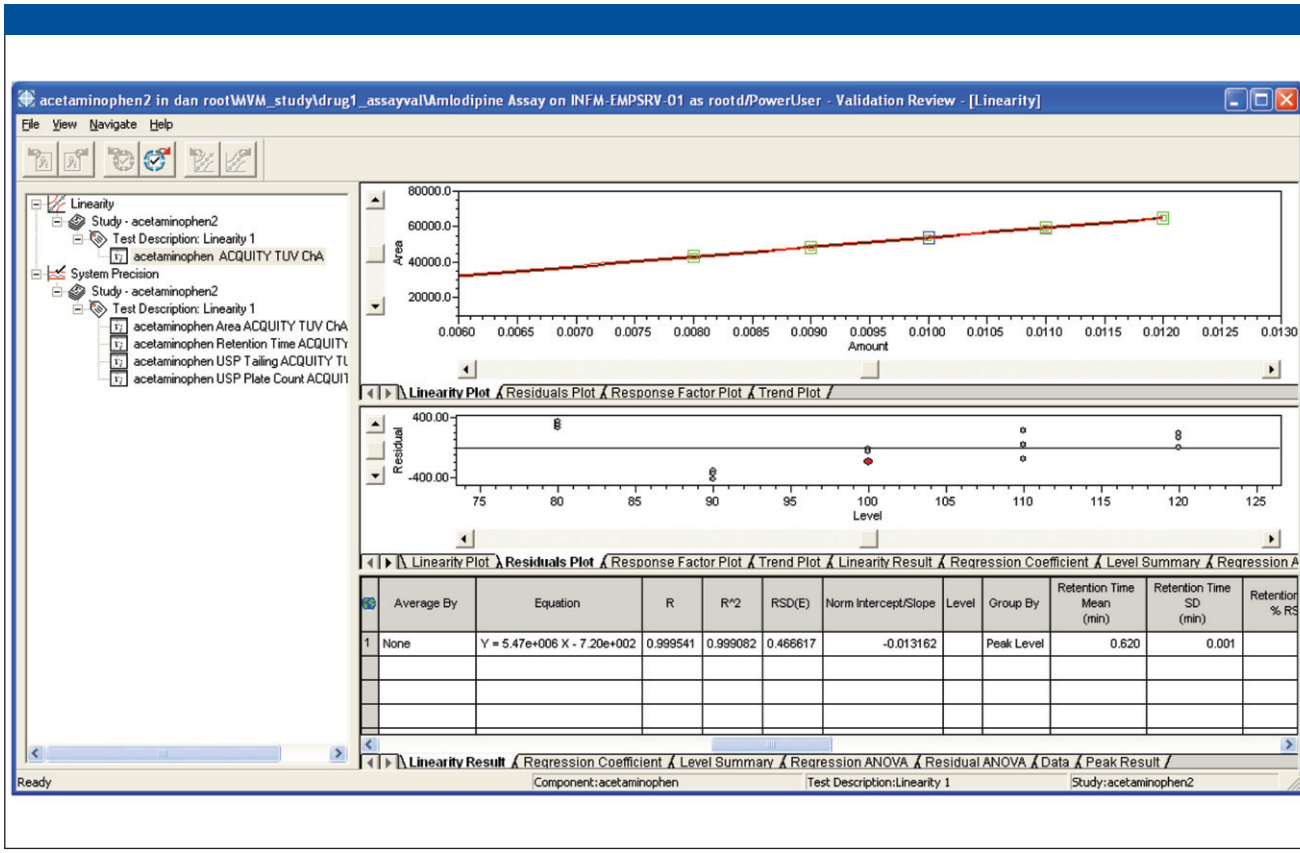


Figure 5: Linearity result shown in the validation result review window.

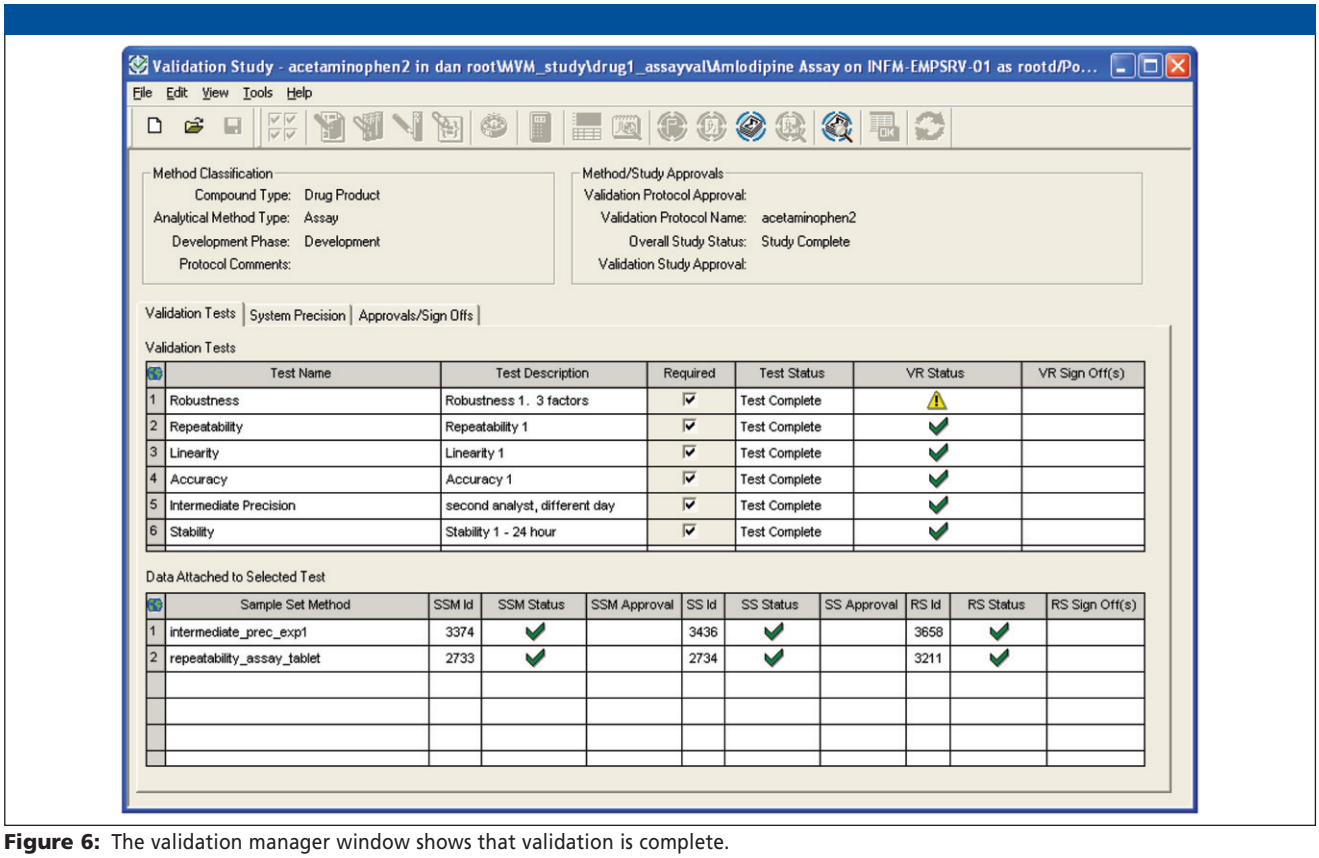


Figure 6: The validation manager window shows that validation is complete.

Repeatability

Repeatability (intra-assay precision) was tested by analyzing six individual sample preparations according to method conditions. The resulting 0.13% RSD for amount easily fell within the acceptance criterion of %RSD ≤ 2.0%, demonstrating that this assay is highly repeatable.

This repeatability result was used in the intermediate precision determination and as the initial time point for the solution stability test. MVM automatically consolidates test result calculations from separate sample set methods.

Intermediate precision

Intermediate precision (ruggedness, inter-assay precision) was evaluated for a different analyst, on a different day, on a different instrument and column. Six individual sample preparations were analyzed according to method conditions. Results were compared with the repeatability determination. A difference of no more than 3.0% in the amount of acetaminophen between the two analysts was an acceptable result. The resulting 2.6% difference demonstrates the ruggedness of this assay.

Accuracy

Accuracy was assessed by analyzing triplicate preparations of mobile phase spiked with acetaminophen RS at 80, 90, 100, 110, and 120% of the target concentration of the method (0.01 mg/mL). The recovery result from the spiked acetaminophen ranged from 99 to 101% and fell within the 95 to 105% acceptance range. The method is very accurate for the range tested.

Linearity

Linearity was evaluated from the same experiment as the accuracy test. The results were linear with slope = 5.47×10^6, R^2 = 0.999, and a y-intercept of −720.3. The method is linear within the range tested (Figure 5).

Stability

Stability was evaluated by the analysis of the repeatability sample preparations (N=6) after 24 hours at room temperature. The repeatability results were used as the time zero condition and were automatically used in the stability data processing. As shown in the validation results, acetaminophen sample preparations are stable for at least 24 hours.

Validation summary

The status and final results for each of the validation tests was clearly displayed in the validation manager window. The green checks indicated tests with acceptable validation results, while the yellow triangle flagged robustness test results that fell outside the acceptance range (Figure 6).

The method for the assay of acetaminophen was analyzed for robustness, repeatability, intermediate precision, accuracy, linearity, and solution stability. This assay was found to be linear, accurate, repeatable, and to be accurately and precisely performed by more than one analyst.

Additionally, samples prepared following the method procedure were documented as stable for 24 hours.

From the robustness testing, altering the column temperature and flow rate was found to significantly affect the accuracy and precision of the method. The method will be revised to clearly state the need to control these two factors.

Conclusion

Empower 2 Method Validation Manager Software effectively streamlines the validation process and integrates smoothly into the validation workflow of the compliant laboratory.

Some of the benefits from the use of MVM are:

- Regulatory compliance: Empower 2 MVM Software easily meets all of the regulatory needs of the compliant laboratory.
- Straightforward validation troubleshooting: The update tool/message center provides an application-directed, timeef ficient troubleshooting process, reducing the time required to get the validation back on track.
- Data traceability: Out-of-specification results are clearly indicated and subsequent investigations are facilitated by the self-contained, completely traceable data management capability of the MVM.
- Reduction of supervisory review: The onus of supervisory review is reduced using MVM, enabling rapid progression in the validation workflow. Potentially error-prone steps such as processing, calculation, and overall data management are all eliminated with the automatic, self-contained design of MVM. The need for any additional third party software pack ages is also eliminated.
- Validation consistency: The ability to create project and sample set method templates ensures consistency of validation protocols with the guidance documents of the laboratory. This reduces errors in the execution of the protocols and increases confidence in the data acquired and the results obtained.

MVM not only effectively organizes and manages the performance of a method validation, it also delivers inarguable confidence in its results. Coupling Empower 2 Method Validation Manager Software to the ACQUITY UPLC System provides an unparalleled solution to the validation needs of a laboratory.

A Rapid and Sensitive SPE-UPLC/MS/MS Method for Determination of Ropinirole in Human Plasma

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Ropinirole is a potent drug with a low oral dose range. A bioanalytical method needs to be developed that can reach a lower limit of quantitation of 0.005 ng/mL. We describe here a method which increases the productivity and efficiency of pk laboratories. The method minimizes solvent composition and time as well as being fully automatable.

Analytical Challenges

Ropinirole (marketed as Requip® in the US and as Adartrel® in Europe) is a non-ergoline dopamine agonist used in the treatment of Parkinson's disease, and it is also the only medication in the United States cleared by the Food and Drug Administration for the treatment of restless leg syndrome. Ropinirole is extensively metabolized by the liver to inactive metabolites through N-despropylation and hydroxylation, primarily by the specific P450 enzyme CYP1A2. Monitoring authorities require that the safety and efficacy of drugs and active metabolites be assessed. Many of the challenges of developing a bioanalytical method revolve around meeting the rigorous criteria set forth in the FDA Guidance for Industry for Bioanalytical Method Validation. Methods need to be acceptable in terms of linearity, sensitivity, accuracy and precision, selectivity, stability and carryover. In addition, sample preparation needs to be as thorough and selective as possible to ensure a robust final method. It is important to obtain the cleanest final extracts and to minimize or eliminate matrix effects. We employ mixed-mode solid-phase extraction (SPE) for this purpose as it relies on both reversed phase and ion exchange to separate more selectively analytes from matrix components.

Solution

A rapid and sensitive method for the determination of ropinirole in human plasma has been developed. The required LLOQ of 0.005 ng/mL was achieved through the combination of sample concentration using Oasis® MCX µElution 96-well plate and the speed and sensitivity of UltraPerformance LC® and Mass Spectroscopy (UPLC® /MS/MS). Standard curves were linear over the range 0.005 to 10 ng/mL.

Sample preparation of human plasma was carried out with Oasis® MCX, as both ropinirole and its internal standard, citalopram, are basic compounds, with pka's of 9.7 and 9.5, respectively. The 96-well µElution plate format was employed to facilitate direct injection and to obtain the analyte concentration necessary to meet the required LLOQ of 0.005 ng/mL. Structures for the analytes are shown in Figure 1.

In addition to enhanced sample preparation, the chromatography was optimized for speed, sensitivity, and resolution. The technology of UPLC takes advantage of smaller chromatographic particles to improve these important parameters. Peak widths, on the order of 2–3 seconds, necessitated the use of a detector capable of high speed data acquisition without loss of data quality. Adequate characterization of chromatographic peaks is critical for accurate quantitation, we therefore utilized the Quattro Premier™ tandem mass spectrometer because of its superior performance in these areas.

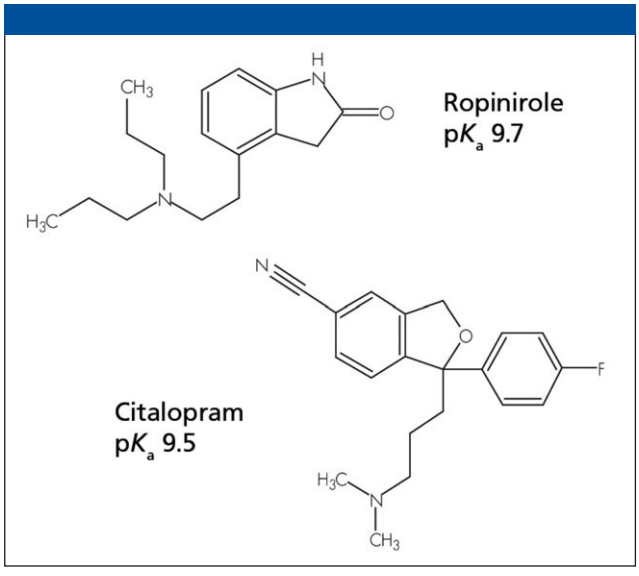


Figure 1: Structures and pk_a's of Ropinirole and Citalopram.

Experimental Conditions

ACQUITY UPLC conditions

Column:		ACQUITY UPLC® BEH C_{18}, 2.1 × 50 mm, 1.7 µm		
Part Number:		186002350		
Mobile Phase A:		10 mM NH_4COOH, pH 9		
Mobile Phase B:		MeOH		
Flow Rate:		0.5 mL/min		
Gradient:	Time (min)	Profile %A	%B	Curve
	0.0	95	5	6
	2.0	2	98	6
	2.5	2	98	6
	2.6	95	5	6
	3.0	95	5	6
Injection Volume:		8.0 µL		
Column Temperature:		45 °C		
Sample Temperature:		15 °C		
Sample Diluent:		100% MeOH + 5% NH_4OH		

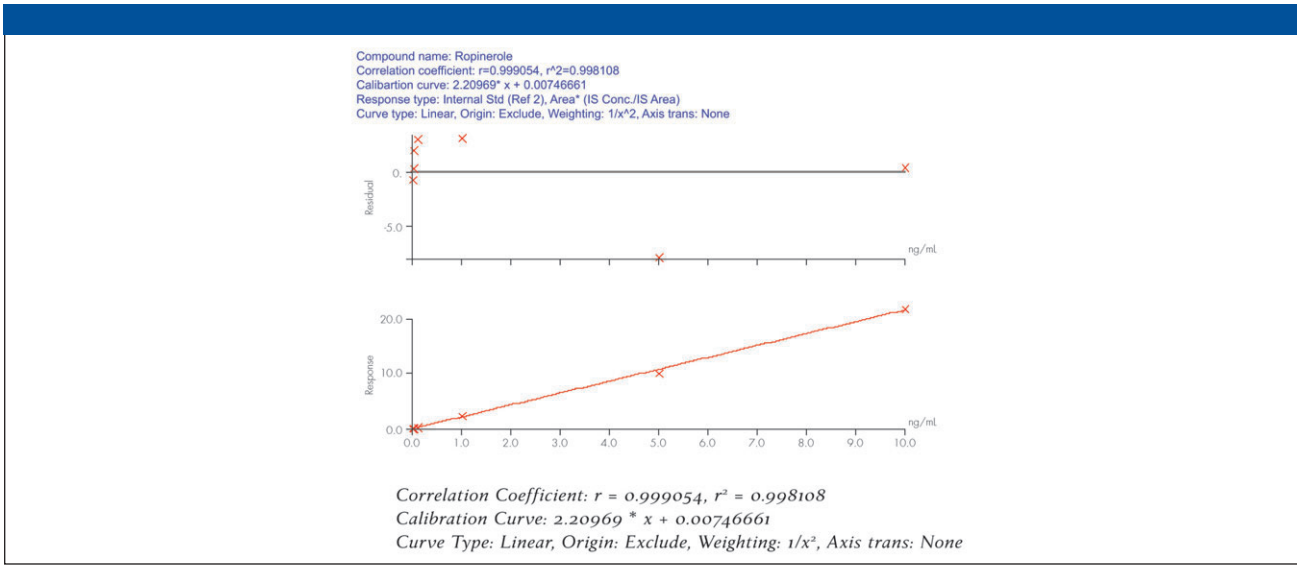


Figure 2: Representative standard curve demonstrating method linearity from 0.005 to 10 ng/mL.

Strong Needle Wash: 60:40 ACN:IPA + 0.5% HCOOH (1200 µL)
Weak Needle Wash: 95:5 H_2O:MeOH (500 µL)
96-well Collection Plate Part Number: 186002481

Quattro Premier conditions

Desolvation Temp: 350 °C
Cone Gas Flow: 50 L/Hr
Desolvation Gas Flow: 700 L/Hr
Collision Cell Pressure: 2.6 × 10(-3) mbar
MRM transitions monitored: Ropinirole m/z 261.2 > 113.95 (ESI+)
Citalopram (IS) m/z 325.2 > 108.85 (ESI+)

For both analytes, the optimal cone voltage was 40 V. For ropinirole and citalopram, the collision energies were 18 eV and 25 eV, respectively.

Standard solutions

Stock solutions (1 mg/mL) of each analyte were prepared by dissolving the appropriate amount of compound in MeOH. Working solutions of ropinirole were then prepared by diluting the appropriate volume of stock solution with 50:50 v/v MeOH:H_2O to give the following concentrations: 0.1, 0.2, 0.4, 2, 20, 100, and 200 ng/mL.

An appropriate volume of citalopram was diluted with 50:50 v/v MeOH:H_2O to give an internal standard working solution of 20 ng/mL. The above concentrations were necessary for a 50 µL per mL spike volume.

Sample preparation procedure

Calibration curves for the determination of ropinirole in human plasma were prepared at the following concentrations: 0.005, 0.01, 0.02, 0.1, 1, 5, and 10 ng/mL.

The internal standard, citalopram was spiked to a final concentration of 1 ng/mL.

Bulk human plasma was fortified as follows for each concentration point: 250 µL internal standard (20 ng/mL) and 250 µL ropinirole standard solution were added to 5 mL human plasma; this was then diluted with 4.5 mL 4% H_3PO_4 in water. The plasma was acidified to disrupt protein binding and diluted to improve flow through the SPE device as well as enhance contact time with the sorbent. These solutions represent the pretreated (acidified and diluted) plasma solutions which are ready for solid phase extraction. The analytes are added as part of the dilution for accuracy and simplicity. Concentrations above refer to concentration in the plasma, not the diluted solution. Plasma blanks were prepared by mixing equal volumes plasma and 4% H_3PO_4 in water.

Extraction procedure

Solid-phase extraction was performed with the Oasis MCX µElution 96-well plate for increased sensitivity, through concentration, without drying down and reconstituting the extract.

Oasis MCX µElution 96-well plate:

Condition the wells with 200 µL MeOH and then equilibrate with 200 µL water.

Load 600 µL diluted plasma sample.

Wash with 200 µL 2% HCOOH in water.

Wash with 200 µL MeOH.

Elute with 1 × 25 µL 5% NH_4OH in MeOH.

The Oasis µElution plate eluates were injected directly, without dilution, onto the ACQUITY UPLC BEH C_{18} column.

SPE recovery

Recoveries of ropinirole and citalopram were assessed by the comparison of peak areas of extracted samples at 0.1, 1, and 10 ng/mL to post-extracted spiked plasma extracts at the same concentrations. Blank plasma was extracted through the plate, dried down, and reconstituted with 50 µL of MeOH containing 0.1, 1, or 10 ng/mL ropinirole and 1 ng/mL citalopram.

The extracted samples (plasma spiked with analytes extracted through the plate) were also dried down and reconstituted in 50 µL of MeOH. A consistent volume of a constant solvent

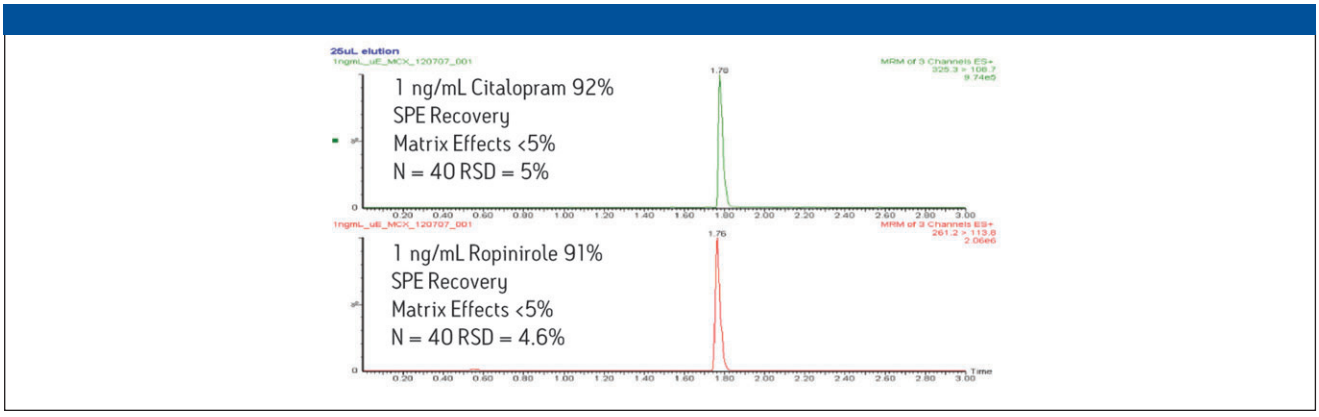


Figure 3: A 1 ng/mL sample of ropinirole and citalopram in human plasma, prepared with Oasis MCX µElution plate SPE.

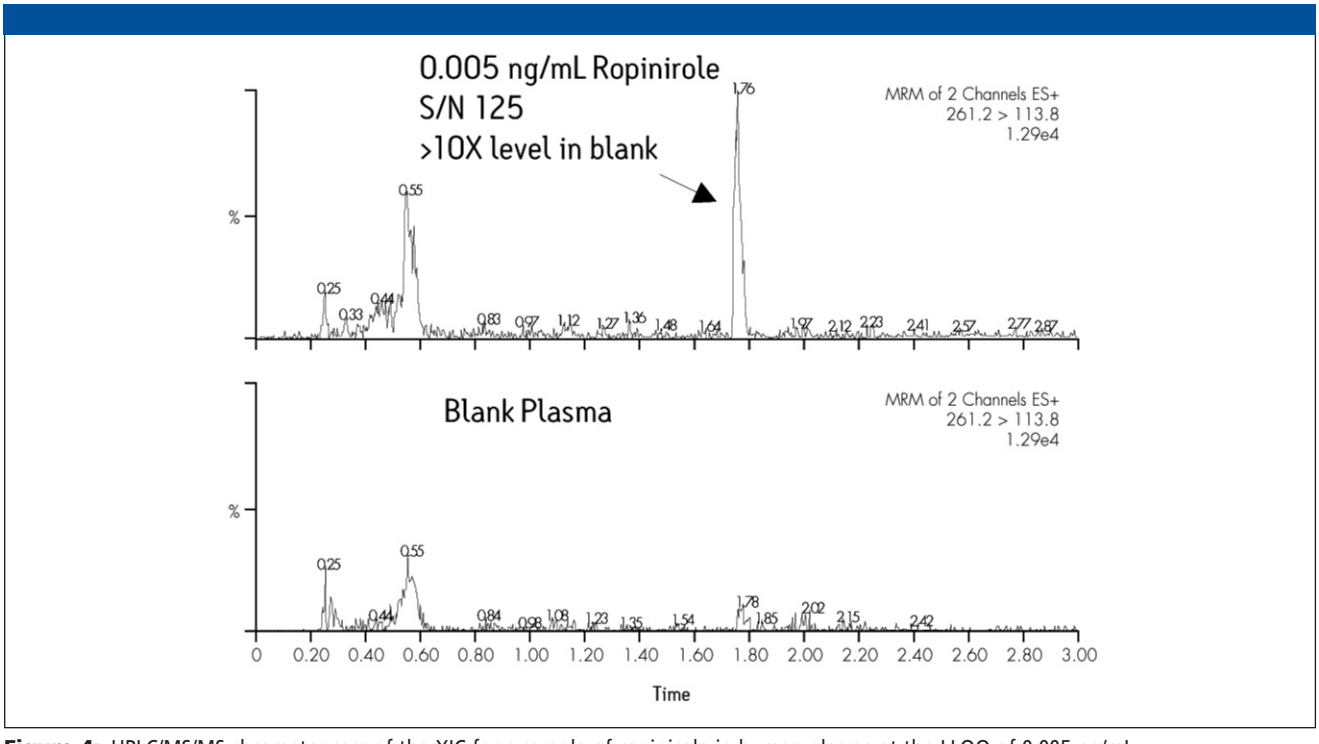


Figure 4: UPLC/MS/MS chromatogram of the XIC for a sample of ropinirole in human plasma at the LLOQ of 0.005 ng/mL.

must be used for both post-extracted spiked and actual extracted samples in order to accurately calculate recovery. The average area counts for each analyte from 8 replicates of the extracted sample were divided by the average area counts in 8 replicates of the post spiked standard. The result was multiplied by 100 to obtain a percentage recovery for each analyte.

Calibration curves

Calibration curves were generated using the QuanLynx™ Application Manager for MassLynx™ Software by plotting the peak area ratio of ropinirole to the internal standard for each calibration concentration. The linear regression was constructed from 0.005 – 10 ng/mL (excluding the origin) and a weighting of $1/x^2$ was applied.

Results and Discussion

The assay was determined to be linear over the required range of 0.005 to 10 ng/mL. For each day of analysis, calibration curves were analyzed in duplicate or triplicate. All calibration curves had an $r^2 > 0.998$ and all calibration points were within the FDA defined criteria for acceptance of ±15% of their theoretical concentration. A representative standard curve is shown in Figure 2.

SPE recoveries for both citalopram and ropinirole were > 90% for all concentrations. A representative chromatogram of ropinirole and its internal standard at 1 ng/mL is shown in Figure 3.

Due to the relatively low dose regime for ropinirole, a lower limit of quantitation in the pg/mL range was required. The combination of UPLC and Oasis µElution SPE provided the sensitivity necessary to achieve a 0.005 ng/mL LLOQ. Chromatography at the LLOQ for a sample prepared by µElution is shown in Figure 4.

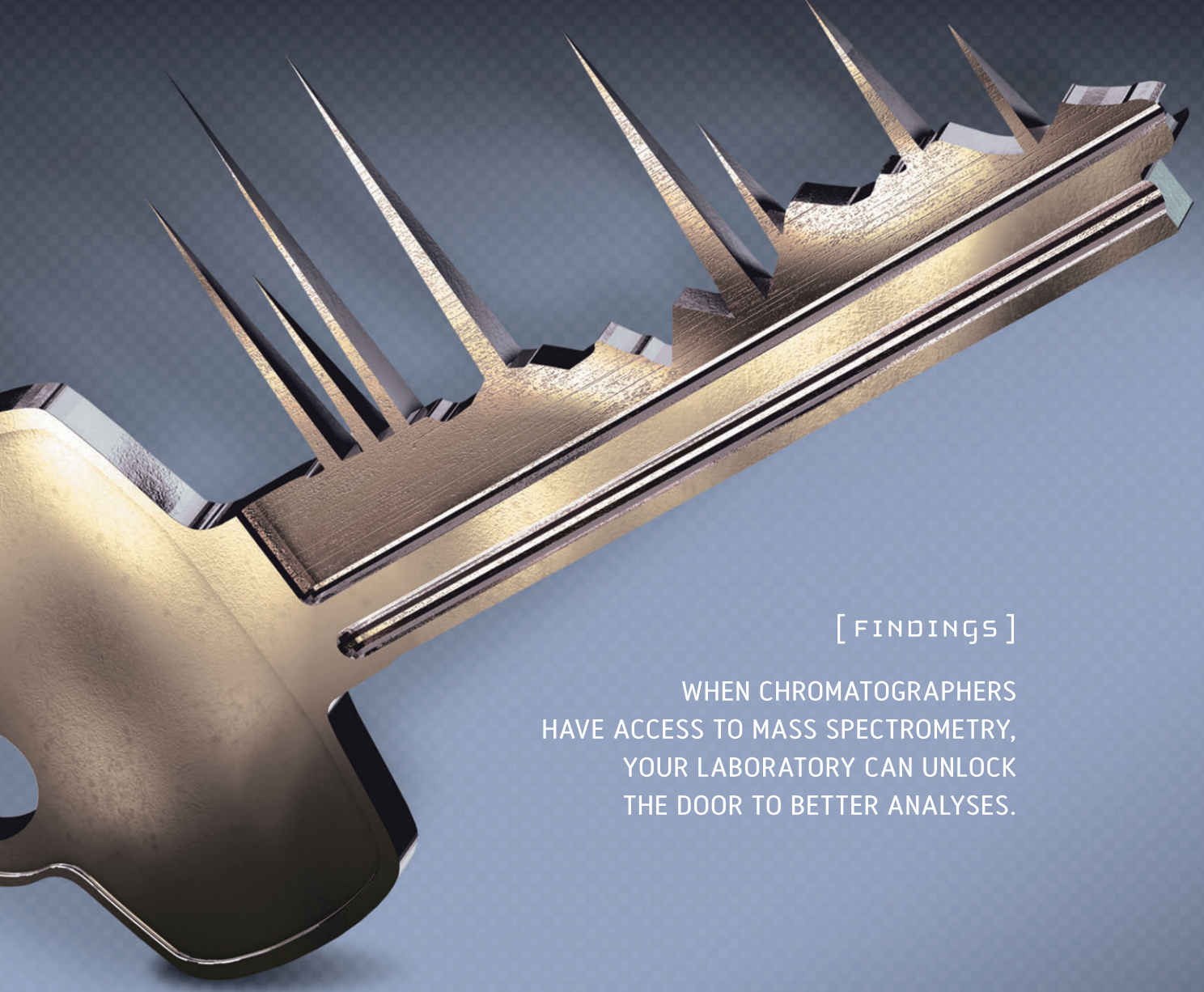
For UPLC, the recommended starting point for strong needle

wash composition is to make it the same organic strength as the end of your gradient. In this case, that would have been 98% MeOH, 2% aqueous. We found it necessary to modify the strong needle wash from 98% MeOH to 60:40 ACN:IPA with 0.5% HCOOH to eliminate carry-over.

Conclusions

A rapid and sensitive method for the determination of ropinirole in human plasma has been developed. The method achieves a S/N of over 100:1 at the required LLOQ of 0.005 ng/mL. The method meets the FDA requirements for linearity and excellent recovery for both analytes is achieved.

The combination of the Oasis MCX µElution plate for easy sample preparation and the speed and sensitivity of the UPLC/Quattro Premier platform facilitated rapid development of a robust bioanalytical method. This combination also enables researchers to obtain higher quality data faster in order to make critical project decisions.



[FINDINGS]

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