[APPLICATION NOTEBOOK]

METHOD DEVELOPMENT

The Path to Successful Drug Development Starts with Purposeful Method Development

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The Path to Successful Drug Development Starts with Purposeful Method Development

The goal of method development in pharmaceutical organizations is to deliver a set of sustainable results that support decisions throughout the drug development process. Achieving those reliable and reproducible methods is the challenge. Method development is time-consuming. It can seem like a semi-random process. It's under pressure to get done faster, which can result in an inefficient and/or inconsistent method. When that happens, significant resources are spent troubleshooting, re-running samples, or re-developing poor methods.

A successful approach to method development is build on understanding and controlling the fundamental influencers of a separation, including chromatographic and instrumentation effects, and considering how processes can be improved to ensure a quality method produces the right results every time.

This compilation of applications demonstrates the use of purposefully integrated analytical technologies, standards, and workflows to produce more robust and reliable methods.

- The powerful ACQUITY UPLC® H-Class System with its Column Manager, Solvent Select Valve, and Auto•Blend Plus™ Technology can speed up method development, provide greater resolution and sensitivity, and reduce errors in mobile phase preparations.
- The easy-to-use ACQUITY® QDa™ Detector enables chromatographers to use UV and mass detection together, simplifying peak tracking and adding confidence in peak identification.
- Waters' range of UPLC® column chemstries offers selectivity choices to separate key components earlier in the development process, and high-pH-tolerant hybrid columns take further advantage of selectivity by enabling scientists to manage separations by pH.
- Move from a successful separation to an insightful answer with more confidence and consistency using data evaluation tools such as ApexTrack™ peak integration and custom calculations and reporting features in Empower® Chromatography Data Software.
- For methods that are developed for quality control, the utility of a Quality-by-Design approach is increasingly valuable; using Design of Experiment software (DoE) allows a scientist to efficiently automate the method development process, building in robustness by using a comprehensive understanding of a method's statistical performance and limitations.

Method Development

Data Evaluation and Processing

Appendix

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Improving Effectiveness in Method Development by Using a Systematic Screening Protocol

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APPLICATION BENEFITS

- Robust UPLC® method development
- Quick and accurate identification of sample components using mass detection with the ACQUITY® QDa™ Detector
- Minimize the need for running individual injections of sample components to confirm the identity of peaks

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY UPLC Columns

ACQUITY QDa Detector

ACQUITY UPLC PDA Detector

Empower® 3 Software

Waters Quality Control Reference Material (QCRM) benchmarking standards

KEY WORDS

UPLC, method development, ApexTrack™ integration, Auto•Blend Plus™, metoclopramide HCl, sub-2-µm column particles, mass spectrometry

INTRODUCTION

Method development involves screening a range of chromatographic parameters to generate sufficient resolution and robust separations. While there are many approaches to method development, such as one factor at a time, systematic, and quality by design (QbD), the goals and factors used for optimizing separations are the same. The parameters that are adjusted include column chemistry, organic solvent, pH, gradient slope, flow rate, temperature, among other factors.

The impact of modifying these parameters are then systematically evaluated during development. Methods from each round of optimization are assessed using specific criteria such as the greatest number of peaks of interest with appropriate retention, resolution, and tailing values. The best method(s) from each step are then selected for further investigation until a suitable method is obtained. Throughout this development process, it is essential to ensure selection of the best conditions at each step and have demonstrable reasons for selection.

Regardless of the optimization strategy selected, it is important to identify and track critical sample components across the conditions investigated. Because peak elution order can change and UV spectra of related substances can be indistinguishable, standards (if available) are sequentially injected under the same conditions to simplify analysis. While ultimately effective, this is a time-consuming process. Using mass detection in addition to optical detection enables unambiguous identification. It also enables analysts to monitor sample components, and to rapidly identify and track coelutions and elution order changes.

In this application, we present the development of a UPLC method for metoclopramide HCl and related substances. We combine UV (PDA) and mass detection, with the user-friendly ACQUITY QDa Detector. A systematic protocol is employed that includes scouting, screening, and optimization steps. Results for each step are analyzed and ranked using custom calculations and reported within Empower 3 Chromatography Data Software to minimize analyst bias in decision making and ensure the overall goals are achieved.

EXPERIMENTAL

Waters reference standard

Packaged in a vial:	LCMS Quality Control Reference Material (QCRM, p/n 186006963)

Method development conditions

LC system:	ACQUITY UPLC H-Class with Column Manager and Solvent Select Valve (SSV)
Columns:	All columns with dimension of 2.1 x 50 mm: ACQUITY UPLC CSH™ C_{18}, 1.7 µm (p/n 186005296) ACQUITY UPLC CORTECS C_{18}+, 1.6 µm (p/n 186007114) ACQUITY UPLC CSH Phenyl Hexyl, 1.7 µm (p/n 186005406) ACQUITY UPLC HSS Pentafluorophenyl (PFP), 1.8 µm (p/n 186005965)
Column temp.:	40, 45, and 50 °C
Injection volume:	1.0 µL
Flow rate:	0.6 mL/min
Mobile phase A:	125 mM Formic acid in water
Mobile phase B:	125 mM Ammonium hydroxide in water
Mobile phase C:	Water
Mobile phase D1:	Acetonitrile
Mobile phase D2:	Methanol

System control, data acquisition, and analysis:

Empower 3 FR2 CDS Software

Separation:	Standard gradient with 5-90% organic solvent over 5 minutes
Wash solvents:	Purge/Sample Wash: 50:50 water/methanol
Seal wash:	90:10 water/acetonitrile
PDA detector:	ACQUITY UPLC PDA
PDA settings:	210-400 nm (derived at 270 nm)
MS detector:	ACQUITY QDa (Extended Performance)
Scan mode:	100-400 *m/z*
Ionization mode:	ESI+, ESI-
Probe temp.:	600 °C
Sampling rate:	10 pts/sec
Capilllary voltage:	0.8 kV (pos/neg)
Cone voltage:	15 V
Data:	Centroid

In this application, we demonstrate how using both UV and mass data allows accurate tracking of all components during development and ensures peak purity in the final method. Overall, following a systematic protocol and utilizing mass detection enables faster and more effective development of a chromatographic method that conforms to the USP standard methodology for robustness and performance verification.[1]

Preparation of Solutions

Sample solution with APIs and related compounds

Separate stock solutions were prepared in methanol at 1.0 mg/mL. An equal volume of each stock solution was transferred to one vial and diluted with water to make a working sample with a final concentration of 0.06 mg/mL of each analyte. The compounds used in this study are listed in Table 1.

Compound	Common Name	Monoisotopic Mass (Da)
API	Metoclopramide	299.14
Imp. A	4-Acetamido-5-chloro-N-(2-(diethylamino) ethyl)-2-methoxybenzamide	341.15
Imp. B	Methyl 4-acetamido-5-chloro-2-methoxybenzamide	257.05
Imp. C	4-Amino-5-chloro-2-methoxybenzoic acid	201.02
Imp. D	Methyl 4-acetamido-2-methoxybenzoate	223.08
Imp. F	4-Amino-5-chloro-N-(2-(hydroxbenzamido)-2-hydroxbenzamide	
Imp. G	2-(4-Amino-5-chloro-2-hydroxbenzamido)-N,N-diethylethanamide oxide	315.14
Imp. H	4-Acetamido-2-hydroxbenzoic acid	195.05
Imp. 9	Methyl 4-amino-2-methoxybenzoate	181.07

Table 1. List of USP specified related substances of metoclopramide HCl for UPLC method development.

RESULTS AND DISCUSSION

Method development systematic protocol

Using a systematic protocol enables a consistent evaluation of major selectivity parameters, which ensures the development of robust and reproducible methods; here, using UPLC for faster and more sensitive analysis.

Column chemistries with different base particles and ligands were selected to reflect a wide selectivity range.

As shown in Figure 1, the protocol is built around a series of steps, each designed to address resolution systematically. The first step in our protocol involves defining our sample, success criteria, chromatographic system, and verifying system performance.

For metoclopramide and its USP-defined related substances, our goal was to separate these components to achieve a minimum USP resolution of ≥2.0 for each peak with a USP tailing of ≤1.5, and a retention factor (k*) ≥3.0. The retention factor of a peak for gradient separations is defined as k/(k+1).

For the greatest flexibility in development, we used the ACQUITY UPLC H-Class System configured with a Column Manager and Solvent Select Valve. To identify all components and possible coelutions, we used both ACQUITY PDA for optical detection and ACQUITY QDa for mass detection. We verified system performance using a LCMS Quality Control Reference Material (QCRM) to confirm system was operating properly prior initiating the study.[3]

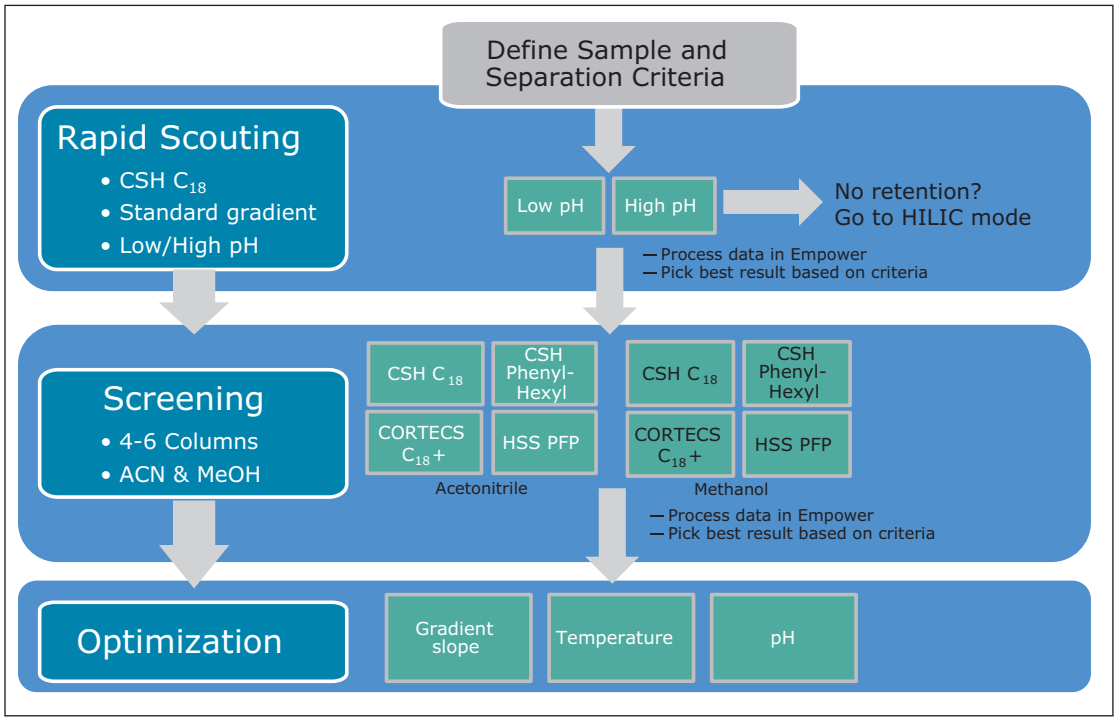


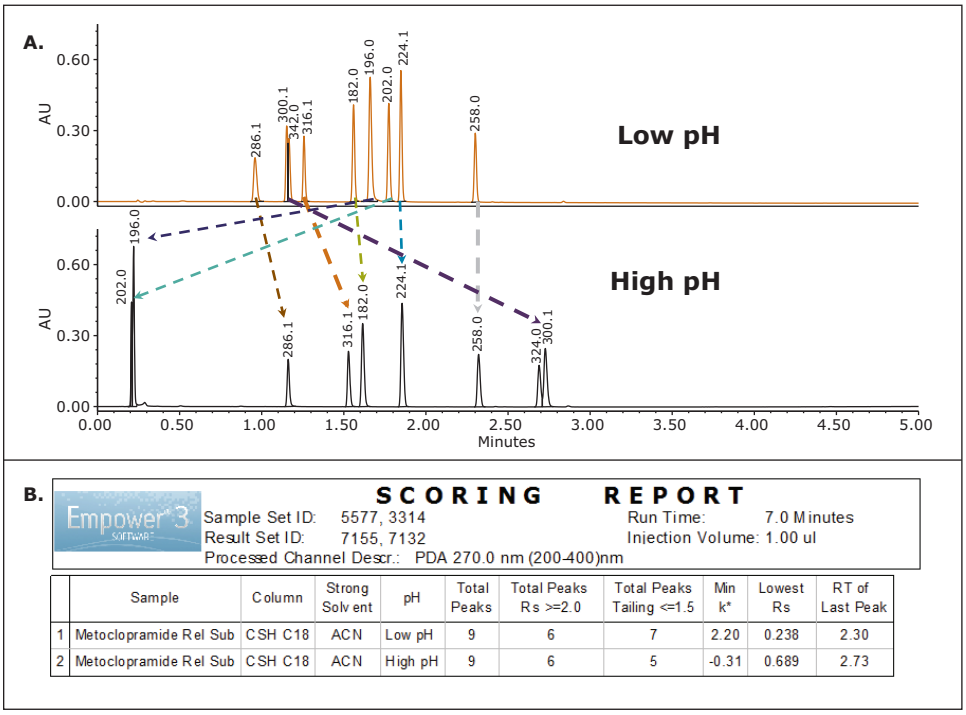
Figure 1. Systematic protocol for development of chromatographic methods.

Rapid scouting

After defining our sample, criteria, and system, we began the systematic protocol with rapid scouting to quickly screen for an acceptable separation condition. The goal of rapid scouting is to select acidic or basic conditions that provide the best retention of the sample components, as well as to identify the best separation mode (reversed-phase or HILIC).

Low and high pH separations were performed using stock solutions of 125 mM formic acid and 125 mM ammonium hydroxide, respectively. For the reversed-phase separation, we used a standard gradient of 5-90% of acetonitrile over 5 minutes. As expected for this basic sample mixture, there were dramatic changes in retention observed between the low and high pH separations (Figure 2). We were also able to track which components are most affected by the pH using the mass data. The chromatographic data was processed in Empower automatically using ApexTrack integration to detect peaks.

To determine the best conditions to move forward, we defined custom calculations and created a customized report in Empower Software. The methods were scored and selected using the best conditions by tracking the number of peaks that meet our defined goals. In this case, the best retention of all components is achieved at low pH, and for this reason, we continued our study with low pH.



	Sample	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Min k*	Lowest Rs	RT of Last Peak
1	Metoclopramide Rel Sub	CSH C18	ACN	Low pH	9	6	7	2.20	0.238	2.30
2	Metoclopramide Rel Sub	CSH C18	ACN	High pH	9	6	5	-0.31	0.689	2.73

Figure 2. Rapid scouting with low and high pH. A. Chromatographic data showing impact of low and high pH on the separation of metoclopramide and related compounds. The sample components that are most affected by the pH were tracked using the mass data. B. Empower 3 scoring report. Criteria for success were defined in Empower as custom calculations, which were then used to create a report. Criteria were ranked so that best method appears first.

Screening

The conditions with best retention selected in the scouting step (low pH condition) did not fully meet our criteria for success. We moved to the screening phase of the protocol with a goal of separating all sample components. Using the Column Manager allowed us to select each column without the need for user intervention. For each separation we used the same standard gradient as in the scouting experiments, but investigated both methanol and acetonitrile eluents.

Again, we used the Empower scoring report to analyze the chromatographic data and select the best separation (Figure 3). As shown, the ACQUITY UPLC CSH C_{18} Column with methanol provides the highest number of peaks and has the highest number of peaks with resolution ≥2.0 and a tailing ≤1.5. For this reason we selected this condition for the final phase of the systematic protocol, optimization.

SCORING REPORT

Sample Set ID: 4001 Run Time: 7.0 Minutes
Result Set ID: 7073 Injection Volume: 1.00 ul
Processed Channel Descr.: PDA 270.0 nm (200-400)nm

	Sample	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Lowest Rs	Min k*	RT of Last Peak
1	Metoclopramide Rel Sub	CSH C18	MeOH	Low pH	9	7	7	1.283	3.22	3.11
2	Metoclopramide Rel Sub	CORTECS C18+	ACN	Low pH	9	7	5	0.769	1.98	2.15
3	Metoclopramide Rel Sub	CSH C18	ACN	Low pH	9	5	7	2.308	2.15	2.30
4	Metoclopramide Rel Sub	CORTECS C18+	MeOH	Low pH	8	7	3	2.094	2.99	2.98
5	Metoclopramide Rel Sub	CSH Phenyl Hexyl	MeOH	Low pH	8	6	8	1.690	2.30	3.13
6	Metoclopramide Rel Sub	CSH Phenyl Hexyl	ACN	Low pH	8	5	5	0.654	0.98	2.21
7	Metoclopramide Rel Sub	HSS PFP	MeOH	Low pH	8	2	2	1.870	6.86	3.44
8	Metoclopramide Rel Sub	HSS PFP	ACN	Low pH	7	2	2	0.108	4.51	2.61

Figure 3. Empower 3 scoring report for screening different columns and organic solvents. The method using the ACQUITY UPLC CSH C_{18} Column and methanol scored highest, indicating the separation had the highest number of peaks with resolution ≥2.0 and a tailing ≤1.5.

Optimization

Although we were closer to the method development goal, the results from screening did not fully meet the criteria for success. We continued through the optimization step to improve the separation. During optimization we investigated the impact of gradient slope, column temperature, and pH. After each step we applied our scoring report to select the best conditions.

The first parameter we investigated was gradient slope by varying the gradient end point using the same gradient time. After applying our report we found that a gradient slope from 5-60% over 5 minutes provided the best separation (Figure 4). With a goal of meeting the criteria for resolution between all the peaks, we then optimized column temperature using the same system setup. Our results indicated that 45 °C yielded the greatest resolution of all components and met all of the goals we set at the start of the development process, Figure 5.

SCORING REPORT

Sample Set ID: 5181 Run Time: 7.0 Minutes
Result Set ID: 7285 Injection Volume: 1.00 ul
Processed Channel Descr.: PDA 270.0 nm (200-400)nm

	Sample	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Lowest Rs	Min k*	RT of Last Peak
1	Metoclopramide Rel Sub, 60%D	CSH C18	MeOH	Low pH	9	7	9	1.727	3.56	4.01
2	Metoclopramide Rel Sub, 70%D	CSH C18	MeOH	Low pH	9	7	8	1.592	3.43	3.63
3	Metoclopramide Rel Sub, 80%D	CSH C18	MeOH	Low pH	9	7	7	1.463	3.31	3.34
4	Metoclopramide Rel Sub, 90%D	CSH C18	MeOH	Low pH	9	7	7	1.346	3.21	3.11

Figure 4. Gradient slope optimization. Different gradient slopes were explored by decreasing the % of organic at the end of the gradient from 5-90% to 80, 70, and 60% over 5 minutes. A gradient with 5-60% of methanol over 5 minutes had the highest score, indicating best separation with highest number of peaks with resolution ≥2.0 and a tailing ≤1.5.

SCORING REPORT

Sample Set ID: 5325, 5232
Result Set ID: 7236, 7205
Processed Channel Descr.: PDA 270.0 nm (200-400)nm
Run Time: 7.0 Minutes
Injection Volume: 1.00 ul

	Sample	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Min k*	Lowest Rs	RT of Last Peak
1	Metoclopramide Rel Sub, 45C	CSH C18	MeOH	Low pH	9	8	9	3.27	2.280	3.87
2	Metoclopramide Rel Sub, 50C	CSH C18	MeOH	Low pH	9	7	9	3.04	1.816	3.79
3	Metoclopramide Rel Sub, 40C	CSH C18	MeOH	Low pH	9	7	9	3.54	1.757	3.99

Figure 5. Column temperature optimization. The temperatures investigated included 40, 45, and 50 °C. Method at 45 °C scored highest with greatest number of peaks with a resolution of ≥2.0, indicating best separation.

At this stage, although we had met all our criteria, we also investigated impact of pH on the chromatographic separation. Often, small changes in pH can have a great impact on the retention of ionizable compounds. We performed separations at pH 2.15, 3.0, and 4.0 using the existing mobile phases defined in the protocol, Figure 6. For pH 3.0 and 4.0, we used Auto•Blend Plus Technology to blend formic acid and ammonium hydroxide solutions, methanol, and water already on the system to deliver mobile phases with constant pH. Our results showed large changes in selectivity as we moved to the higher pH and that, ultimately, pH 2.15 yielded the best separation, Figure 7.

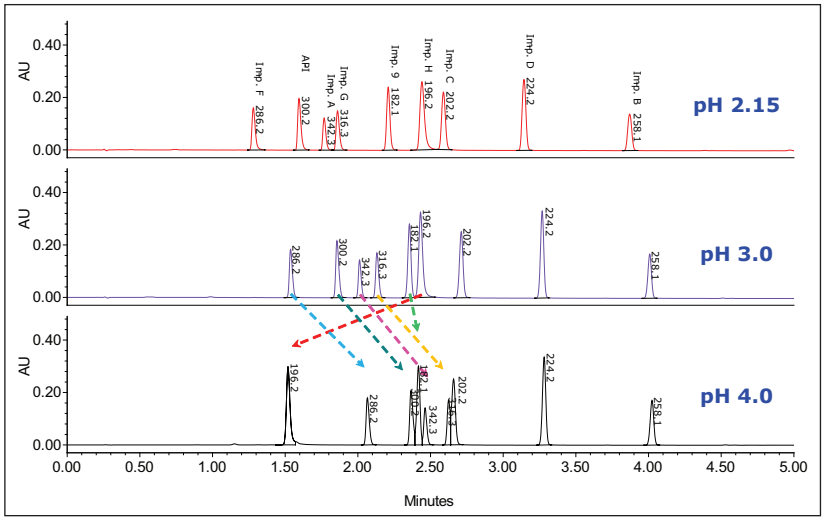


Figure 6. pH optimization to study the impact of pH on the separation of metoclopramide and related compounds. Peaks were tracked by mass detection using an ACQUITY QDa Detector. The best separation conditions were found to be at a pH of 2.15.

SCORING REPORT

Sample Set ID: 5325, 6461
Result Set ID: 7263, 7273
Processed Channel Descr.: PDA 270.0 nm (200-400)nm
Run Time: 7.5, 7.0 Minutes
Injection Volume: 1.00 ul

	Sample	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Min k*	Lowest Rs	RT of Last Peak
1	Metoclopramide Rel Sub	CSH C18	MeOH	Low pH	9	8	9	3.27	2.280	3.87
2	Metoclopramide Rel Sub, pH 3.0	CSH C18	MeOH	pH = 3.0	9	7	9	4.12	1.687	4.01
3	Metoclopramide Rel Sub, pH 4.0	CSH C18	MeOH	pH = 4.0	9	5	4	4.06	0.632	4.02

Figure 7. pH optimization. The method with a mobile phase pH of 2.15 scored highest, indicating best separation conditions.

Final UPLC method conditions

LC System:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC CSH C_{18}, 1.7-µm, 2.1 x 50 mm
Column temp.:	45 °C
Injection volume:	1.0 µL
Flow rate:	0.6 mL/min
Mobile phase A:	125 mM Formic acid in water
Mobile phase C:	Water
Mobile phase D2:	Methanol
Separation:	Gradient

Step	Time (minutes)	Solvent A (%)	Solvent C (%)	Solvent D2 (%)
1	Initial	10	85.0	5.0
2	5.0	10	30.0	60.0
3	5.5	10	30.0	60.0
4	5.6	10	85.0	5.0
5	7.0	10	85.0	5.0

Wash solvents:	Purge/Sample wash: 50:50 water/methanol
	Seal wash: 90:10 water/acetonitrile
PDA detector:	ACQUITY UPLC PDA
PDA settings:	210-400 nm (derived at 270 nm)
MS detector:	ACQUITY QDa (Extended Performance)
Scan mode:	100-400 *m/z*
Ionization mode:	ESI+, ESI-
Probe temp.:	600 °C
Sampling rate:	10 pts/sec
Capilllary voltage:	0.8 kV (pos/neg)
Cone voltage:	15 V
Data:	Centroid

System control, data acquisition, and analysis:

Empower 3 FR2 CDS Software

Final UPLC method

To verify performance of the developed UPLC method, we evaluated repeatability of replicate injections of the sample. The system suitability of five replicate injections was determined according to specifications defined in the USP General Chapter, <621> Chromatography.[2] Results of the method system suitability for each component are shown in Table 2.

The retention times and area repeatability were well below the USP specification of less than 2.0% RSD. The USP resolution between all the peaks was ≥2.5, which is above the general USP requirements of ≥1.5. The system suitability results of replicate injections were excellent. Further validation testing can be done automatically using Empower Method Validation Manager (MVM) Software.

Empower 3 SOFTWARE

Report Method: System Suit_Sum Report
Sample Set ID: Sample Set Id 2622
Result Set ID: Result Set Id 2660
Channel Name: PDA 270

	Name	# of Inj.	%RSD RT	%RSD Peak Areas	Ave USP Resolution	Ave USP Tailing
1	Imp. F	5	0.07	0.19		1.2
2	API	5	0.06	0.22	6.7	1.3
3	Imp. A	5	0.06	0.21	3.4	1.2
4	Imp. G	5	0.07	0.23	2.5	1.2
5	Imp. 9	5	0.06	0.19	9.2	1.1
6	Imp. H	5	0.06	0.19	4.2	1.4
7	Imp. C	5	0.06	0.31	2.5	1.1
8	Imp. D	5	0.05	0.21	9.0	1.1
9	Imp. B	5	0.04	0.21	13.0	1.1

Table 2. System suitability results for five replicate sample injections acquired using an ACQUITY UPLC H-Class System.

CONCLUSIONS

Following a systematic protocol, we have successfully developed a UPLC method for the separation of metoclopramide and related compounds. The criteria for success with a goal of separating all nine components, achieving a resolution of ≥2.0, tailing of ≤1.5, and retention factor (k*) ≥3.0, were met.

Using the ACQUITY QDa Detector in conjunction with UV detection and the ACQUITY UPLC H-Class System streamlined the method development process by removing the need for multiple chromatographic runs to confirm the identity of peaks by retention times.

Using a single injection, instead of nine individual sample injections, we were able to quickly identify components and track elution order of peaks during the method development study.

Finally, the use of ApexTrack in Empower Software enabled consistent evaluation of chromatograms for fair comparison across the development process. Empower custom calculations and reporting allowed us to generate a scoring report to easily identify the best conditions at each step in our protocol.

Overall, using a defined systematic protocol with the UPLC system, detectors, and its column chemistries enables analytical laboratories to quickly and efficiently develop chromatographic methods. Methods developed in this manner are typically more reproducible, which allows laboratories to have a higher validation success rate.

References

1. General Chapter, <1226>, Verification of Compedial Method, USP36-NF31, The United States Pharmacopeia Convention, official December 1, 2013.
2. USP General Chapter, <621>, Chromatography, USP36-NF31, The United States Pharmacopeia Convention, official December 1, 2013.
3. Berthelette KD, Summers M, Fountain KJ. Ensuring Data Quality by Benchmarking System Performance Using Waters Neutrals Quality Control Reference Material. Waters Corp. 2013; 720004622en.

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[TECHNOLOGY BRIEF]

Method Development for Impurity Analysis Using ACQUITY UPLC H-Class System with an ACQUITY QDa Detector

Margaret Maziarz

GOAL

To demonstrate the use of qualitative mass spectral data in method development, using an ACQUITY UPLC® H-Class System with ACQUITY® QDa™ Detector to confirm the identity of ziprasidone HCl and related compounds.

BACKGROUND

Method development typically involves screening chromatographic parameters such as columns, organic solvents, buffers, gradient slope, flow rate, temperature, and so on. Any of these parameters may be modified to alter the resolution to achieve the required analytical quality.

Small modifications in pH often alter the relative retention (elution position) of compounds in a reversed-phase separation. As these separation variables are investigated, it is essential to track changes in chromatographic behavior for each of the sample components. At the same time, recognition of coeluting species is required.

Without accurate and complete peak tracking, development times can be prolonged and significant impurities may be unrecognized. In addition, incorrect identification or failure to identify impurities may compromise the safety and efficacy of the end pharmaceutical product. Utilizing a mass detector enables the analytical laboratory to correctly monitor peak retention by mass spectrometric detection.

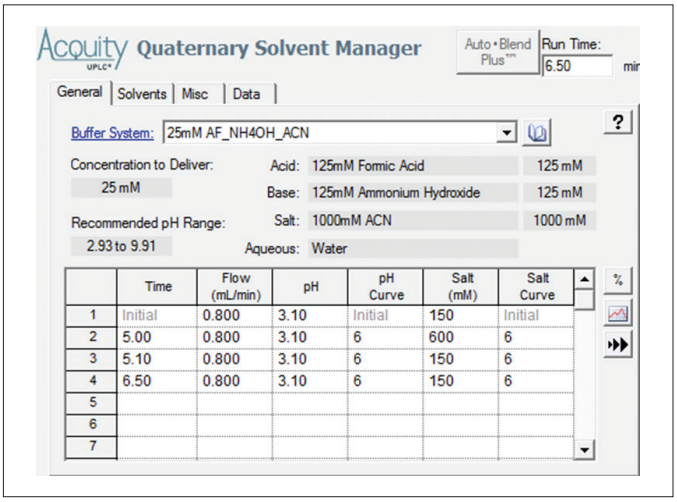
The ACQUITY QDa Detector aids in the development of efficient and robust screening methods by minimizing the need for standard runs to confirm the identity of peaks by retention time.

In this study, we take advantage of qualitative mass spectral data acquired using an ACQUITY QDa Detector to track the elution of ziprasidone HCl and its USP-specified related compounds over a series of different mobile phase pH experiments. This method development process was also facilitated by using the ACQUITY UPLC® H-Class with Auto•Blend Plus™ Technology to control pH.

THE SOLUTION

Auto•Blend Plus, which is included with the ACQUITY UPLC H-Class System, was used to program the blending of acid and base stock buffers with organic and aqueous solvents to deliver a mobile phase with a constant pH. The ACQUITY QDa Detector was used to confirm identity of the ziprasidone HCl and related compounds.

In this pH screening study, 125 mM formic acid and ammonium hydroxide stock solutions, acetonitrile, and water were programmed for mixing by the quaternary pump to deliver mobile phases with pHs of 3.1, 4.0, and 5.0. The Auto•Blend Plus method, set at pH 3.1, is shown in Table 1. The impact of pH on the separation of ziprasidone HCl and the related compounds is displayed in Figure 1.



	Time	Flow (mL/min)	pH	pH Curve	Salt (mM)	Salt Curve
1	Initial	0.800	3.10	Initial	150	Initial
2	5.00	0.800	3.10	6	600	6
3	5.10	0.800	3.10	6	150	6
4	6.50	0.800	3.10	6	150	6
5						
6						
7						

Table 1. Auto•Blend Plus gradient programming for method development to deliver a mobile phase with a pH of 3.1.

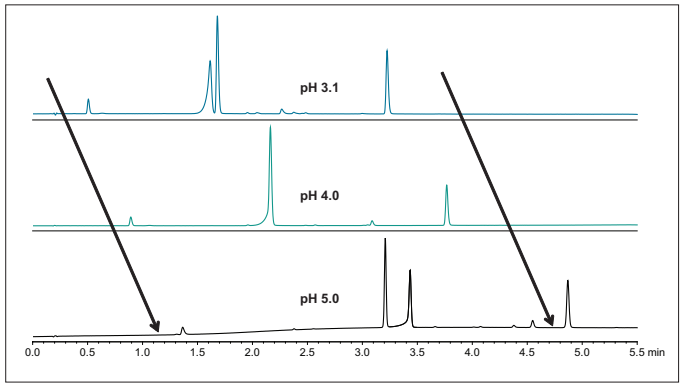


Figure 1. UV data at 254 nm. pH screening in method development using the ACQUITY UPLC H-Class System with an ACQUITY UPLC CSH C_{18} 2.1 x 50 mm, 1.7-µm Column. Column temperature and flow rates were set to 30 °C and 0.8 mL/min, respectively. Injection volume was 0.5 µL.

As shown in Figure 1, an increase in pH results in a higher retention of all the peaks. Fewer peaks were observed with pH 4.0 mobile phase than with 3.1 or 5.0. Tracking and identification of the peaks over the method developments runs with different pHs was performed using an ACQUITY QDa Detector.

Tracking the elution of the peaks by mass detection is displayed in Figure 2. The mass spectra analysis confirmed the identity of the peaks and complemented tracking the elution order of peak 2 with the UV data.

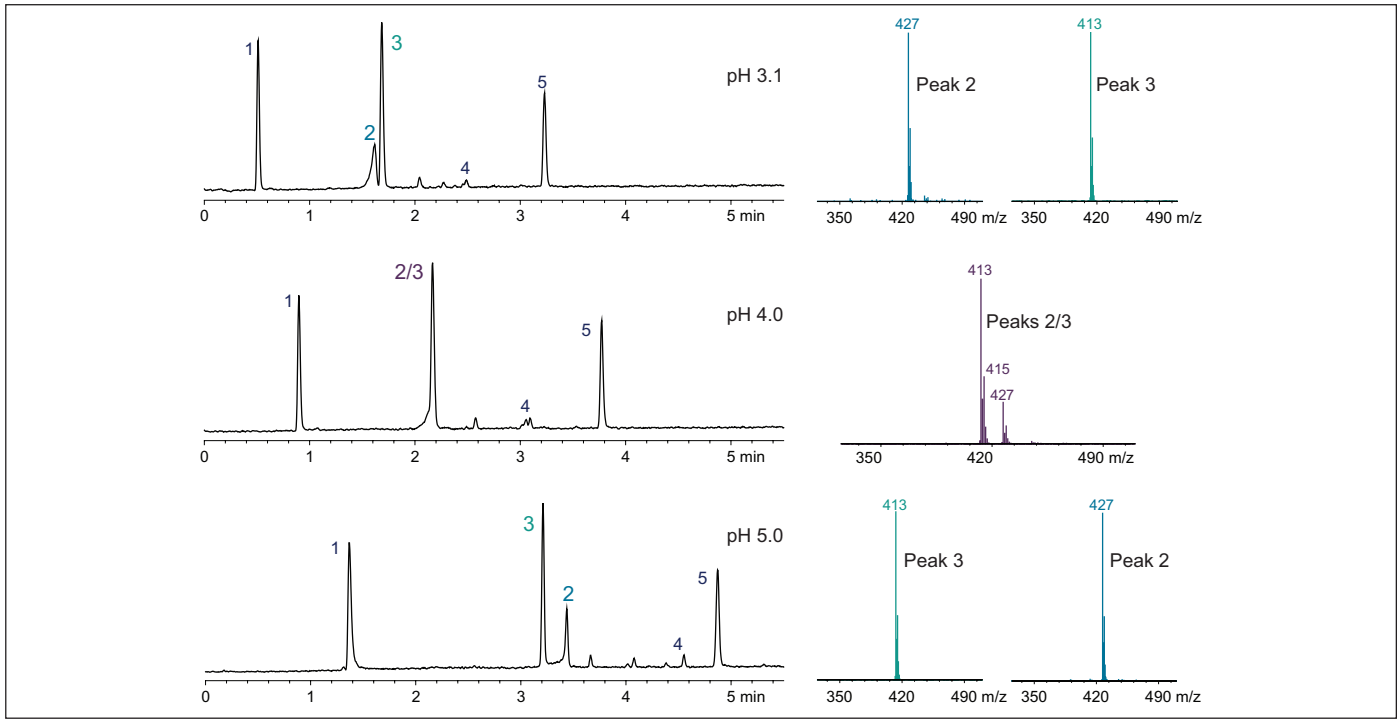


Figure 2. Peak tracking with the ACQUITY QDa Detector. Total ion chromatograms (TIC) and molecular mass determination to track retention of peaks 2 and 3 over the chromatographic runs with different pHs.

Peak 1: Related compound A

Peak 2: Related compound B (MW: 426.92 m/z)

Peak 3: Ziprasidone HCl (free base MW: 412.94 m/z)

Peak 4: Related compound C

Peak 5: Related compound D

In summary, the ACQUITY QDa Detector is a synergistic element of the chromatographic system that provides mass spectral molecular information for analytical scientists in a quick manner, without the need for high-end mass spectrometry. It streamlines development of efficient and robust methods by minimizing the need for standard runs to confirm the identity of peaks by retention times.

When used in conjunction with Empower® 3 Software, which integrates optical and mass data processing, the mass spectral data can be interrogated in the same workflow as the ACQUITY UPLC PDA Detector data.

SUMMARY

The ACQUITY QDa Detector was used to track sample components during development of the UPLC® method for the separation of Ziprasidone HCl and its USP-specified impurities. The ACQUITY QDa Detector was designed to complement the optical data with the enhanced qualitative mass spectral data to confirm the identity of components using an orthogonal detection technique.

Overall, the ACQUITY QDa Detector coupled with the ACQUITY UPLC H-Class System and and Auto•Blend Plus Technology provides complete and rapid chromatographic separation and characterization of compounds, streamlining a laboratory's workflow in the analysis of pharmaceutical products.

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Combining Mass and UV Spectral Data with Empower 3 Software to Streamline Peak Tracking and Coelution Detection

Paula Hong and Patricia R McConville
Waters Corporation

APPLICATION BENEFITS

- Combining mass and UV spectral analysis allows for tracking peaks and identifying coeluting peaks in a single run
- Flexible software combined with the quaternary-based ACQUITY UPLC H-Class System allows for mobile phase pH manipulation with Auto•Blend Plus
- Software allows the analysis of UV and mass spectral data in a single workflow, streamlining the data analysis

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System with Auto•Blend Plus™

ACQUITY UPLC PDA Detector

ACQUITY® QDa Detector

Empower® 3 Software FR2

KEY WORDS

Peak identification, coeluting peaks, Quaternary Solvent Manager, Auto•Blend Plus, analgesics

INTRODUCTION

Methods development for reversed-phase liquid chromatographic (RPLC) separations typically requires many time-consuming steps, including extensive data processing. While many screening protocols rely on UV detection, a single detection technique provides insufficient information for missed or coeluted peaks.

Isobaric compounds can be difficult to distinguish with a mass detector. In addition, minor components may be missed against background, and some important sample components may not ionize. Peak tracking with UV is not possible for compounds that lack a chromophore. Spectra may not be reliably distinguishable. It is not generally easy to recognize which spectra have been "summed" in a coelution. Due to extreme differences in concentration, the minor peak spectrum may simply disappear in a coelution. Finally, UV spectra may change with solvatochromatic effects, in particular with changes in pH.

To address some of these challenges, multiple detectors can be used for analysis of a single sample with each detection technique dependent on a different physical or chemical property of the molecule. Combining detector responses into a single software interface allows streamlined data analysis in a simplified platform.

EXPERIMENTAL

Sample description

Set of analgesics containing acetylsalicylic acid, acetaminophen, 2-acetamidophenol, acetanilide, phenacetin, and caffeine were prepared at 0.2 mg/mL in 90:10 water/acetonitrile.

UPLC conditions

LC system:	ACQUITY UPLC H-Class System with ACQUITY Isocratic Sample Manager (ISM)
UV detector:	ACQUITY UPLC PDA
Column:	ACQUITY UPLC BEH C_{18}, 1.7-µm, 2.1 x 50 mm
Column temp.:	30 °C
Sample temp.:	20 °C
Solvent A:	125mM formic acid in water
Solvent B:	125 mM ammonium hydroxide in water
Solvent C:	Acetonitrile
Solvent D:	Water
Composition:	Prepared using Auto•Blend Plus
Wash solvent:	50:50 water/ acetonitrile with 0.05% formic acid
Purge solvent:	90:10 water/methanol
Seal wash:	90:10 water/methanol
Flow rate:	0.6 mL/min
Gradient:	2-10% acetonitrile in 0.5 min, 10-50% acetonitrile in 0.3 min
Wavelength:	245 nm
Sampling rate:	20 pts/sec
Time constant:	Normal (0.1s)
ISM solvent:	90:10 water/acetonitrile 0.1% formic acid
ISM split:	10 (UPLC)
ISM flow rate:	0.5 mL/min
Injection volume:	1 µL

MS conditions

Mass detector:	ACQUITY QDa
Ionization mode:	ESI+, ESI-
Acquisition range:	100-250 *m/z*
Sampling rate:	5 pts/s
Capillary voltage:	0.8 kV
Cone voltage:	10 V
Probe temp.:	600 °C

Chromatography data management

Empower 3 FR2

RESULTS AND DISCUSSION

In the following study, a mixture of analgesics was analyzed on an ACQUITY UPLC H-Class System with both ACQUITY QDa and ACQUITY UPLC PDA detectors. Empower 3 Software was used as the operating software. The initial screening, at mobile phase pH of 5, resulted in the separation of five of the six components in the standard (Figure 1).

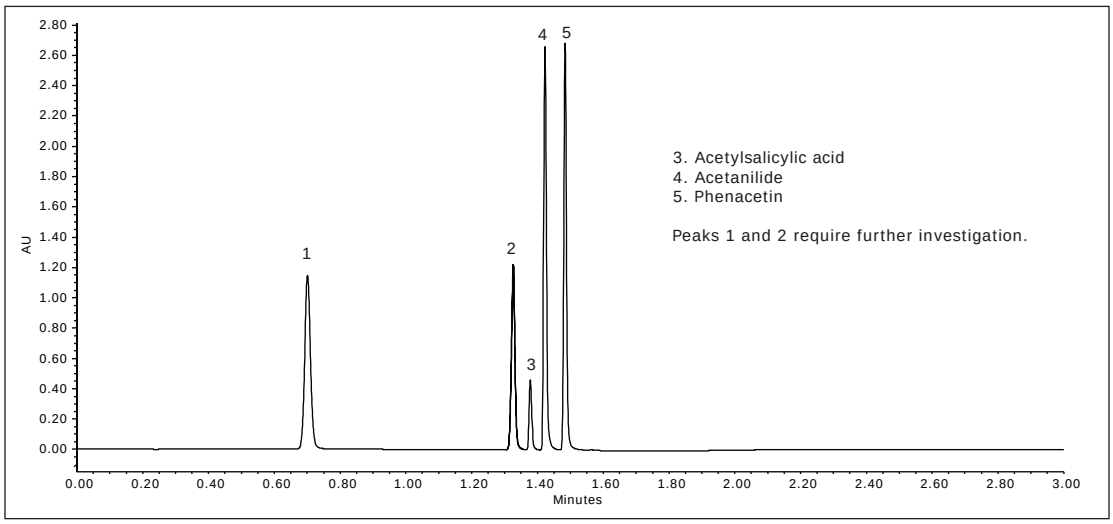

Figure 1. UV chromatogram of analgesic sample at pH 5 UV at 245 nm.

For peak tracking in the separation and to determine the presence of any coelutions, mass and UV spectral data were analyzed in tandem. The mass spectral data was used to identify those components possessing a unique mass in the mixture. For example, tentative identification of peak 4, acetanilide (*m/z* 136.0) and peak 5, phenacetin (*m/z* 180.0) could be made. Peak 3 was present in negative ionization mode and was also identified by a unique mass, acetylsalicylic acid (fragment ion at *m/z* 137.0). Assignment of peak 1 and 2, however, required further investigation of both mass and UV spectral data.

Two isobaric compounds (acetaminophen and 2-acetamidophenol) were present in the mixture (monoisotopic mass of 152.1). In the separation (Figure 1), both peak 1 and peak 2 contain the corresponding mass. In addition, peak 2 contained additional prominent ions, indicating potential coelutions. Therefore, while UV detection is required for peak tracking of the isobaric species, to confirm the identification, the components need to be fully resolved from all other analytes in peak 2.

To evaluate the purity of peak 2, the mass analysis window in Empower 3 Software was used (Figure 2). Evaluation of the peak (1.3 min) reveals *m/z* 152 and *m/z* 110 to be present in the leading edge of the peak (Figure 3). In contrast, the trailing edge, while showing the presence of all three masses, reveals a different ion ratio than that of the apex. The presence of the three ions is suggestive of multiple analytes eluting together. Specifically, the different ion ratios and the absence of *m/z* 195 at the leading edge suggests partial chromatographic resolution. If the ratios had been constant across all time segments, the possibility of fragmentation in the source would have been likely. Given the known composition of the mixture, the predominant mass at the trailing edge of the peak can be identified as caffeine (*m/z* 195.0). The leading edge contains both the parent ion of the isobaric compounds (2-acetamidophenol or acetaminophen) as well as the common fragment ion *m/z* 110.0.[1]

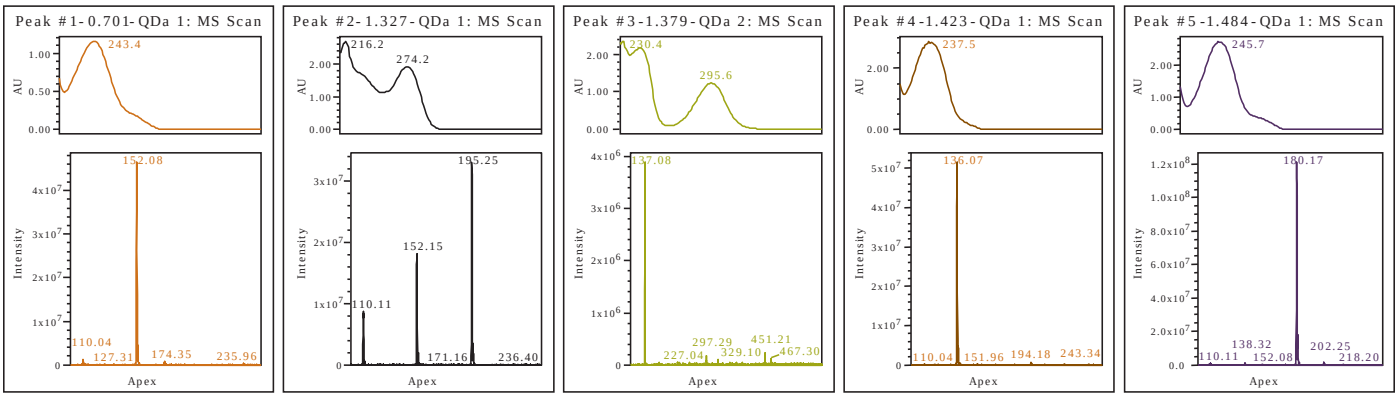

Figure 2. Mass and UV spectral combined view in of analgesics mix in the Empower 3 Mass Analysis window. All mass apex spectrum contain a single predominant mass with the exception of peak 2, which reveals three prominent ions.

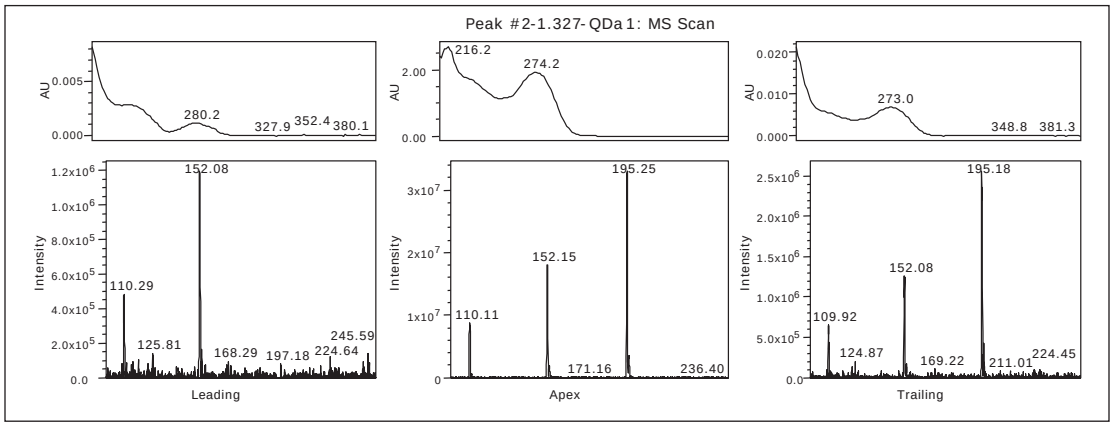

Figure 3. Peak purity view in Empower 3 showing mass and UV spectral data of peak 2 in the Mass Analysis window. Comparison of leading, apex and trailing portions of peak indicate different ion ratios, suggestingcoeluting species.

In order to improve the resolution of the coeluting species in peak 2, the effect of mobile phase pH was evaluated. Using flexible software, the reversed-phase gradient was entered directly in pH units using Auto•Blend Plus.[2] This process allowed for manipulation of pH without the need for preparing new buffer bottles.

Increased mobile phase pH altered the selectivity of the two coeluting compounds (Figure 4). Caffeine (peak B) was found to be predominant mass (*m/z* 195) in the later of two unresolved peaks at pH 6. At pH 7, the two species were baseline-separated with caffeine (peak B) eluting later (peak A) at 1.2 min.

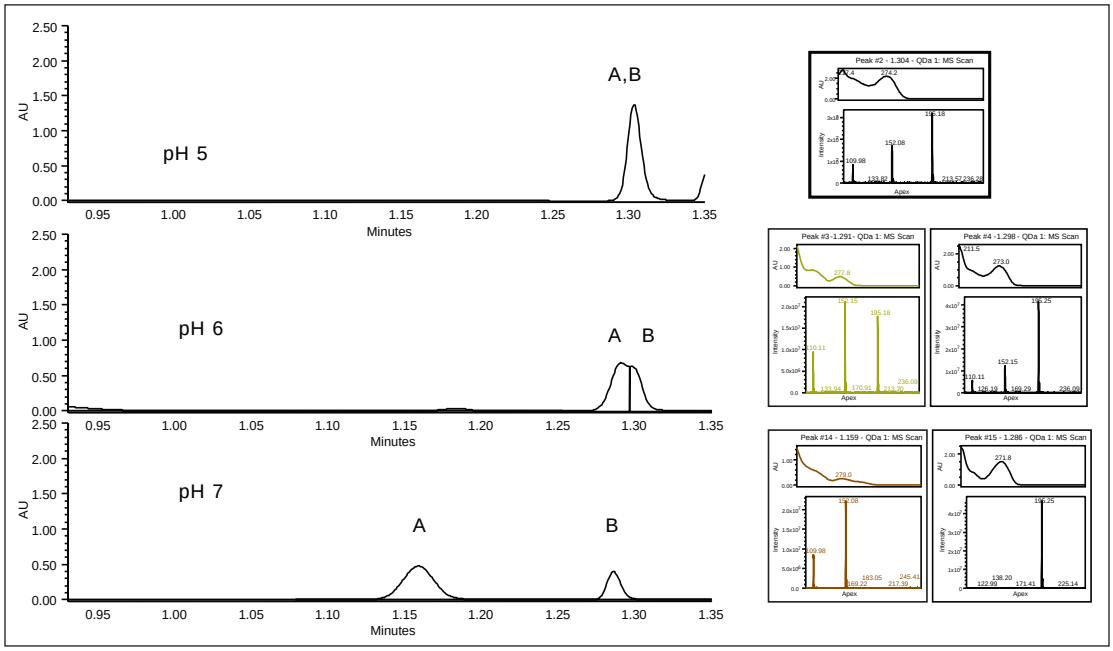

Figure 4. Effect of mobile phase pH on the separation of peak 2. Higher mobile phase pH increased resolution between caffeine and 2-acetamidophenol. Baseline separation was achieved at pH 7.

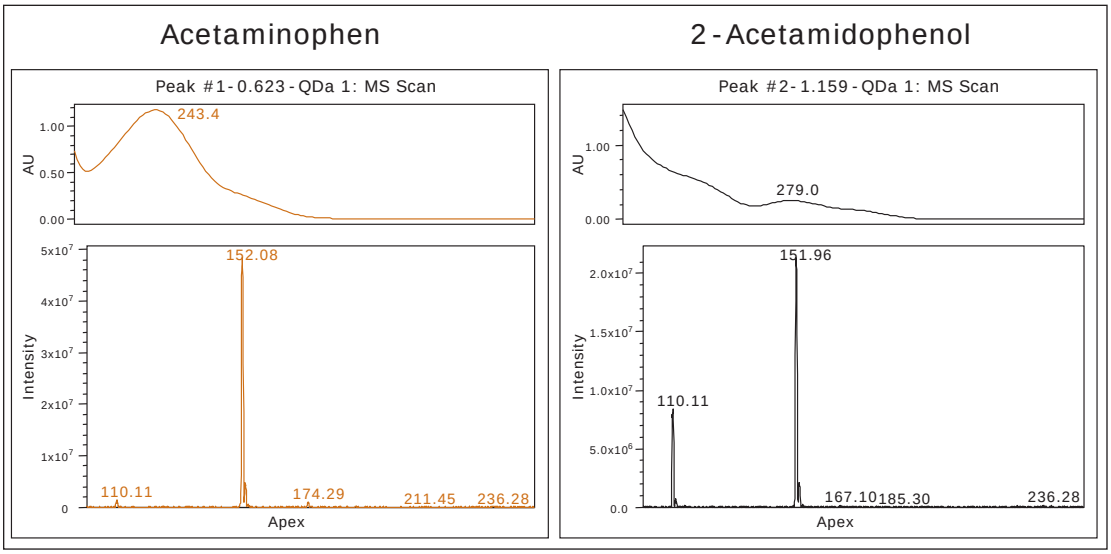

Figure 5. Peak purity view in Empower 3 showing mass and UV spectral data of isobaric compounds in Mass Analysis window. Isobaric compounds can be identified based on UV spectrum.

The baseline separation of one of the isobaric compounds (peak A) and caffeine (peak B) at mobile phase pH 7 (Figure 4) enables the use of the UV spectrum for peak tracking of both isobaric compounds. While the predominant mass for both peak 1 (Figure 1) and peak A (Figure 4) is the same, the peak A contains a fragment ion at 110 *m/z* (Figure 5). Comparison of the mass and UV spectrum for the isobaric compounds provides the information needed for peak identification: the UV spectrum of peak 1 corresponds to that of acetaminophen (λ max of 243 nm). The later eluting peak A could thus be identified as 2-acetamidophenol.

CONCLUSIONS

Methods development using a single detection technique can be challenging. UV spectral data can assist in peak purity and identification, however, it is difficult to perform peak tracking in the presence of coelutions. Mass spectral data can be used to match chromatographic peaks to items on a short list of compounds, but the presence of isobaric compounds can require additional information: complete separation is typically required and not only allows for immediate identification based on the UV but also more reliable quantification.

Typically, identifying these phenomena requires further analyses. For simplified methods development, a chromatographic system using both UV and mass detection and a streamlined software platform can be combined to evaluate peak purity and assist in the identification of coelutions in a single run.

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Rapid Method Development through Proper Column Selection

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APPLICATION BENEFITS

- Improved separations in less time using different UPLC® Column chemistries
- Early selection of the optimal stationary phase results in faster method development
- Automated sample screening with multiple column chemistries using an ACQUITY UPLC® H-Class Column Manager

WATERS SOLUTIONS

ACQUITY UPLC H-Class System with Column Manager

ACQUITY UPLC BEH, CSH™ and HSS Columns

Waters Column Selectivity Chart

Empower™ 3 CDS Software

KEY WORDS

Method development, HPLC, UPLC, stationary phase, selectivity, reaction products, screening, degradation, natural products

INTRODUCTION

Column selection for chromatographic analysis is an important step in method development that can have significant consequences to the effectiveness of the separation. If the wrong column is chosen, the length of time and effort to develop and optimize the separation may be unnecessarily long. Many labs have limited column selection and may base their methods on one core column chemistry, such as a conventional endcapped C_{18} column. However, with advances in column technology, there is an increase in the availability of different base particles and ligand chemistries to screen for alternate selectivity and achieve improved separations.

This application note highlights the importance of selecting an optimal column stationary phase, by demonstrating changes in selectivity of various types of samples including synthetic mixtures, forced degradation reactions and natural product extracts, across different columns. Sample screening across multiple column chemistries was automated using an ACQUITY UPLC H-Class System with a column manager, and shifts in the compound elution order were monitored by UV and mass spectrometric detection. Proper column selection is essential in quickly establishing an effective method and minimizing the need for further extensive method development and optimization.

EXPERIMENTAL

ACQUITY UPLC H-Class Conditions

Mobile Phase:	A: water with 0.1% formic acid, B: acetonitrile with 0.1% formic acid
Gradient:	2 to 98% B over 5 minutes, hold for 1 minute, re-equilibrate at 2% B
Detection:	UV at 254 nm
SQD:	ESI+ mode, mass range 100-600 amu
Needle Wash:	90:10 acetonitrile:water
Sample Purge:	90:10 water:acetonitrile
Seal Wash:	50:50 methanol:water
Flow Rate:	0.8 mL/min
Column Temp.:	30 °C
Injection Volume:	2 µL
Columns:	ACQUITY UPLC, 2.1 x 50 mm, 1.7 – 1.8 µm
Stationary Phases:	BEH C_{18}, part number 186002350 BEH Shield RP18, part number 186002853 CSH C_{18}, part number 186005296 CSH Fluoro-Phenyl, part number 186005351 CSH Phenyl-Hexyl, part number 186005406 HSS Cyano, part number 186005986 HSS PFP, part number 186005965 HSS T3, part number 186003538

Data Management

Empower 3 CDS

Sample Preparation

Nadolol and 3,4-dihydroxy phenylacetic acid: Samples representative of synthetic reaction products were prepared by acetylating 10 mg of each compound. Compounds were first dissolved in pyridine and dichloromethane. Acetic anhydride was added, the reaction was heated to 40 °C and stirred for one hour. Samples were concentrated by rotary evaporation and resuspended in acetonitrile for injection.

Ziprasidone base degradation: A 1 mg/mL solution of ziprasidone was prepared in methanol. To this was added 0.1N NaOH and the reaction was heated at 80 °C for two hours. The reaction was neutralized with 0.1N HCl and transferred to a vial for injection.

Ashwagandha root: 1200 mg of ashwagandha root (*Withania somnifera*) was extracted with 2 mL of methanol, stirring at room temperature overnight. The extract was centrifuged at 12,000 rpm for 10 minutes to remove any particulates prior to injection.

RESULTS AND DISCUSSION:

Selection of the proper column early in the method development process is crucial to obtain an optimal separation. If a separation is developed on a generic column chemistry (perhaps based on column availability in the lab) the chromatography may not be ideal, resulting in further method development that may be unnecessarily complicated and highly time consuming. Instead, if several different column stationary phases are screened to rapidly identify a column providing the best separation, subsequent method development may be minimal or even unnecessary. To maximize the selectivity differences of comparative separations, columns with very different stationary-phase properties can be identified using the Waters Column Selectivity Chart (www.waters.com/selectivitychart). Sample screening on various columns is streamlined and automated using the ACQUITY UPLC H-Class System with a column manager and Empower 3 Software.

A variety of samples were prepared to examine selectivity differences between columns. Although pH is a great effector of peak shape and selectivity in method development, only the low-pH method condition is compared here to clearly monitor the effects of changing only the column stationary phase. Compound identification for every peak in each sample was not performed due to the complexity of the samples, instead, the base peak mass of the major peaks were used to track changes in selectivity.

Forced Degradation Reaction

The base degradation sample of ziprasidone was analyzed on a number of different column chemistries to examine the effects of the base particle and bonded phase chemistry on the separation (Figure 1). Significant changes in elution order and retention of this sample are seen with different column chemistries. The ACQUITY UPLC BEH (Ethylene-Bridged Hybrid) C_{18} Column is a

very robust column frequently used in UPLC. In this case, the BEH column provides an adequate separation, but lacks baseline resolution between the peaks 1 and 2. The CSH C_{18} column has the same ligand but the chromatography shows a completely different elution order and increased resolution between all peaks, due solely to the applied charge on the surface of the CSH particle.

The HSS (High-Strength Silica) Cyano column provides similar retention but increased resolution compared to the BEH C_{18} column, whereas the HSS PFP column separation shows increased retention of all components, particularly minor components 2 and 3. The HSS T3 column has a C_{18} ligand on an HSS particle but has lower ligand density resulting in a slight increase in retention and change in elution order compared to BEH C_{18}, with a co-elution of peaks 1 and 2. Finally, chromatography on the BEH Shield RP18 column shows a change in elution order compared to BEH C_{18} with baseline resolution of peaks 2 and 3. There is also less retention of all components due to the fewer interactions with the shielded silanol groups on the base particle. Overall, the ziprasidone base degradation sample shows very different selectivity when analyzed on a variety of column particles and ligands. Initial use of a BEH C_{18} or HSS T3 column would require additional method optimization to fully resolve the components. By rapidly screening a wide range of columns and selecting a column that demonstrates good resolution early, the need for further method development in such cases can be avoided. In this example, the CSH (Charged-Surface Hybird) C_{18} column may ultimately be chosen for its sharp peak shapes and improved resolution of impurities away from the API peak.

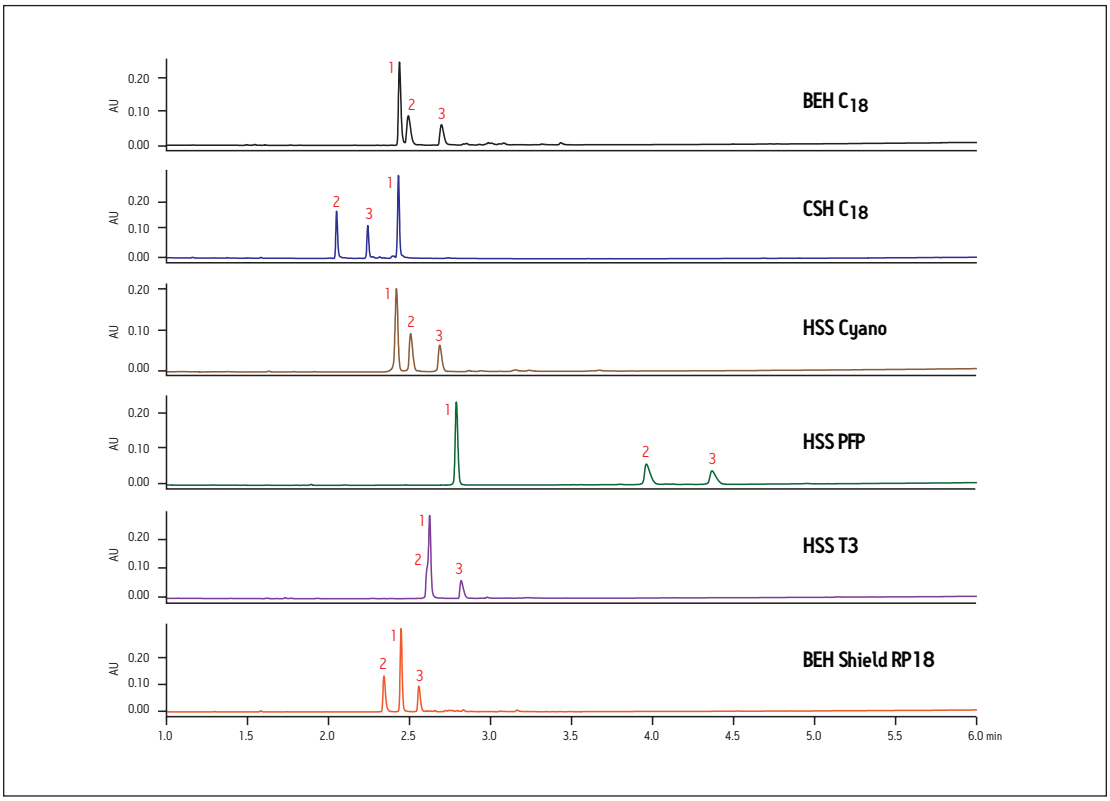

Figure 1. Selectivity differences of a ziprasidone base degradation sample on various columns. Masses of labeled peaks (m/z): (1) 445 (impurity), (2) 413 (ziprasidone API), (3) 417 (impurity).

Synthetic Reaction Mixtures

Synthetic reaction mixtures may contain unreacted starting materials, reagents, reaction side-products and target compounds that require separation. In situations where it may be important to identify or resolve targeted components or product impurities, proper assessment of the separation on various column stationary phases is essential. Changes in selectivity may provide increased resolution of the targeted peak of interest, facilitating identification and purification should the separation be scaled up to a larger diameter column. The separation of acetylation reaction products of 3,4-dihydroxy phenylacetic acid is shown in Figure 2, where the CSH C_{18} column shows shifts in retention time and elution order compared to the BEH C_{18} column. These shifts are due to the effect of the charged surface of the CSH particle on ionizable analytes in the sample. The Fluoro-Phenyl ligand on the CSH particle shows elution order differences and overall less retention compared to the CSH C_{18} and BEH C_{18} columns. Since some analytes in this reaction mixture have aromatic properties, interactions between the analytes and a Phenyl-Hexyl ligand on a CSH particle results in shifts in elution order and altered selectivity. Interactions between the analyte and the short cyano ligand on the HSS Cyano column results in overall reduced retention of hydrophobic analytes and different selectivity compared to all other columns screened.

The HSS PFP column has the same fluoro-phenyl ligand as the CSH Fluoro-Phenyl column but is bonded to a HSS particle instead of the CSH particle. The difference in the properties of the base particles results in very different elution order and retention between the two columns. Finally, the HSS T3 column has a similar elution order to the BEH C_{18} column, but gives improved resolution between peaks 5/6 and 7/8. In this example, the BEH C_{18} column gives adequate resolution for all 8 compounds, but if we focus on peak 6 as the target peak of interest, the best resolution and peak shape is obtained on the HSS T3 column.

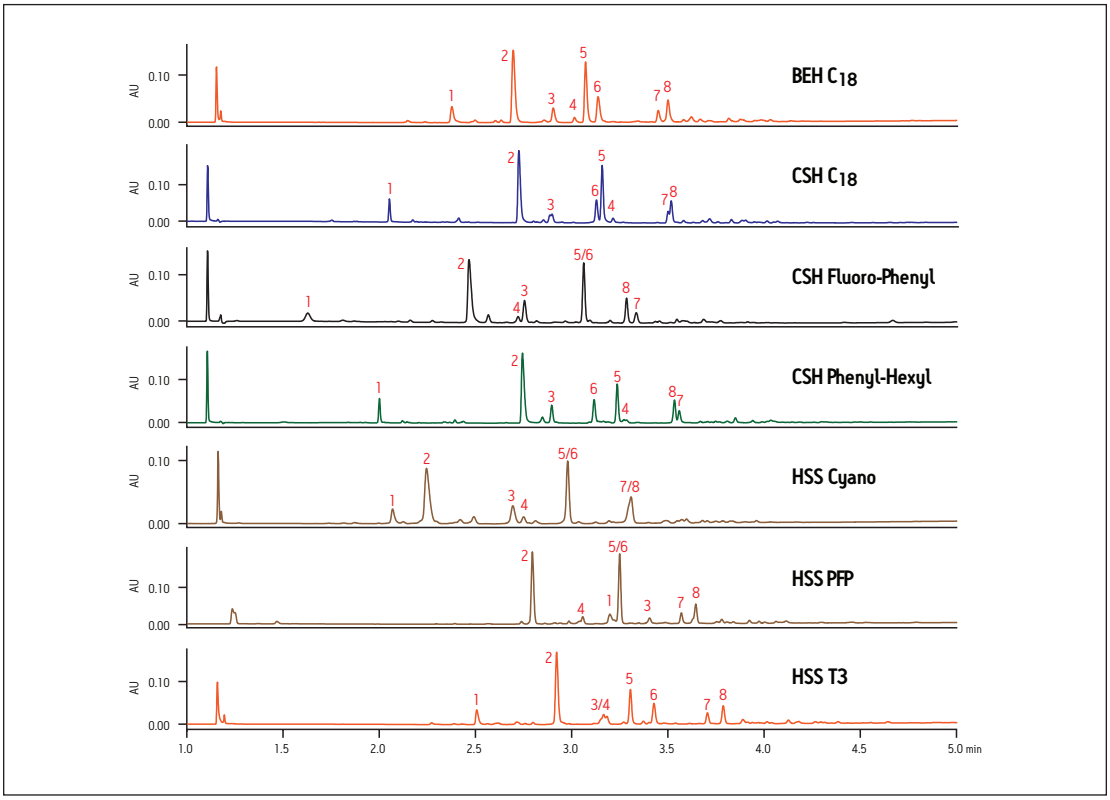

Figure 2. Selectivity differences of acetylation products of 3,4-dihydroxy phenylacetic acid on various columns. Masses of labeled peaks (m/z): (1) 286, (2) 270, (3) 268, (4) 300, (5) 284, (6) 165, (7) 481, (8) 476.

Another important consideration when selecting a column is the loading capacity of the stationary phase. While basic compounds often have better loading and peak shape at high pH on compatible hybrid particle columns such as BEH or CSH, they tend to have worse peak shape and loading on traditional C_{18} columns in low-ionic-strength mobile phases, such as formic acid.[2] However, a CSH column can provide better loading of basic compounds at low pH using formic acid, resulting in sharper peak shapes and enhanced sensitivity of detection. Loading limitations are demonstrated in the analysis of acetylation products of nadolol, where the reaction products labeled as peaks 1 and 2 show overloaded peak shape on the BEH C_{18} column (Figure 3). By contrast, these peaks are considerably sharper, with enhanced loading and sensitivity on the CSH C_{18} column. At low pH, greater sensitivity and peak shape for these basic compounds allows faster identification of impurities on the analytical scale, and facilitates isolation of desired peaks at the preparative scale.

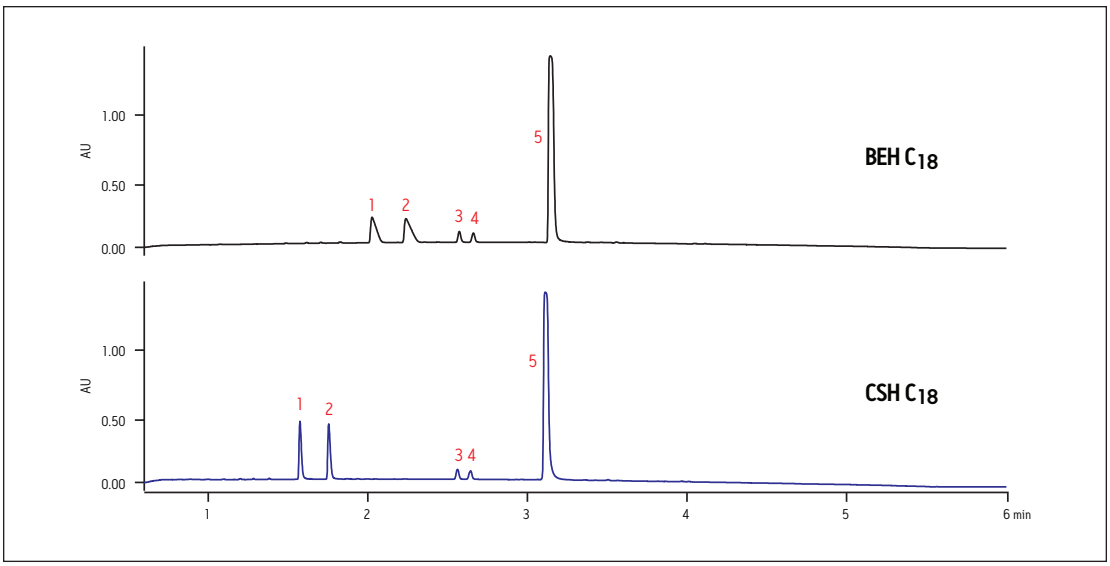

Figure 3. Loading differences of nadolol acetylation products on BEH and CSH C_{18} columns at low pH using formic acid. Masses of labeled peaks (m/z): (1) 394, (2) 406, (3) 436, (4) 418, (5) 478.

Screening Natural Product Extracts

When screening natural product extracts that contain many different types of compounds, it is particularly important to screen a wide selectivity range of columns. Selectivity can vary greatly when running extracts on various column chemistries and identification of minor components from complex crude extracts may be easily missed without proper screening. In Figure 4, the chromatographic profile of an extract of ashwagandha root is compared on four different column chemistries that were identified as having a wide selectivity range using the Waters Column Selectivity Chart (www.waters.com/selectivitychart).

The BEH C_{18} column shows increased retention for hydrophobic compounds compared to the CSH Fluoro-Phenyl, HSS Cyano or HSS PFP columns (Figure 4). There are also considerable peak elution order and retention differences observed across all columns, especially in the regions of peaks 2 to 4. Note again the significant difference in selectivity between the CSH Fluoro-Phenyl and HSS PFP columns. Although they both have the same ligand chemistry, they display significantly different chromatography due to the differences in base particle, making these two columns particularly good orthogonal choices for column screening. In this example, peak 7 is clearly resolved using the BEH C_{18} column, whereas the separation and identification of peak 2 is

more readily achieved using the HSS Cyano column, thus illustrating the utility of screening across different column chemistries. Early screening of extracts using columns with a wide range of selectivity facilitates rapid identification of minor components in complex mixtures by providing a better chance of resolving peaks of interest and enabling more accurate compound identification using mass spectrometry.

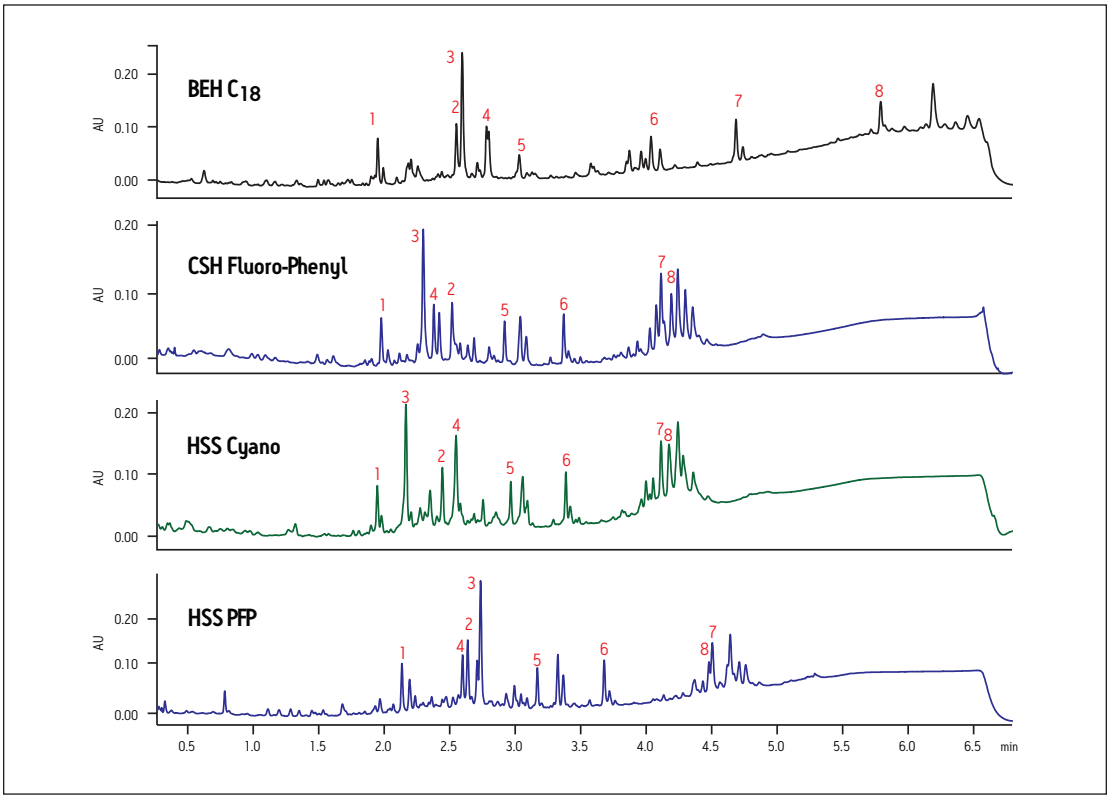

Figure 4. Screening an ashwagandha extract across columns with a wide selectivity range. Masses of labeled peaks (m/z): (1) 422, (2) 471, (3) 263, (4) 418, (5) 576, (6) 362, (7) 425, (8) 481..

CONCLUSIONS

Proper column selection considering appropriate base particle and bonded-phase chemistry is an important tool in rapidly developing methods for effective separations. Poor column choice early in the development of a new method can result in costly and unnecessary secondary optimization experiments. With advances in column technology, there are increasing choices of columns with different base particles and ligands to provide optimal chromatography. For the separation of components in any matrix, sample screening across a wide range of column chemistries should be considered. Columns with diverse chemical properties can be easily selected using the Waters Column Selectivity Chart. Screening of samples across columns is automated using the ACQUITY UPLC H-Class System with a column manager and Empower 3 Software. Using these tools, rapid screening on a variety of columns can be performed for each sample, resulting in faster and more efficient method development with improved separations.

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2. McCalley, D. V. *Anal. Chem.* **2006**, *78*, 2532.

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A UPLC Method for Analysis of Metformin and Related Substances by Hydrophilic Interaction Chromatography (HILIC)

Michael D. Jones[1] and Ben William[2]
[1] Waters Corporation, Milford, MA, USA
[2] Valeant Pharmaceuticals International, Steinbach, Manitoba, Canada

APPLICATION BENEFITS

- Reduced development time facilitated by instrument flexibility
- A 6x reduction in chromatographic run time resulting in a 3.5x reduction of total required analysis time resulting in faster throughput and reduced solvent consumption in routine sample analysis
- Utilizing ACQUITY UPLC results in a savings of $5800/per 1000 injections by reducing solvent consumption

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class system
- ACQUITY UPLC BEH Amide column and pre-column filter
- Empower™ 2 CDS software
- ACQUITY UPLC Columns Calculator

KEY WORDS

Method transfer, polar basic compounds, method development, melamine, cyanoguanidine, pharmaceutical impurities

INTRODUCTION

Metformin hydrochloride is an anti-diabetic drug typically administered orally while treating non-insulin dependant (type 2) diabetes mellitus.[1] It is one of the most widely-used oral antidiabetic drugs worldwide, with over 48 million generic formulation prescriptions filled in the United States alone.[2] Liquid chromatographic analysis of metformin and related impurities presents a challenging task due to the highly polar characteristics of the molecules and the low UV absorbance of the analytes. These challenges limit the options to manipulate selectivity during method development, especially with reversed-phase (RP) chromatography. Numerous methods are cited utilizing a variety of techniques such as ion chromatography, hydrophilic interaction chromatography (HILIC), and RP LC methodologies reporting run times up to 30 minutes.[1,3,4]

In this application note, a method was developed on the ACQUITY UPLC H-Class system using an ACQUITY UPLC BEH Amide sub-2 µm particle stationary phase in HILIC mode to successfully resolve and analyze metformin and six related substances. Development of the method was facilitated through the column and solvent switching capabilities of the ACQUITY UPLC H-Class which allows automated changes of stationary phase, ionic strength, cation buffer, pH and temperature. The major contributors to the successful separation of metformin and the related substances are discussed. A routine use evaluation study was performed to determine feasibility of the method for use in QC laboratories. Informatics provided visualization of trending results with intent to identify deficiencies regarding the developed methodology. The final method will provide cost reduction improvements in method robustness for routine analysis.

EXPERIMENTAL

Sample Description

Samples were provided by a pharmaceutical collaborator. Stock solutions of metformin hydrochloride, as well as impurities A, B, C, D, E and I were prepared in water. Working standards were prepared as per the previous HPLC methodology (70:30 acetonitrile:water). A working standard mixture was prepared whereas impurity concentrations were in respect to the metformin concentration: Impurity A was prepared at 0.05% of metformin and Impurities B, C, D, E and I were prepared at 0.1% of metformin. A mixture of the impurities without addition of metformin was also prepared at the same concentration as the impurities working standard. In addition to the working standard and impurity working standard, two separate preparations consisting of vials prepared with metformin with and without impurities spiked into the matrix. Individual standards were also prepared for each of the analyte constituents.

Method Conditions

Instrument:	ACQUITY UPLC H-Class configured with CM-A, CM-AUX, SSV, PDA
Buffer:	20 mM potassium phosphate, pH 2.3
Mobile Phase:	80:20 acetonitrile:buffer
Separation Mode:	Isocratic
Detection:	UV at 218 nm
Column:	ACQUITY UPLC BEH Amide, 2.1 x 150 mm, 1.7 µm, part number 186004802
Needle Wash:	90:10 acetonitrile:water
Seal Wash:	90:10 water: methanol
Sample Diluent:	70:30 acetonitrile:water
Flow Rate:	0.5 mL/min
Column Temp.:	40 °C
Injection Volume:	1.0 µL
Data Management:	Empower 2 CDS

RESULTS AND DISCUSSION

Method Transfer

The originally supplied HPLC methodology for metformin utilized isocratic conditions with a low-pH sodium phosphate buffer and acetonitrile mobile phase.[1] An Atlantis® HILIC 4.6 mm x 250 mm, 5 µm column was used with an approximate flow of 2 mL/min resulting in a run time of 30 minutes. Sample injection volume was 10 µL. The HPLC method resolves metformin and all six impurities (not shown).

The HPLC methodology was transferred to a 2.1 x 100 mm, 1.7 µm ACQUITY UPLC BEH HILIC column using the ACQUITY UPLC Columns Calculator. It should be noted that the ACQUITY UPLC BEH HILIC stationary phase does not have exactly the same selectivity as the Atlantis HILIC stationary phase due to differences in the base particle, although the Waters Column Selection application indicated that the two stationary phases have similar selectivity. In this application, there were observed selectivity differences between the Atlantis HILIC column and BEH HILIC column. The chromatogram in Figure 1 shows coelutions and a lack of overall retentivity on the BEH HILIC column using UPLC® technology. Slight changes in organic composition were not successful in resolving the impurity peaks. In some instances, as organic composition was increased, salt in the mobile phase precipitated due to mixing a high buffer concentration with a high composition of organic mobile phase. The precipitated salt resulted in increased pressure and baseline absorbance issues. It was then determined that a small amount of redevelopment would be needed to resolve the metformin and related substances by exploring suitable variables.

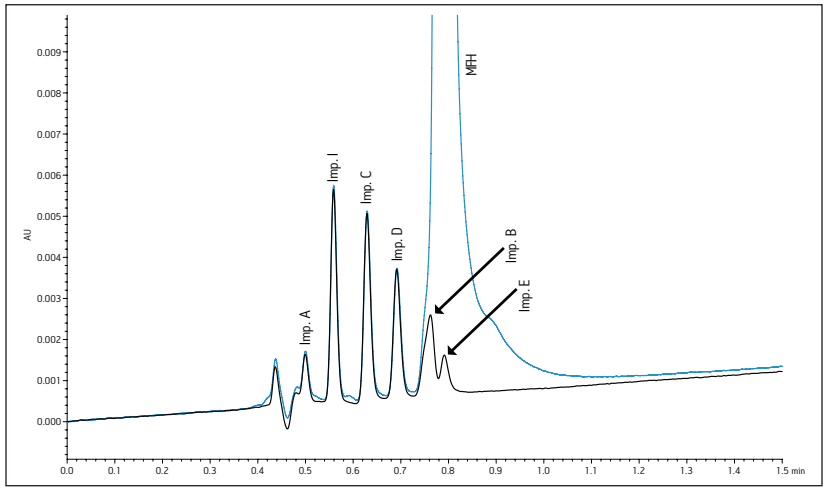

Figure 1. Working standard (blue) and Impurity mix (black) overlay. Direct scaling to a BEH HILIC column was unsuccessful in resolving two of the impurities from the API. The mobile phase was 84:16; acetonitrile: 28 mM sodium phosphate buffer pH 2.2, respectively. The flow rate was scaled to 0.736 mL/min and maintained at a temperature of 20 °C. The injection volume was 0.8 µL.

Manipulating Selectivity on HILIC

A method development scheme to analyze metformin and related substances presents a challenging task. Limitations regarding the low UV spectral absorbance of the analytes at 218 nm inhibit the use of typical MS-friendly buffers such as ammonium formate and ammonium acetate, since their UV cutoff approaches 230 nm. Reversed-phase LC is unsuccessful in retaining the analytes due to the polar basic characteristics of the compounds.

A method development scheme was employed to investigate two HILIC stationary phases: ACQUITY UPLC BEH HILIC and ACQUITY UPLC BEH Amide. Ionic strength, buffer cation selection, and temperature were determined as the remaining options to alter selectivity. Based on the poor retentivity and resolution of these compounds using the ACQUITY UPLC BEH HILIC stationary phase, the ACQUITY UPLC BEH Amide column was investigated.

The working standard was injected onto the ACQUITY UPLC BEH Amide 2.1 x 100 mm, 1.7 µm column. The resulting chromatogram in Figure 2 resolved all compounds with the exception of a slight co-elution between Impurity B and Impurity D. Desired improvements in peak shape and sensitivity were seen for Impurity E. Due to the isocratic conditions, changes in flow rate and temperature were explored individually to improve on these critical impurity peaks of interest. An experiment utilizing higher mobile-phase pH was explored but the results yielded little to no retention of many of the impurity peaks. The following relationships were observed during development of the separation on the amide column:

- Increases in organic content increased retention of Impurity D and B to co-elute with impurity E. A flow rate of 400 µL/min at 25 °C was determined to be optimal when using sodium phosphate as the aqueous mobile phase (Figure 2).
- As temperature increased, retention of impurity E decreased and co-eluted with impurity B (Figure 3).
- As flow rate increased, resolution of Impurity B, D, and E decreased.

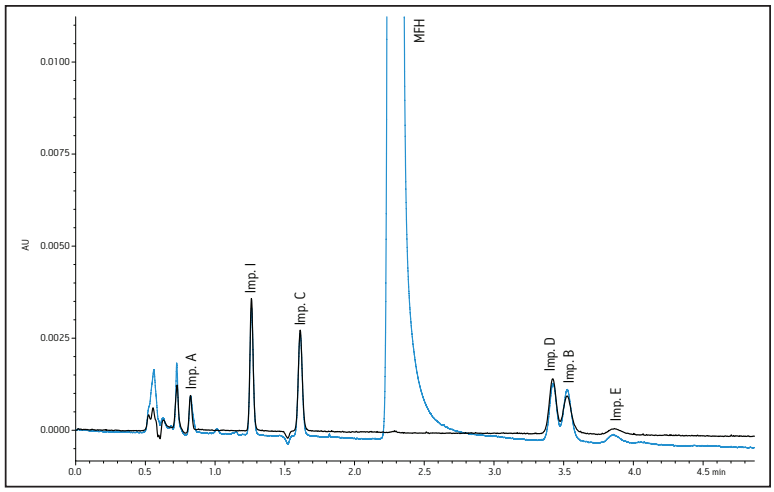

Figure 2. Injection of WS and Impurity mixture on ACQUITY UPLC BEH Amide column. A generic method was used to begin redevelopment. The conditions were derived from initial method conditions. The mobile phase was 80:20; acetonitrile: 30 mM sodium phosphate buffer pH 2.2, respectively. The flow rate was to 0.4 mL/min and maintained at a temperature of 25 °C. The injection volume was 1.0 µL.

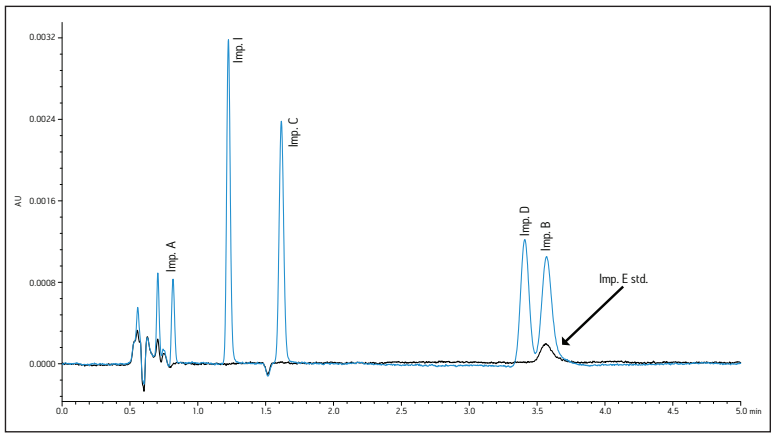

Figure 3. An Increase in temperature to 30 °C shifted retention time of Impurity E to decrease and co-elute with Impurity B. The mobile phase was 80:20; acetonitrile: 30 mM sodium phosphate buffer pH 2.2, respectively. The flow rate was to 0.4 mL/min and maintained at a temperature of 30 °C. The injection volume was 1.0 µL.

The proper selection of cation in the buffer can help control the ionic interactions on the surface of the column and in some instances, alter selectivity. The 30 mM sodium phosphate buffer was substituted with a 30 mM potassium phosphate buffer while maintaining a pH of 2.2. Optimal conditions were determined by combining the immediate improvement of changing the buffer cation to potassium. Combining the change in cation with a slight adjustment in flow rate and temperature, a desired resolution of the critical peaks was achieved. The chromatogram in Figure 4 shows better peak shape and resolution for Impurity peaks B, D, and E. Also, Impurity E shifted retention and elutes before Impurities B and D. Since the baseline noise was higher with the method in Figure 4, the buffer strength was decreased to minimize the potential for salt precipitation. The effect of decreasing the ionic strength to 10 mM resulted in co-elution of Impurities B and D. A concentration of 20 mM potassium phosphate resulted in acceptable peak shape and resolution (Figure 5).

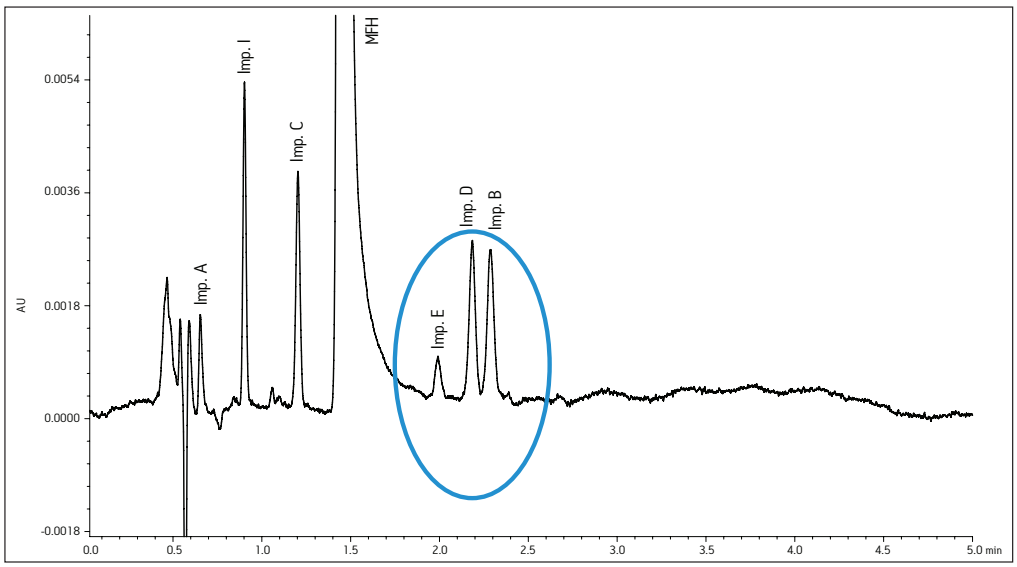

Figure 4. Changing the cation from Na+ to K+. Immediate improvement of resolution and peak shape were observed for Impurities E, D, and B. The mobile phase was 80:20; acetonitrile: 30 mM potassium phosphate buffer pH 2.2, respectively. The flow rate was to 0.5 mL/min and maintained at a temperature of 40 °C. The injection volume was 1.0 µL.

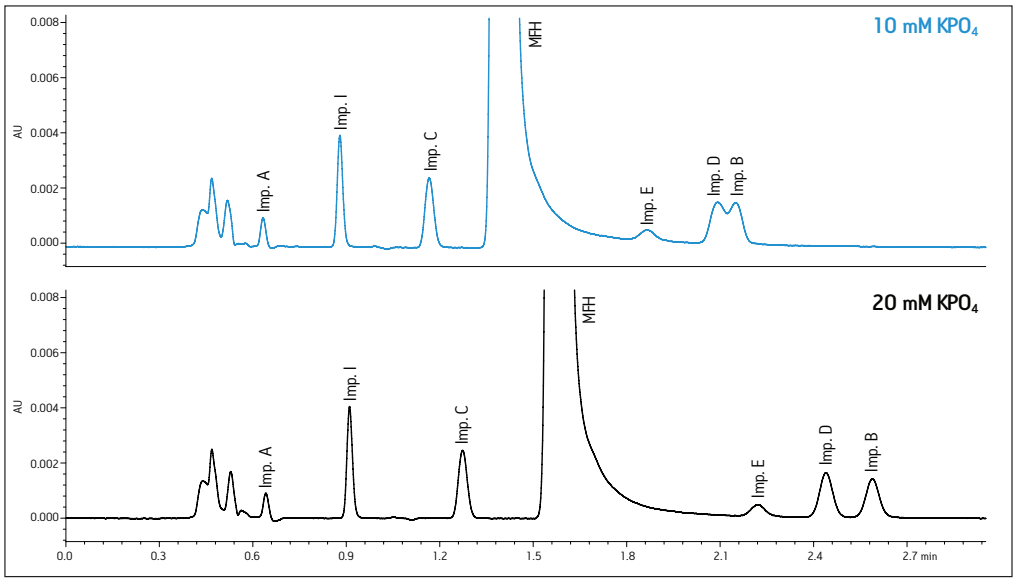

Figure 5. Effect of buffer concentration.
Note: Impurities D and B affected by ionic strength of buffer.

The effects of temperature were explored to determine the effect on selectivity when using the potassium phosphate buffer. Temperature was increased from 20 °C to 50 °C in 10 °C increments. Resolution increased for Impurities B, D, and E as temperature increased. A temperature of 40 °C was determined to provide adequate resolution of the critical pairs (Figure 6). The use of higher temperature resulted in lower column pressure, which allowed the use of a longer column to improve the resolution for the final methodology (Figure 7).

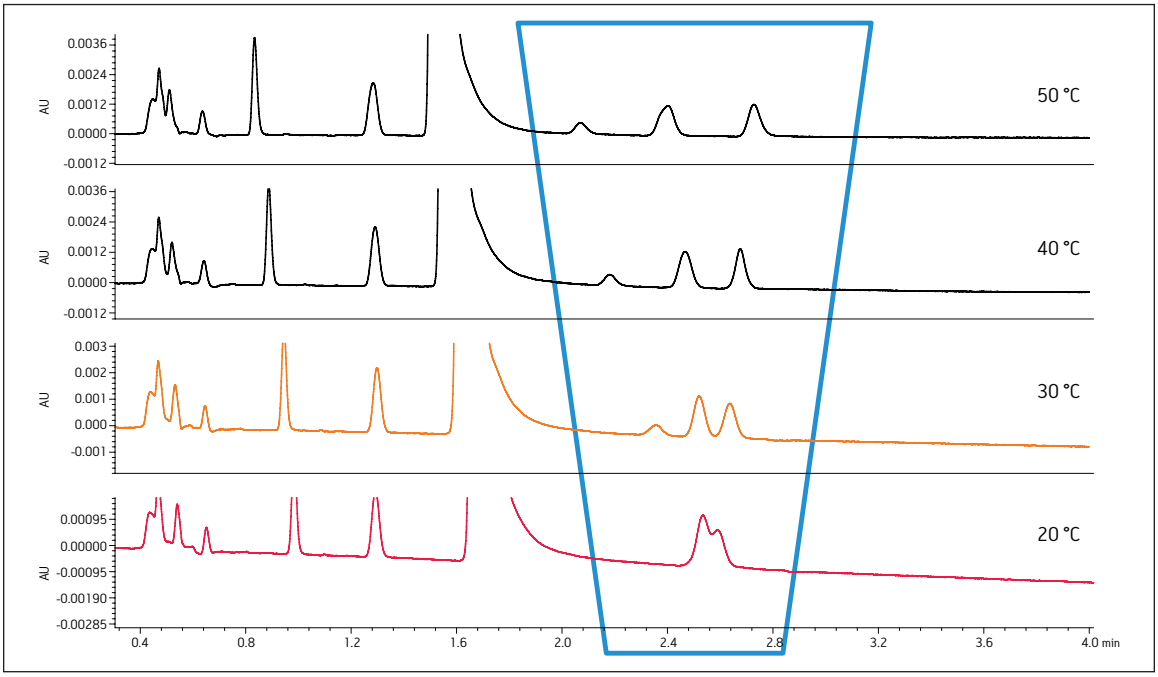

Figure 6. Effect of temperature on selectivity. As temperature increased, resolution between each of the critical pair analytes increased. Note: Impurities E, D and B resolution are affected by temperature, or perhaps linear velocity.

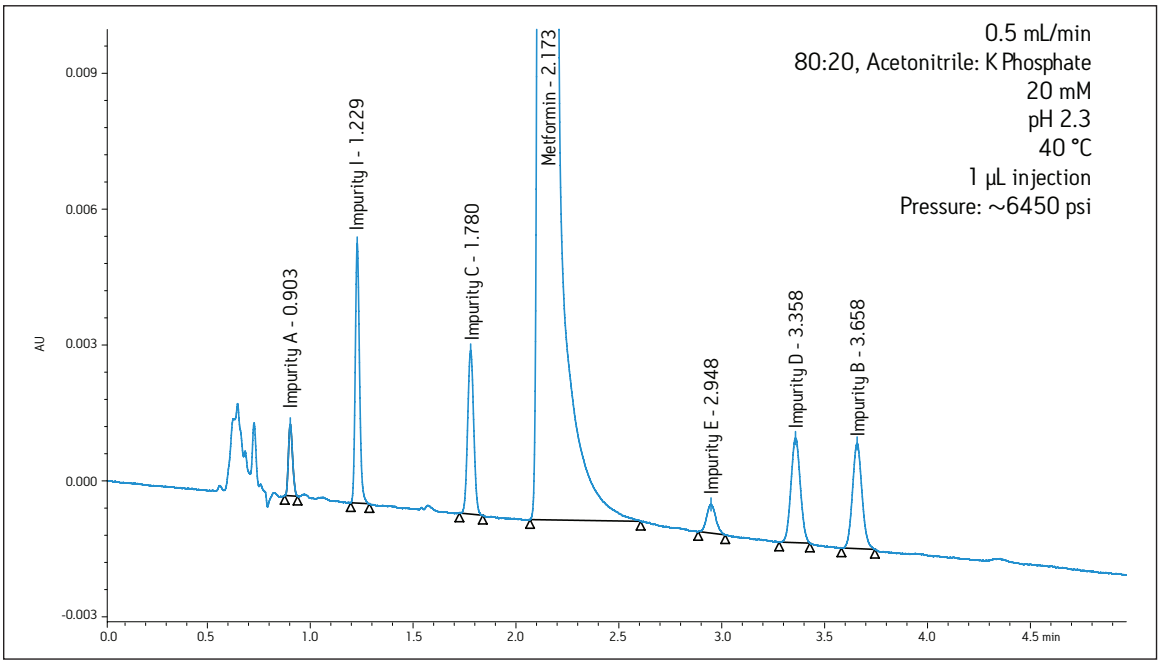

Figure 7. Final conditions. ACQUITY UPLC BEH Amide 2.1 mm x 150 mm, 1.7 µm with pre-column filter. The mobile phase was 80:20; acetonitrile: 20 mM potassium phosphate buffer pH 2.2, respectively. The flow rate was to 0.5 mL/min and maintained at a temperature of 40 °C. The injection volume was 1.0 µL of working standard.

ROUTINE USE STUDY

In order to evaluate the effects of using the potassium phosphate buffer at high organic composition, injections of the standards and samples were performed over a period of time to replicate routine use of the method in a QC laboratory. The sample set consisted of a bracketing procedure constructed with the working standard preparations, individual standard preparations, as well as the spiked and unspiked sample matrix formulations totaling over 360 injections for a given experimental run. A single bracket consisted of 30 injections, which was repeated 60 times to achieve 1800 injections to complete the study designed to replicate practices within a quality control testing laboratory. A pre-column filter was installed for preventative and investigative purposes in the event of a pressure increase over the time of the study.

In an effort to understand how the data was trending, custom calculations and custom reports were created in Empower 2 CDS, whereby processed data could be visualized in the form of trend plots without exporting to spreadsheets. Initial pressure readings were approximately 6500 psi and increased steadily to approximately 6700 psi over the first 860 injections, as indicated by the summary pressure trend plot in Figure 8a. Closer investigations of the summary trend data showed further trending within the bracketed sample set (Figure 8b). The trend plots generated in Empower 2 CDS showed increases in system pressure once the matrix samples were injected. This indicated a deficiency in the original sample preparation procedure. The sample preparation procedure was altered to include a longer centrifugation time and the use of 0.2 µm filter disks in place of 0.4 µm filter disks. The filter disks were used to filter the supernatant as it was added to the sample vial. As a result, pressure increases due to the sample preparation was eliminated and extended to a point where the method was suitable for validation.

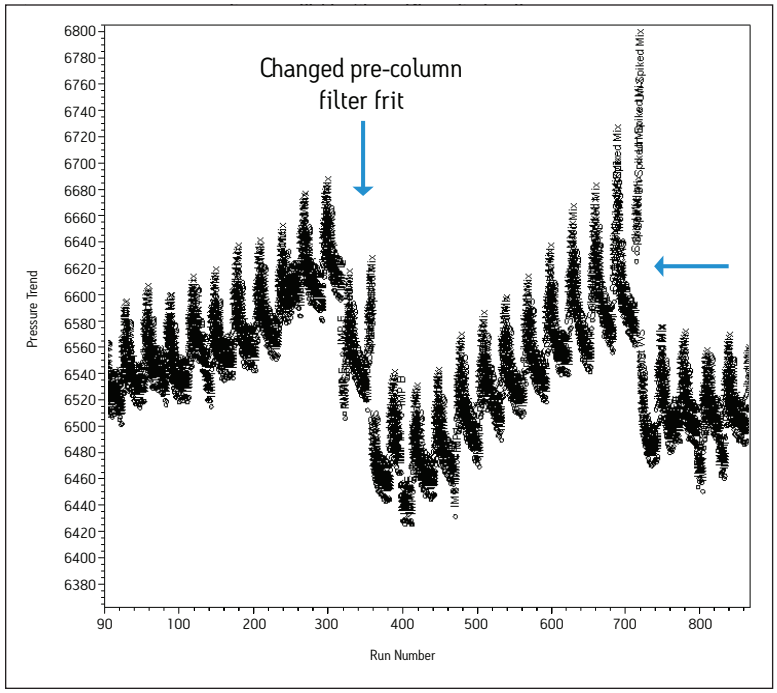

Figure 8a. Pressure trend for 860 injections. Pressure increased steadily over time. Replacement of the pre-column filter frit was successful in returning to initial pressure; however, pressure would steadily increase again.

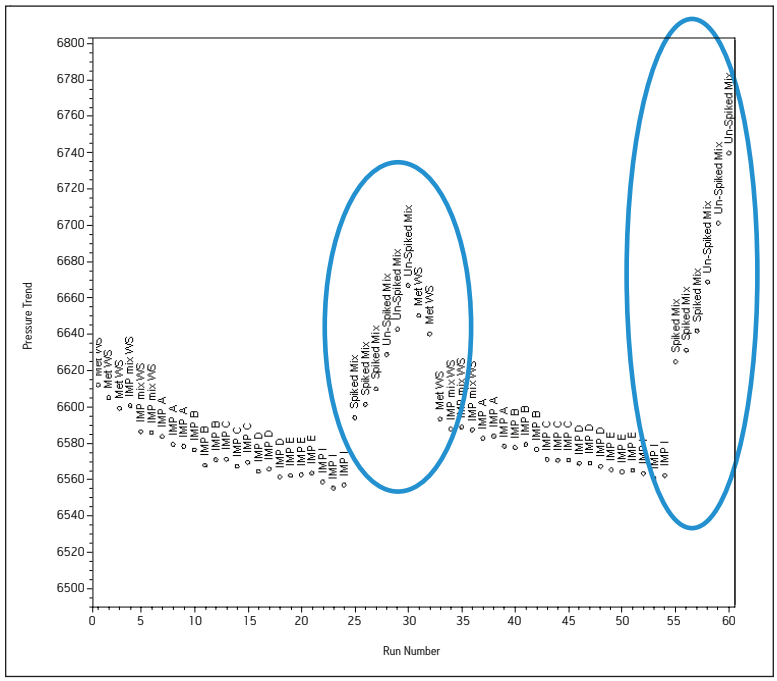

Figure 8b. Further investigation of the trend data revealed a primary trending within the bracket sample set. Injections of the matrix samples were the root cause of the total pressure increasing over time. The circled regions indicate the pressure readings of the matrix injections.

CONCLUSIONS

The complete solution consisting of informatics tools, flexible instrumentation, and a selection of chemistries resulted in a method providing a six-fold reduction in analysis time compared to the HPLC methodology. Altering the buffer cation provided a selectivity change between Impurity E and the Impurity pair; B and D. Ionic strength of the buffer influenced the retentivity of Impurities B and D. Temperature was a useful selectivity influence for HILIC method development. The informatics solutions within Empower provided trending insight to effectively troubleshoot issues relating to poor sample preparation. The use of pre-column filters also contributed towards achieving excellent column performance of over 1500 injections.

In retrospect, a Routine Use Study of the original HPLC methodology would be costly. Comparing the mobile-phase consumption during 1500 injections on HPLC versus UPLC; HPLC would utilize approximately 65 liters compared to 11 liters consumed using UPLC. At an average cost of $165 per liter acetonitrile, the resulting methodology would save approximately $8800 in solvent consumption. Implementing UPLC technology results in a time savings of 26 days per 1500 injections, or roughly an 80% reduction in analysis time in which the resources can be better utilized to increase profitability.

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

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APPLICATION BENEFITS

- Fast method scouting assays can be performed by combining UPLC® with generic gradients, as compared to HPLC
- By using short UPLC columns in method development, many column chemistries can be screened rapidly and automatically using the ACQUITY UPLC® Column Manager
- Using UPLC with mass spectra data facilitates peak tracking during method optimization and complemented UV data for confirming impurities
- Empower® Software's custom calculations reports expidite data mining

WATERS SOLUTIONS

ACQUITY UPLC System

ACQUITY UPLC HSS, BEH, Phenyl and Shield RP Columns

Empower 2 Software

KEY WORDS

Method development, simvastatin, impurities, screening

INTRODUCTION

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 β-hydroxy acid form, which acts as an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme involved in the in vivo synthesis of cholesterol.[2]

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 minutes. 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 for efficient method development of impurity profiles.

In this application, we show how the HPLC method for simvastatin has been redeveloped on UPLC and is compatible for both UV and mass detection. 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.

EXPERIMENTAL

Method development UPLC conditions

LC system:	ACQUITY UPLC System with Column Manager
Column dimensions:	2.1 x 50 mm, 1.7 µm (1.8 µm for HSS)
Column 1:	ACQUITY UPLC HSS C_{18} (p/n 186003538)
Column 2:	ACQUITY UPLC BEH C_{18} (p/n 186002350)
Column 3:	ACQUITY UPLC Phenyl (p/n 186002884)
Column 4:	ACQUITY UPLC Shield RP_{18} (p/n 186002853)
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)

Data processing and management

Empower 2 CDS Software

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

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). A variety of 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 Chromatography Data Software (CDS). The ACQUITY UPLC Column Manager was employed to allow for the simple automated selection of four different columns.

RESULTS

Mining the data

Empower 2 was employed to mine data without the need 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).

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.

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 minutes and RT=2.385 minutes. 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 minutes and RT=2.328 minutes.

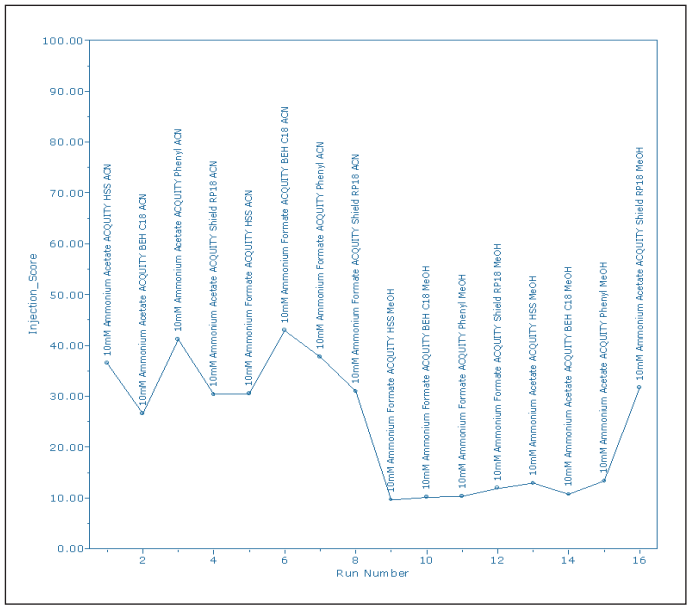


Figure 2. Custom summary plot of calculated "Best set of injections" based on the summary bar charts from Figure 1.

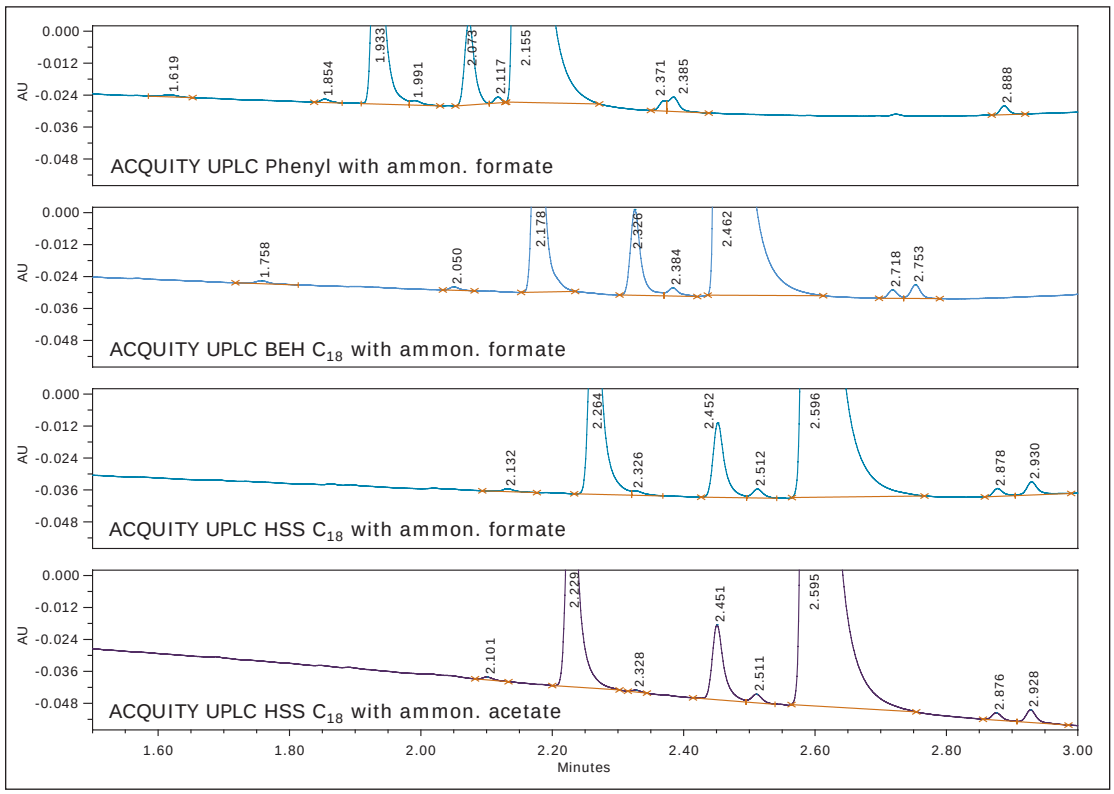


Figure 3. Overlay of the four highest-rated chromatograms of the method scouting process.

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).

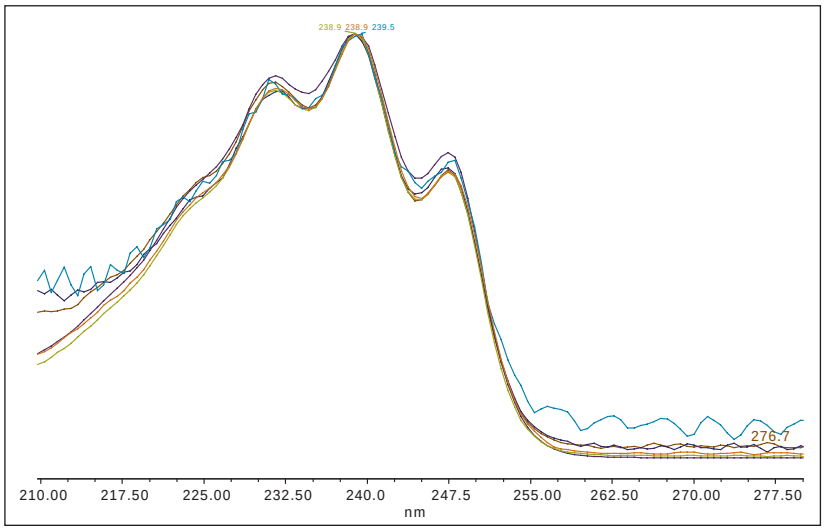


Figure 4. Normalized UV PDA spectral data for the simvastatin and impurity peaks. The data illustrates spectral similarity between the API and identified peaks, suggesting a related structural origin.

The mass spectra data generated by the mass detector, the ACQUITY SQ, facilitated peak tracking during the method optimization process. Furthermore, the mass 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.

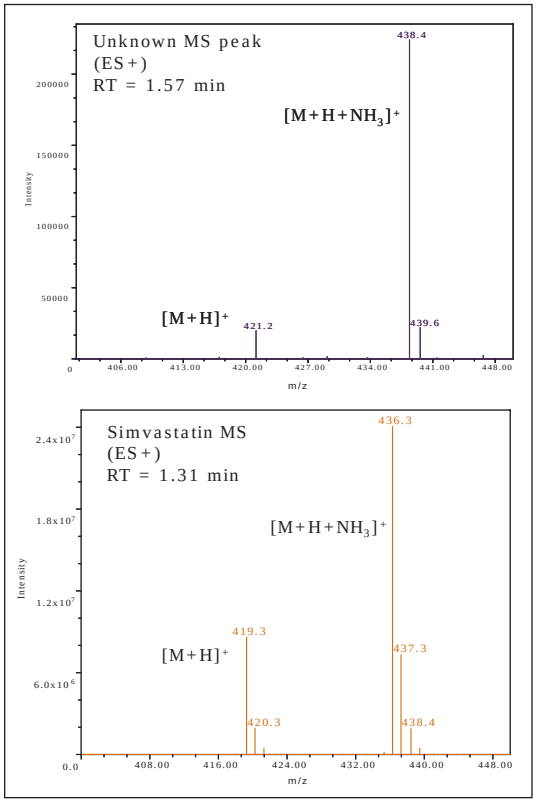


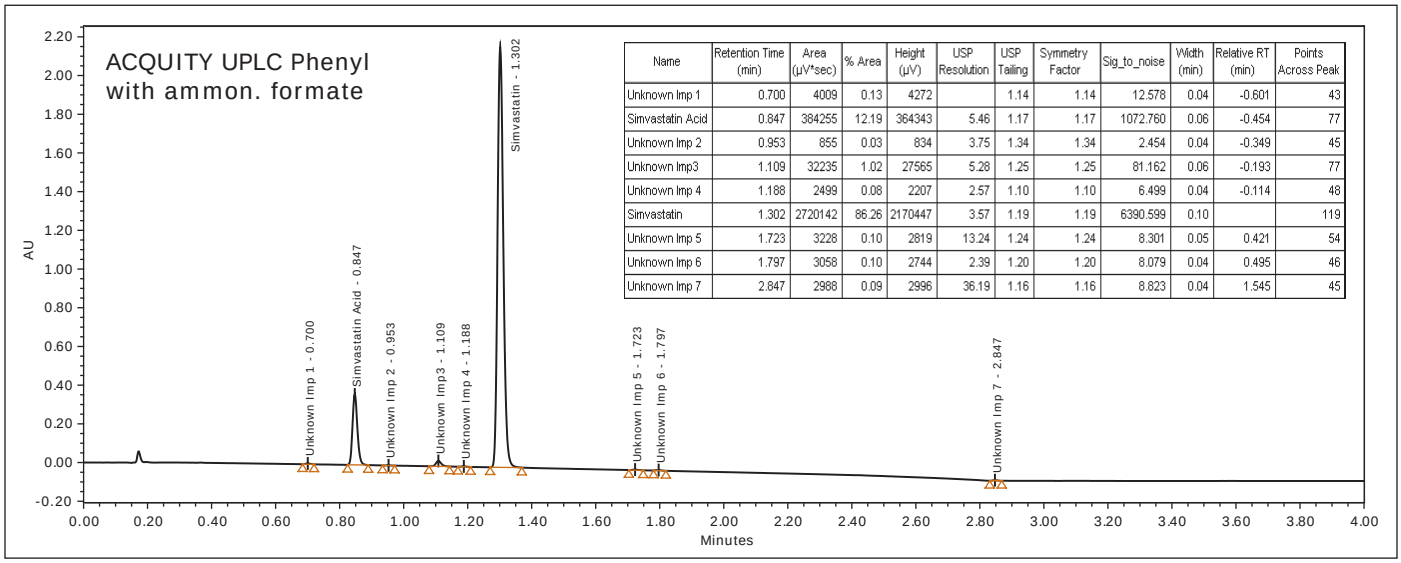
Figure 5. MS spectra of the unknown peak at RT=1.566 minutes. The adduct formation was consistent of observations of the simvastatin MS spectra (RT = 1.31 min).

Method optimization

The UPLC method was optimized for the 1.8-µm ACQUITY UPLC HSS T3 2.1 x 50 mm column with ammonium acetate pH 4.0 and acetonitrile as the mobile phase. Mass 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 (5 and 10 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 x 50 mm column to maximize chromatographic speed 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 6-7.



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 x 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.

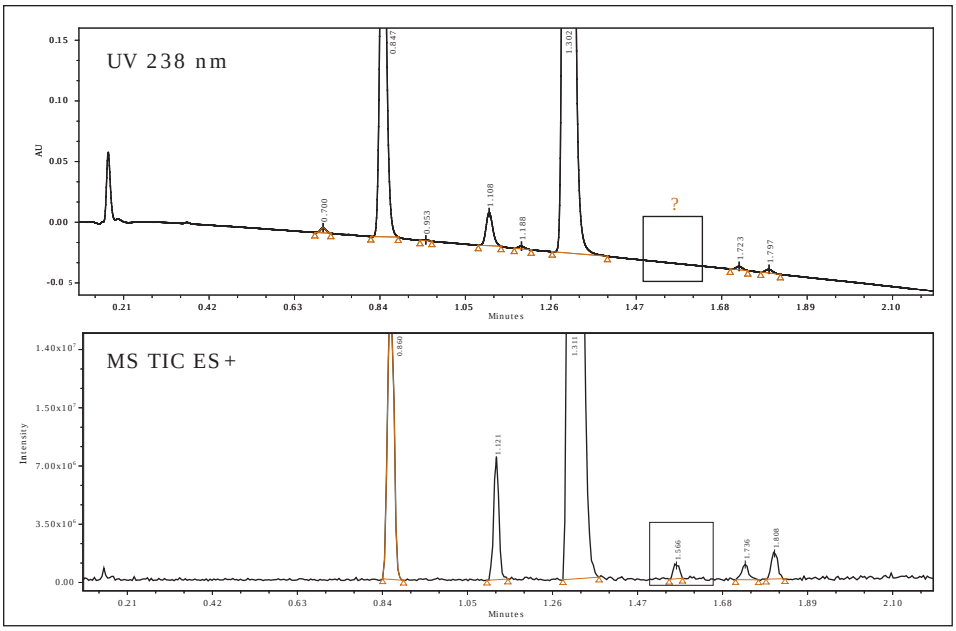


Figure 7. Zoomed baseline of the UV-MS final method. The presence of the unknown peak at 1.566 in the MS TIC was not present in the UV range.

Final method, UPLC conditions

LC system:	ACQUITY UPLC
Column dimensions:	2.1 x 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:	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 ACQUITY UPLC System with Column Manager spectral analysis within Empower 2 Software, which facilitated peak tracking (mass data), simvastatin relation (UV/mass 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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A Systematic Approach Towards UPLC Methods Development

Christopher J. Messina, Eric S. Grumbach, and Diane M. Diehl
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Demonstrates a systematic approach to method development
- Selectivity is manipulated using pH, column chemistry, and organic modifier
- UPLC provides a 6-fold improvement in throughput, reducing time and cost per sample in the analysis

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC BEH, BEH Shield, BEH Phenyl, and HSS T3 Columns

KEY WORDS

Method development, paroxetine hydrochloride and related compounds, pH, column chemistry, organic modifier, optimization, gradient slope, temperature

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

LC Conditions

System:	Waters ACQUITY UPLC
Columns:	ACQUITY UPLC® BEH C_{18} 1.7 µm, p/n 186002350 ACQUITY UPLC BEH Shield RP18 1.7 µm p/n 186002853 ACQUITY UPLC BEH Phenyl 1.7 µm, p/n 186002884 ACQUITY UPLC HSS T3 1.8 µm, p/n 186003538
Dimensions:	2.1 x 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)	Profile %A	%B
0.0	95	5
5.0	10	90
5.01	95	5
5.5	95	5

Injection vol.:	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

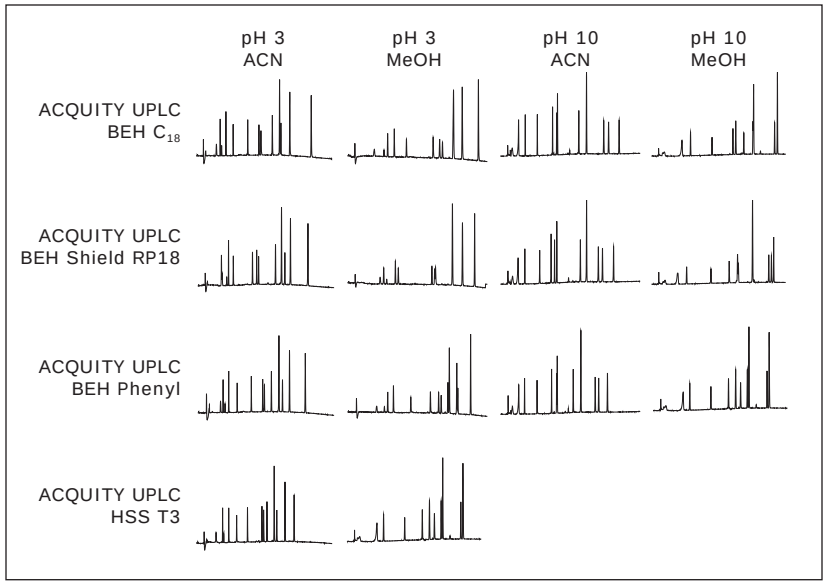

Figure 1. UPLC Methods Development Experimental Matrix.

RESULTS AND DISCUSSION

As depicted in Figure 1, a result matrix of 14 chromatograms is generated by evaluating three Bridged Ethylene 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 potential for resolving all components. The ACQUITY UPLC BEH C_{18} was selected to carry out the separation.

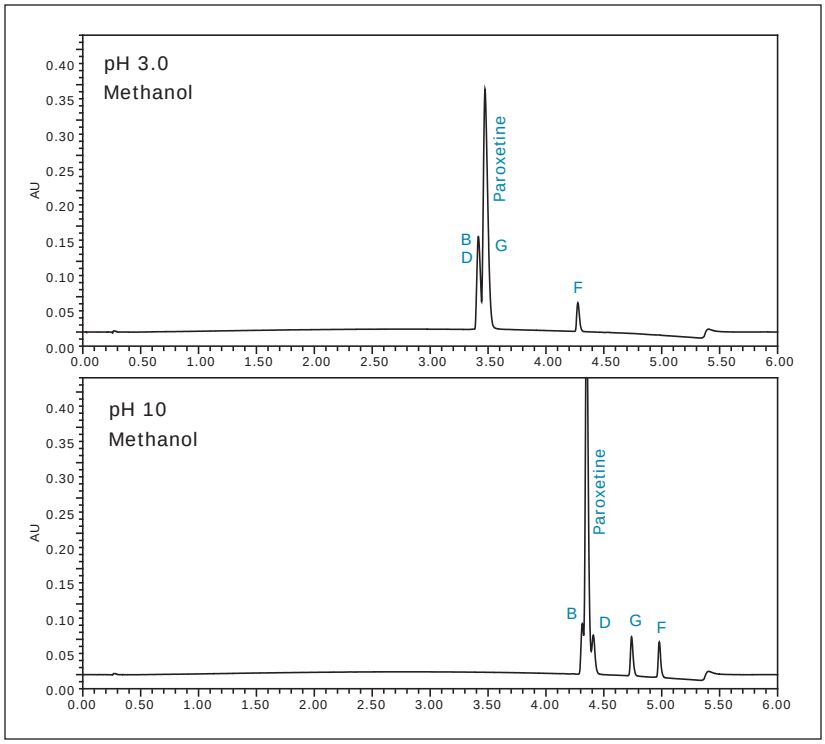

Figure 2. Evaluation of pH selectivity on ACQUITY UPLC BEH C_{18}.

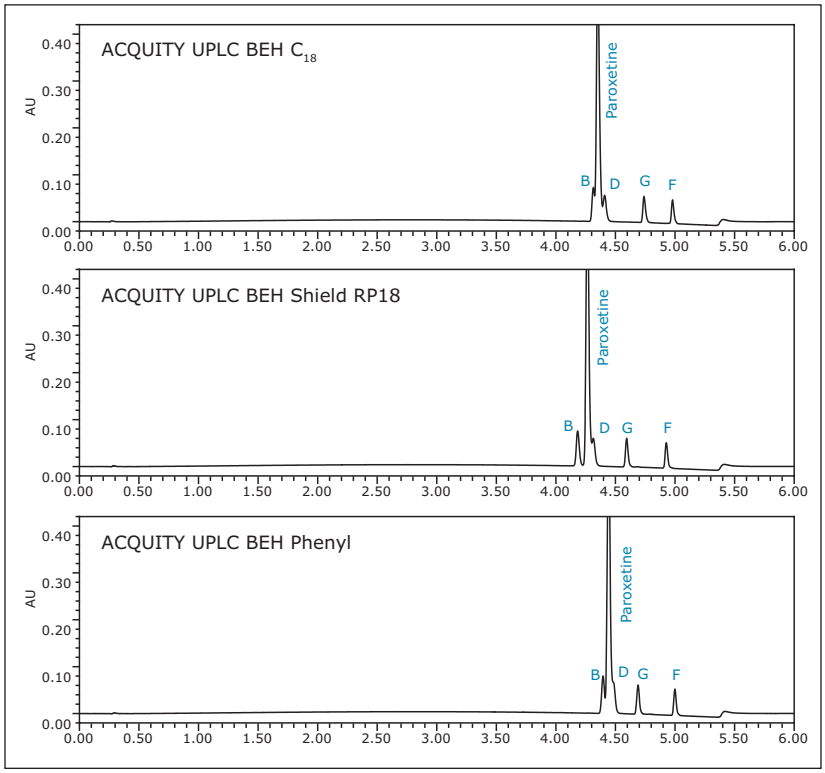

Figure 3. Comparison of column selectivity in methanol at alkaline pH.

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.

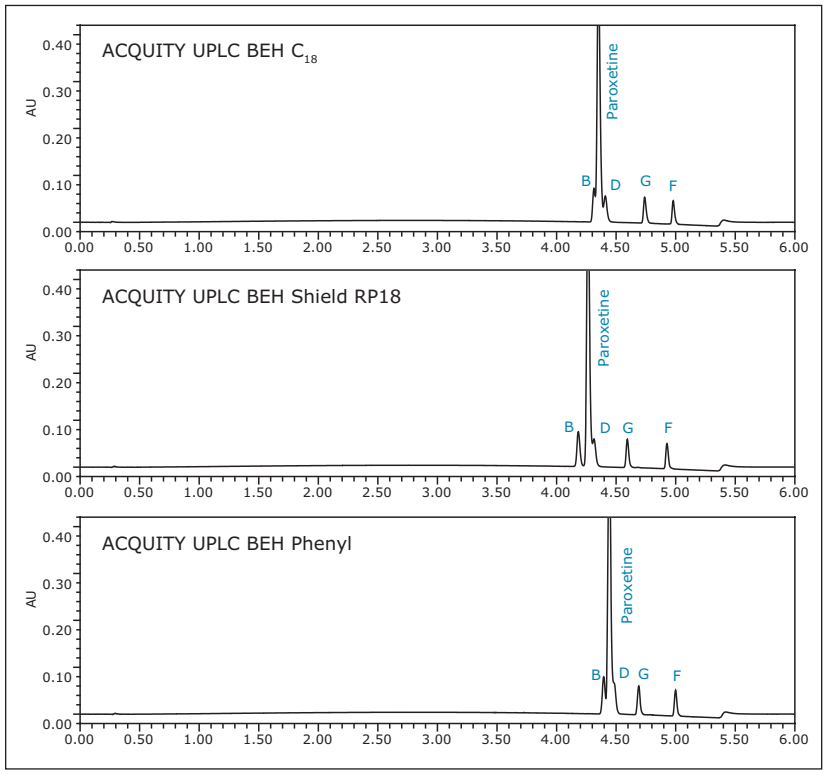

Figure 4. Evaluation of solvent selectivity on ACQUITY UPLC BEH C_{18}

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.

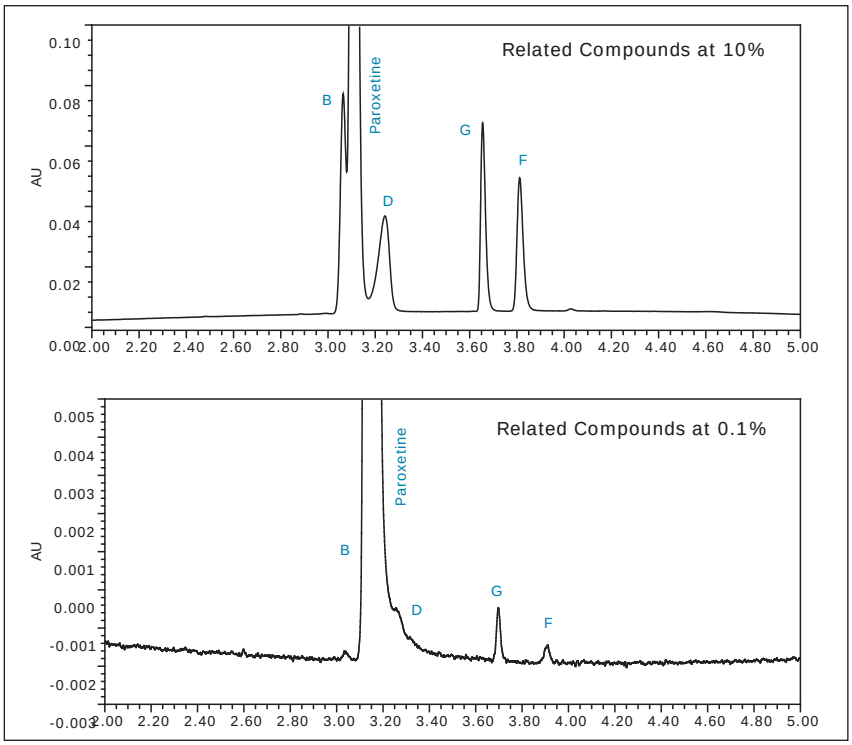

Figure 5. Related compounds at 10% vs. 0.1% of paroxetine.

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.

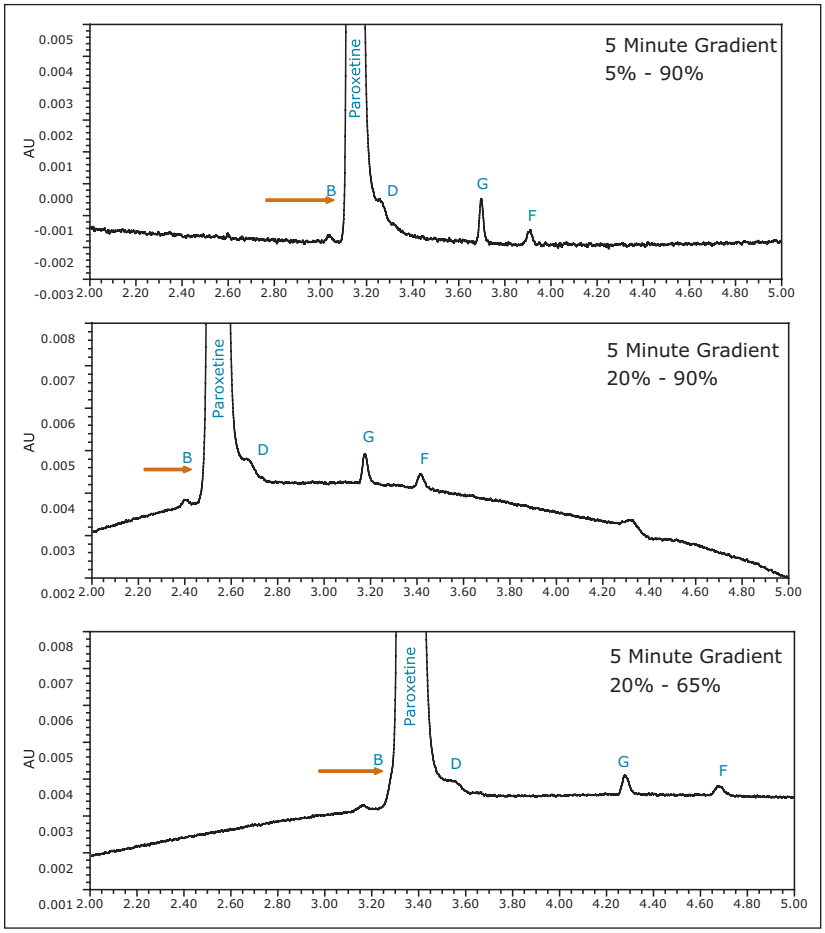

Figure 6. Monitoring influence of gradient slope reduction.

Optimization: Temperature

Temperature affects every chemical process that occurs. Analyte diffusivity, sample loadability and peak shape dramatically improved with increasing temperature. At 60 °C, adequate separation of related compounds from paroxetine was achieved; therefore, no further optimization was necessary.

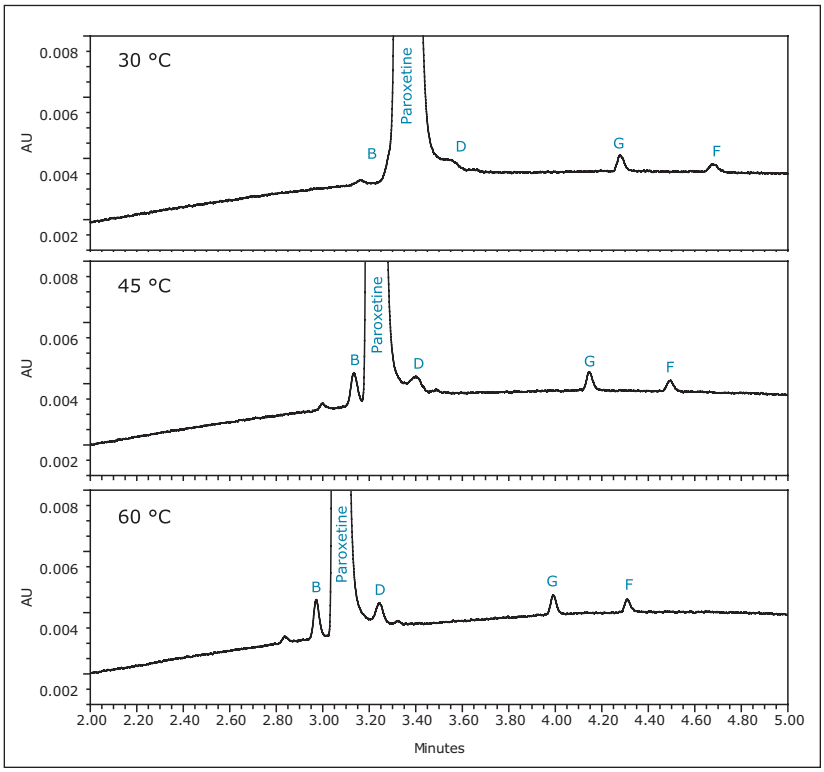

Figure 7. Influence of temperature on separation.

Final conditions

Separation was performed on a ACQUITY UPLC BEH C_{18} 2.1 x 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.

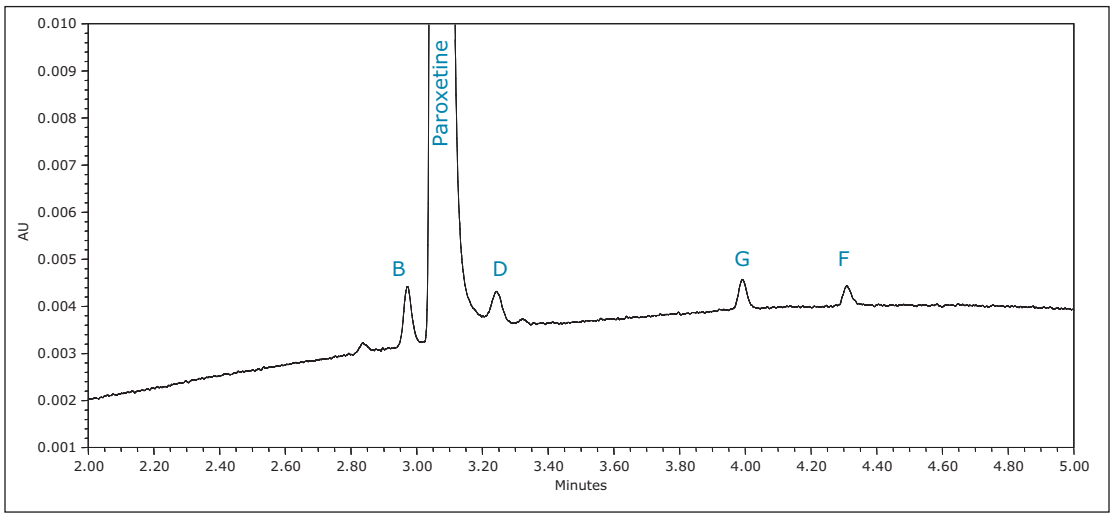

Figure 8: Final separation of Paroxetine and related compounds B, D, G, and F at the 0.1% level.

BUSINESS IMPACT

Productivity improvements associated with employing UPLC technology for methods development are depicted below in Table 1. 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 one work day opposed to one work week with conventional HPLC.

Methods Development Time					
UPLC Technology 2.1 x 50 mm, 1.7 µm, 0.5 mL/min				**Conventional HPLC** 4.6 x 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**			**pH 3**	**methanol**	
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**			**pH 10**	**acetonitrile**	
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**			**pH 10**	**methanol**	
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 Column	6.1 Hours
Total Screening Time	**7 Hours**	**Total Screening Time**	**43 Hours**

Table 1. Comparison of productivity between UPLC Technology and HPLC for methods development.

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.

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A Quality by Design (QbD) Based Method Development for the Determination of Impurities in a Peroxide Degraded Sample of Ziprasidone

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APPLICATION BENEFITS

- Faster, efficient separations and data management using an ACQUITY UPLC® H-Class with Column Manager and Solvent Select Valve in conjunction with Empower™ 2 software.
- Built-in method robustness using a Quality by Design (QbD) approach to generate a method that is amenable to continuous improvement without re-validation in the future.
- Streamlined method transfer from UPLC® to HPLC using the ACQUITY UPLC Columns Calculator and Method Transfer Kits facilitates transfer of methods to labs that may not equipped with UPLC.
- Significant time savings using a statistical design of experiments approach to method development to generate efficient sample sets that cover a wide experimental space.

WATERS SOLUTIONS

ACQUITY UPLC H-Class system
ACQUITY UPLC CSH C_{18} Columns
XSelect™ CSH™ C_{18} Columns
Empower 2 CDs
Fusion AE™ Method Development Software (S-Matrix)

KEY WORDS

Method development, UPLC, method transfer, Quality by Design, ziprasidone, degradation, CSH, ACQUITY UPLC Columns Calculator

INTRODUCTION

Method development can be a time-consuming process that can be repeated many times thoughout a drug development pipeline. Methods are commonly developed using a one-factor-at-a-time (OFAT) approach where one variable is changed sequentially until a suitable method is produced. This type of development may create an adequate method but provides a limited understanding of method capabilities and method robustness. Rather, a systematic screening approach that evaluates a number of stationary phases, pH ranges and organic modifiers provides a more thorough approach to method development. A Quality by Design (QbD) approach to method development uses statistical design of experiments (DoE) to develop a robust method 'design space'. The design space defines the experimental region in which changes to method parameters will not significantly affect the results. This approach builds-in robustness to the method as the method is being developed[1].

A better understanding of the overall method capabilities and limitations in development ensures a greater chance of successful downstream method validation, transfer and routine use. Software-driven method development affords considerable time savings for the scientist and the use of QbD can produce a significantly more robust and quality submission to regulatory authorities.

In this application note, a QbD approach to method development and subsequent method transfer to HPLC is presented on a forced degradation sample of ziprasidone, an anti-psychotic drug. Method development was performed using an ACQUITY UPLC H-Class system equipped with a column manager and solvent select valve to allow for automated exploration of a wide range of conditions, while obtaining efficient separations with shorter chromatographic run times. Fusion AE Method Development software was used in conjuction with Empower 2 to facilitate a more comprehensive QbD approach to method development.

EXPERIMENTAL

ACQUITY UPLC H-Class Conditions

Mobile phase:

A: Acetonitrile

B: Methanol

D1: Water with 0.1% Formic Acid (pH 2.5)

D2: Water with 0.1% Ammonium Hydroxide (pH 10.5)

Columns (All 2.1 x 50 mm, 1.7 µm)

1. ACQUITY UPLC CSH C_{18}
2. ACQUITY UPLC CSH Fluoro-Phenyl
3. ACQUITY UPLC BEH Shield RP18
4. ACQUITY UPLC HSS C_{18} SB
5. ACQUITY UPLC HSS T3
6. ACQUITY UPLC HSS Cyano

Needle Wash:	10:90 Water:Methanol
Sample Purge:	90:10 Water:Methanol
Seal Wash:	90:10 Water:Methanol
Detection:	UV at 254 nm

SCREENING (PHASE 1)

Flow Rate:	0.6 mL/min
Injection Volume:	2 µL
Column Temp.:	30°C
Gradient Time:	5 min
Variables:	stationary phase, mobile phase, gradient endpoint % organic, mobile phase pH 2.5 to 10.5

OPTIMIZATION (PHASE 2)

Column:	ACQUITY UPLC CSH C_{18}, 2.1 x 50 mm, 1.7 µm
Mobile Phase:	A: Acetonitrile
	D1: Water with 0.1% Formic Acid (pH 2.5)
Gradient endpoint:	87.5% Acetonitrile
Variables:	gradient time, column temperature, injection volume, flow rate
Data Management:	Empower 2 CDS Fusion AE Method Development Software (S-Matrix)

SAMPLE PREPARATION

Ziprasidone peroxide degradation sample:

To 0.4 mg/ml ziprasidone in 50:50 water:methanol, add one equal volume of 3% hydrogen peroxide solution in water, heat at 80°C for 30 min. Dilute to 0.1 mg/mL final concentration with water.

RESULTS AND DISCUSSION:

Phase 1: Screening

Method development was performed using an ACQUITY UPLC H-Class system, Empower 2 and Fusion AE Method Development software. The H-Class was equipped with a 6-position column manager and a solvent select valve to enable full method development capability in one system. The initial screening varied column chemistries having CSH, BEH and HSS base particles for maximum selectivity. Organic modifier (acetonitrile or methanol) was screened varying the gradient endpoint from 50% to 100% organic, over a mobile phase pH range from 2.5 to 10.5.

Using these parameters, an experimental design was generated within Fusion AE, including randomization and replicate injections. The design generated encompassed the entire knowledge space defined by the constants and variables entered during the experimental setup. A partial factorial statistical design was selected by the software to obtain the maximum amount of information with the least number of experimental runs. The experimental design was transmitted to Empower2 software where all methods, method sets and sample sets were automatically generated and ready to run.

After initial integration and processing, results from the screening analysis for ziprasidone were imported back into Fusion AE and processed to generate an initial method for subsequent optimization. For the ziprasidone peroxide degradation sample, a water/acetonitrile gradient at pH 2.5 with an 87.5% acetonitrile gradient endpoint on a CSH C_{18} column was found to be optimal. The method developed is compatible with mass spectrometric detection and was directly transferred to LCMS to rapidly identify the ziprasidone forced-degradation products (Figure 1).

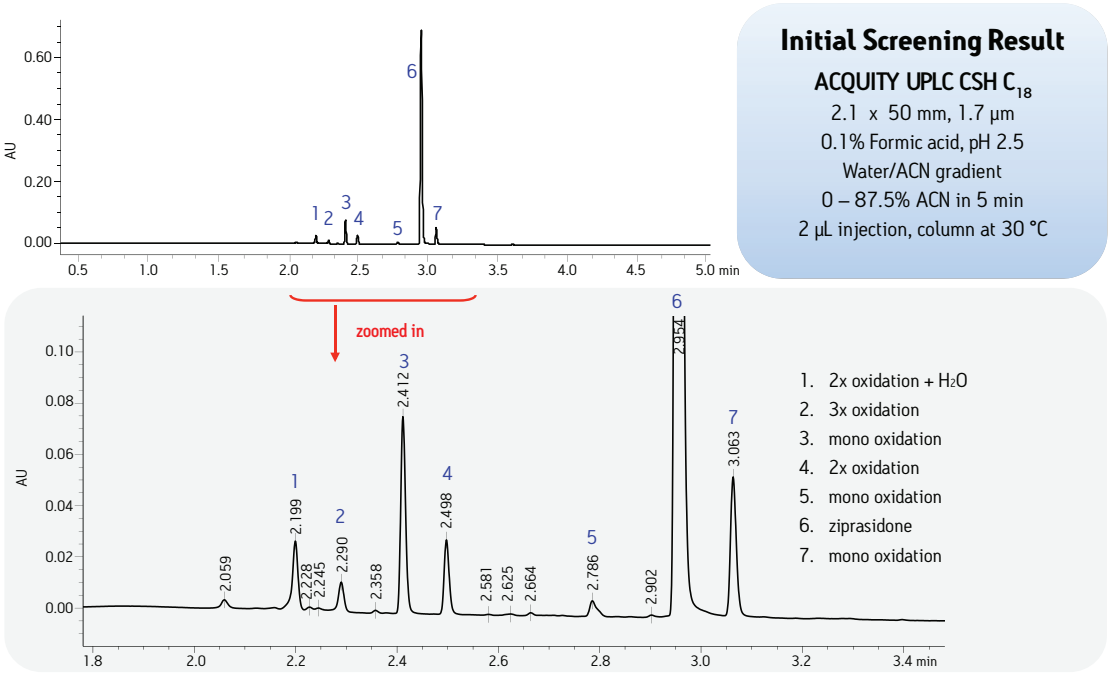

Figure 1. Initial method from screening experiments for ziprasidone peroxide degradation.

Phase 2: Method Optimization

The initial method was further optimized in a second experiment where secondary effectors such as column temperature, injection volume, gradient slope (modified using gradient time) and flow rate were varied. A new experimental design was generated by Fusion AE and new methods and sample sets were automatically created within Empower 2.

After processing data in Fusion AE, the final optimized method was generated, demonstrating the method that best meets the success criteria defined by the user. In the case of the ziprasidone peroxide degradation separation (Figure 2), an improvement in the tailing of peak 2 is seen along with better resolution of baseline impurity peaks and a newly resolved impurity is observed at 2.075 min.

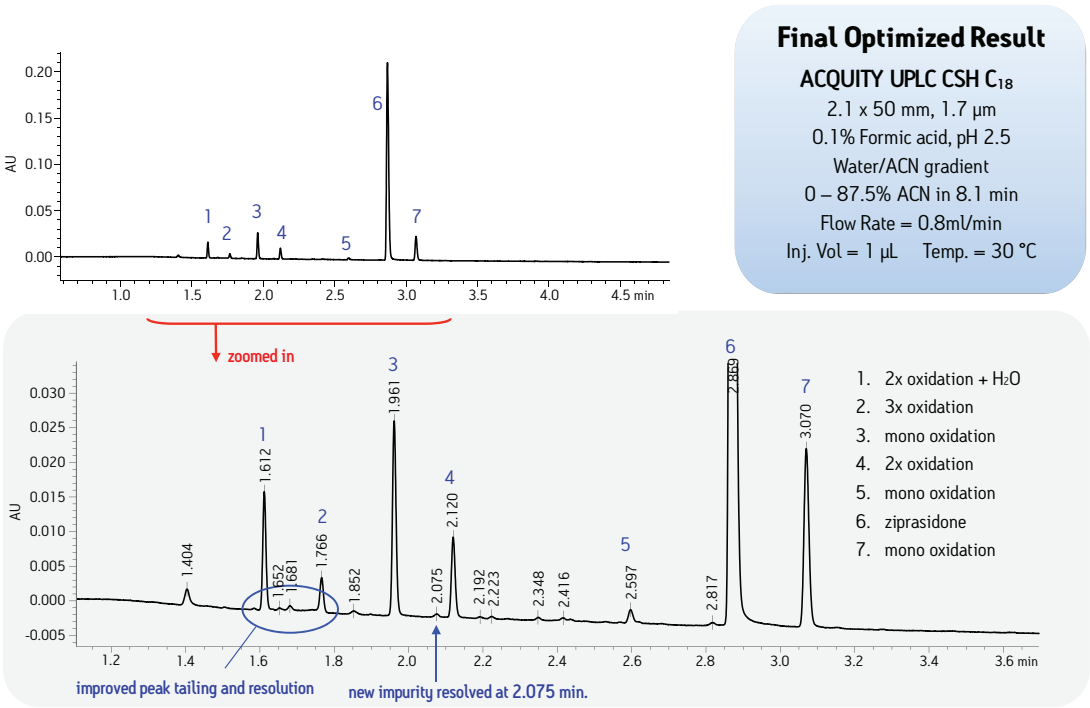

Figure 2. Final optimized method for ziprasidone peroxide degradation showing improved peak tailing and resolution.

Multi-dimensional plots in Fusion AE facilitates visualization of the effect of each factor on the separation (Figure 3). The white region of the 2D contour plot depicts the design space, which defines the robust region of the method where results are within designated criteria. By changing the factors on each axis, the design space can be explored in detail and method robustness can be fully understood.

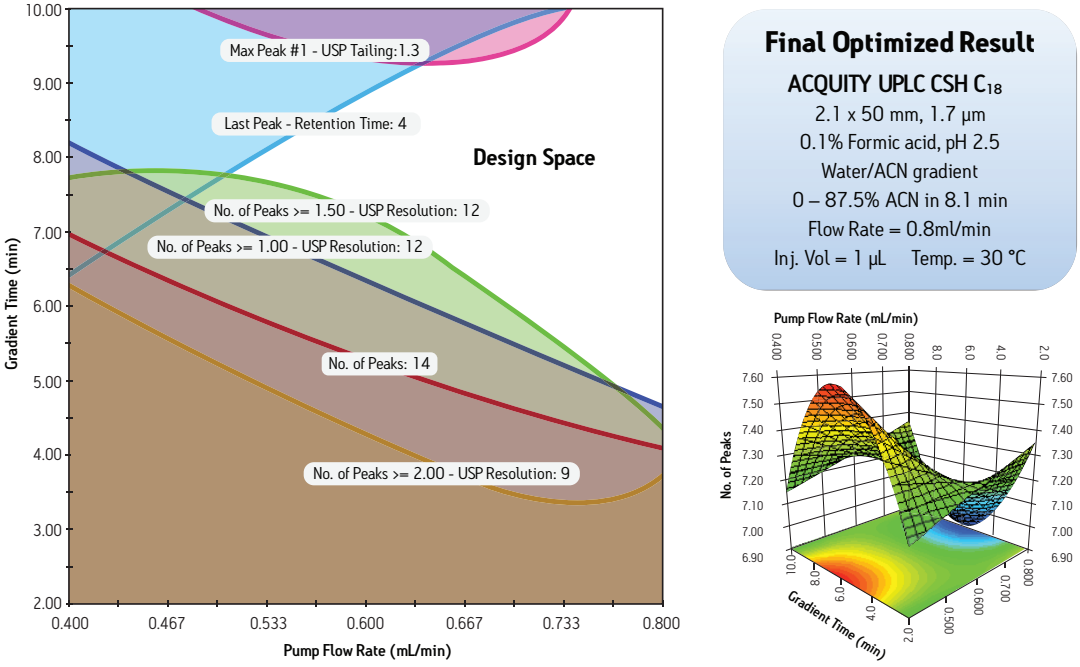

Figure 3. The design space region showing the independent effects of gradient time and pump flow rate on method success. Data can also be visualized in 3D plots as shown.

Method Transfer

The UPLC method developed using Fusion AE software was transferred to HPLC to demonstrate transferability from a method development laboratory to a quality control (QC) laboratory that might not be equipped with UPLC. Method transfer was performed using the ACQUITY UPLC Columns Calculator and Method Transfer Kit, scaling for particle size[2]. The method was scaled from the ACQUITY UPLC CSH C_{18} 2.1 x 50 mm 1.7 µm particle column to the corresponding XSelect CSH C_{18} 4.6 x 150 mm 5 µm HPLC column. A comparison of the UPLC and HPLC separation demonstrates that the peak profile and resolution is maintained when scaling to HPLC conditions from method development on UPLC.

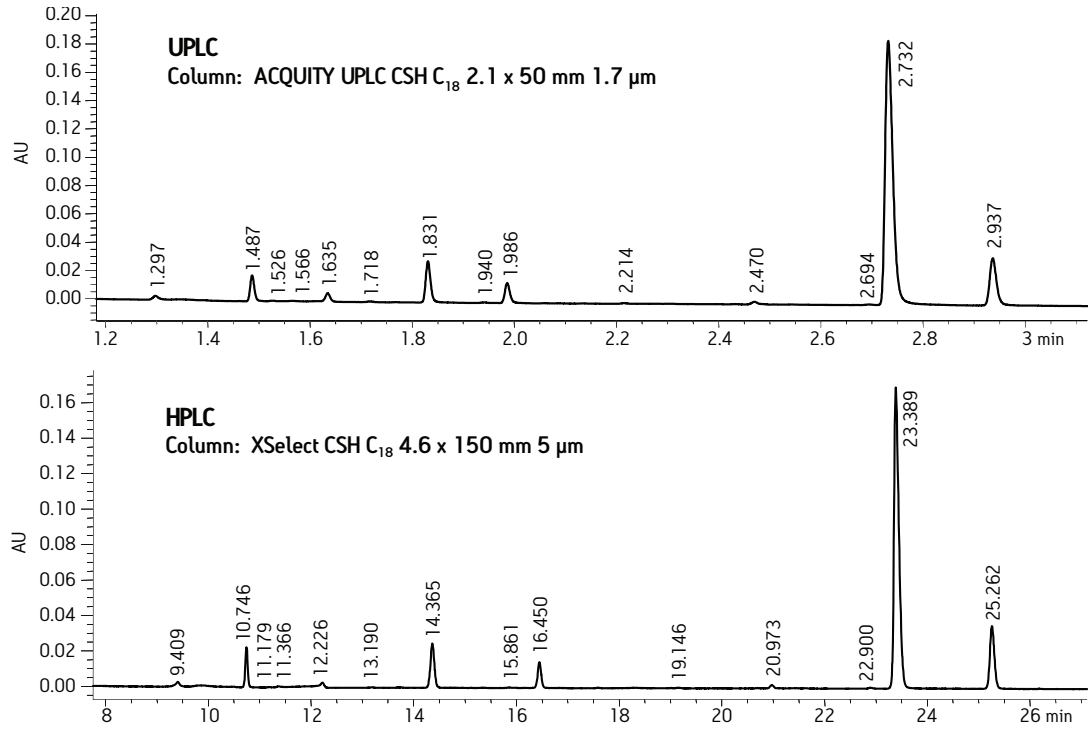

Figure 4. Method transfer from UPLC to HPLC for ziprasidone peroxide degradation.

CONCLUSIONS:

- A robust method for ziprasidone peroxide degradation was developed in two days using a Quality by Design approch on an ACQUITY UPLC H-Class system running Empower 2 and Fusion AE Method Development software.
- QbD method development software in conjunction with ACQUITY UPLC H-Class system automation allows for rapid screening and optimization across a wide range of column chemistries, mobile phases and pH ranges, while evaluating the effects of secondary factors such as column temperature, flow rate, injection volume and gradient slope on the separation.
- A comprehensive method development experiment can be rapidly performed by combining fast separations using UPLC with efficient experimental designs by Fusion AE Method Development software.
- The UPLC method developed for ziprasidone peroxide degradation was transferred to HPLC in one step using a Method Transfer Kit and ACQUITY UPLC Columns Calculator, demonstrating ease of transfer of developed methods to labs that may not be equipped with UPLC.

REFERENCES:

1. **Alden PG, Potts W, Yurach D. A QbD with Design-of-Experiments Approach to the Development of a Chromatographic Method for the Separation of Impurities in Vancomycin. Waters Application Note 70003719EN. 2010 Sept.**
2. **Jones MD, Alden P, Fountain KJ, Aubin A. Implementation of Methods Translation between Liquid Chromatography Instrumentation. Waters Application Note 720003721EN. 2010 Sept.**

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[TECHNOLOGY BRIEF]

Improving Decision Making during Method Development Using Empower 3 CDS Software

GOAL

To demonstrate the capabilities of Empower® 3 Software for scoring chromatographic separations using custom calculations and custom reports.

BACKGROUND

During the development of chromatographic methods, it is important to set separation goals and evaluate data in an unbiased manner. Typically as different variables are tested in method development, the scientist may rely on visual inspection of chromatograms or criteria to select the best conditions to move forward. In addition to being time consuming, this technique imparts skill level and expertise bias to the evaluation, easily resulting in the optimal result being overlooked. By contrast, a well-defined chromatogram scoring method allows all users to choose the best conditions for their method regardless of their level of experience, relying on metrics instead of judgement alone.

Empower 3 Software is a flexible chromatographic data system that allows users to perform many calculations within the data system itself, minimizing user errors in transcription and enabling the laboratory and its users to maintain compliance. Coupling custom calculations with customized reporting allows users to calculate and view only those parameters that are important to them, and to select the appropriate method conditions to move forward.

Empower 3 Chromatography Data Software streamlines method development with custom calculations and custom reporting.

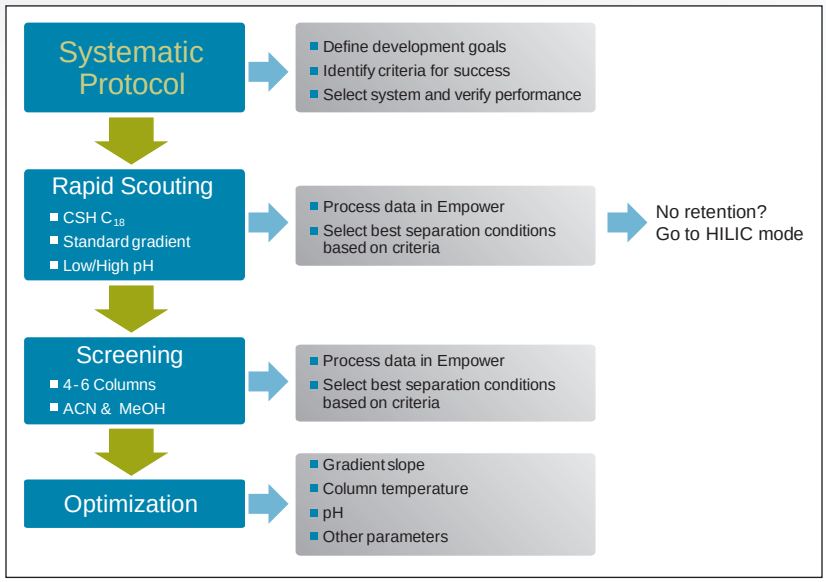

Figure 1. Systematic screening protocol for chromatographic method development.

This technology brief illustrates the use the ACQUITY UPLC® H-Class System with PDA and ACQUITY® QDa detectors for method development using a systematic protocol. Results from each phase of the development process were scored using Empower Custom Calculations and Custom Reports for easy selection of optimal conditions for further study.

THE SOLUTION

A systematic screening protocol was used for developing chromatographic methods (Figure 1). The protocol is designed to address factors of retentivity and selectivity by adjusting parameters to achieve optimal resolution of the components in a mixture.

We selected metoclopramide and its USP-defined related substances assay to demonstrate this protocol and to highlight the use of Empower Custom Calculations and Custom Reporting. Shown in Figure 2 are the chromatograms for the screening phase in the protocol. From our previous steps, we had selected a low pH region, and in this phase we were selecting a column chemistry and elution solvent. For each separation, Empower identified each integrated peak and calculated system suitability parameters.

Using Custom Calculations we were able to automatically tally the total number of peaks, the number of peaks with a USP resolution ≥2.0, and the total number of peaks with USP tailing ≤1.5 for each separation. Using a Custom Report, Figure 3, we tabulated and ranked each separation using these criteria. We also included additional important data, such as lowest resolution, k* a measure of retention in reversed-phase chromatography, and the retention time of the last eluting peak. This automated scoring allowed us to quickly identify the conditions that met our criteria and removed analyst variability and bias in decision making in our method development process.

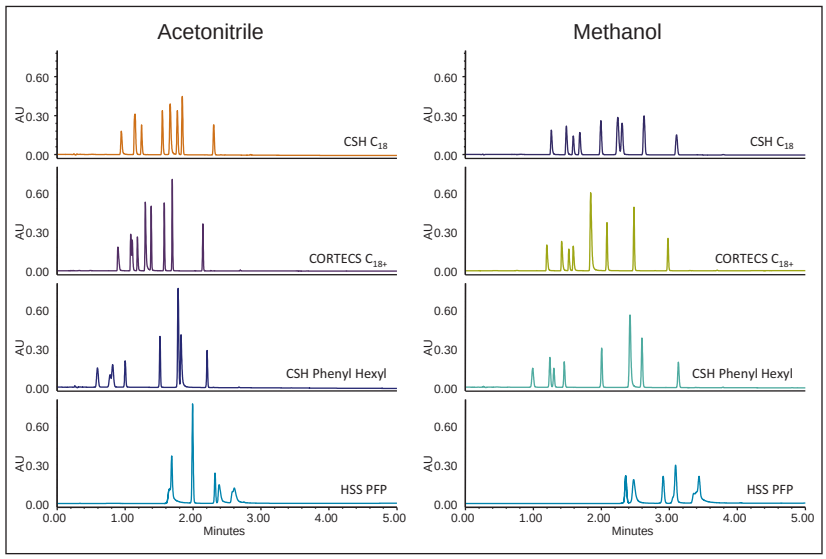

Figure 2. Separation of metoclopramide and USP-defined related substances. Both column chemistry and organic modifier were screened using a generic gradient.

SCORING REPORT

Empower 3

Sample Set ID: 4001
Result Set ID: 6805
Processed Channel Descr.: PDA 270.0 nm (200-400)nm
Run Time: 7.0 Minutes
Injection Volume: 1.00 ul

	Sample	Column	Strong Solvent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Lowest Rs	Min k*	RT of Last Peak
1	Low pH, MeOH	CSH C18	MeOH	Low pH	9	7	7	1.283	3.22	3.11
2	Low pH, ACN	CORTECS C18+	ACN	Low pH	9	7	5	0.769	1.98	2.15
3	Low pH, ACN	CSH C18	ACN	Low pH	9	5	7	2.308	2.15	2.30
4	Low pH, MeOH	CORTECS C18+	MeOH	Low pH	8	7	3	2.094	2.99	2.98
5	Low pH, MeOH	CSH Phenyl Hexyl	MeOH	Low pH	8	6	8	1.690	2.30	3.13
6	Low pH, ACN	CSH Phenyl Hexyl	ACN	Low pH	8	5	5	0.654	0.98	2.21
7	Low pH, MeOH	HSS PFP	MeOH	Low pH	8	2	2	1.870	6.86	3.44
8	Low pH, ACN	HSS PFP	ACN	Low pH	7	2	2	0.108	4.51	2.61

Figure 3. Example of a scoring report generated by Empower 3 Software. Score was based on maximizing total peaks, total peaks with Rs ≥2.0, and total peaks with tailing ≤1.5. Additional data reported for informative purposes and secondary ranking if necessary.

CONCLUSION

Using Empower 3 Software and its Custom Calculations and Custom Reporting functions enables users to quickly and automatically evaluate chromatographic data. Reports can be configured to allow users to choose optimal conditions based on metrics rather than analyst judgment, minimizing analyst bias and ensuring a comprehensive assessment of data and best method conditions.

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Validation of a Method for the Separation of Ziprasidone and Its Degradants using Empower 2 with Method Validation Manager (MVM)

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APPLICATION BENEFITS

- Easy monitoring of the validation study and complete traceability of data in one location, instead of error-prone data tracking using multiple spreadsheets and paper trails
- Efficient sample set designs save sample preparation and instrument time
- Robustness testing analyzing multiple factors at a time results in considerable time savings when compared to a one-factor-at-a-time (OFAT) approach
- Testing multiple column batches made with different stationary-phase batches ensures long-term method ruggedness

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class system
- ACQUITY UPLC CSH™ C_{18} and XSelect™ CSH C_{18} columns
- Method Validation Kits (MVKs)
- Empower™ 2 with Method Validation Manager (MVM) software

KEY WORDS

Method validation, UPLC, batch, reproducibility, ziprasidone, degradation, CSH

INTRODUCTION

Method validation is an essential part of analytical method development in a regulated environment. The process of running a validation study, from defining tests, specifications and acceptance criteria to sample testing and analysis, is a data-intensive and time-consuming process. The use of additional spreadsheets and paper print-outs to process data and track results are error-prone processes that can be remedied using a validation software package such as Empower 2 with Method Validation Manager (MVM).[1]

In this application note, the validation of a method developed for the separation of ziprasidone and associated impurities from a forced degradation is demonstrated using Empower 2 with Method Validation Manager (MVM). While a variety of tests can be set-up using MVM, the validation tests performed in this example include accuracy, linearity, robustness, repeatability, limit of detection (LOD) and limit of quantitation (LOQ) determinations. Intermediate precision was also tested using a Method Validation Kit, to ensure reliability of the method across three different batches of the same column chemistry. The use of efficient sample set designs, along with automated data processing using Empower 2 with MVM, allowed these six validation tests to be performed and processed in one day with simple tracking of the validation study.

EXPERIMENTAL

ACQUITY UPLC H-Class Conditions

Mobile Phase:	A: acetonitrile
	D1: water with 0.1% formic acid (pH 2.5)
Columns:	ACQUITY UPLC CSH C_{18}, 2.1 x 50 mm, 1.7 µm, part number 186005296
Needle Wash:	10:90 water:methanol
Sample Purge:	90:10 water:methanol
Seal Wash:	90:10 water:methanol
Detection:	UV at 254 nm
Flow Rate:	0.8 mL/min
Injection Volume:	1 µL
Column Temp.:	30 °C
Gradient Time:	1 to 45% acetonitrile over 4.3 min., re-equilibrate at starting conditions
Data Management:	Empower 2 CDS with Method Validation Manager (MVM)

Sample Preparation

Ziprasidone peroxide degradation sample:

To 0.4 mg/mL ziprasidone in 50:50 water:methanol, add one equal volume of 3% hydrogen peroxide solution in water, heat at 80 °C for 30 minutes. Dilute to 0.1 mg/mL final concentration with water.

RESULTS AND DISCUSSION

The method for the ziprasidone peroxide degradation separation was previously developed using a Quality by Design (QbD) approach on an ACQUITY UPLC H-Class system running Empower 2 and Fusion AE Method Development Software.[2] Since all components eluted before 4 minutes in the Fusion developed method, the original 12-minute run time was shortened to 6 minutes by adjusting the gradient from 0 to 87.5% acetonitrile over 8.1 minutes (original method) to 1% to 45% acetonitrile over 4.3 minutes. The slope of the original gradient was kept constant to maintain the same separation selectivity in both methods. Standards for the impurities generated from the ziprasidone peroxide degradation were not readily available and therefore, a full impurities method validation was not performed. Instead, select validation tests were performed on the ziprasidone active pharmaceutical ingredient (API) and four impurities from the ziprasidone peroxide degradation separation are shown in Figure 1 to demonstrate the utility of Empower 2 with MVM.

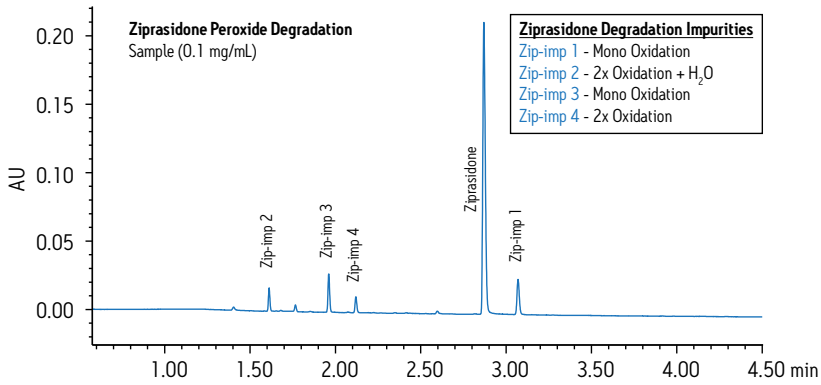

Figure 1. The ziprasidone peroxide degradation separation developed using Quality by Design (QbD).

Accuracy, Linearity and Repeatability, LOD/LOQ

Accuracy was evaluated using triplicate preparations at five levels (80, 90, 100, 110, 120%) of the target concentration of ziprasidone API (0.1 mg/mL). Linearity was assessed using the same range as the accuracy testing protocol. Repeatability was tested using six preparations of 100% target concentration of ziprasidone API. Ziprasidone impurities were not evaluated for these tests as appropriate standards were not readily available.

The acceptance criteria for accuracy, linearity and repeatability tests were specified when setting up the validation protocol in MVM. One sample set was written in Empower 2 to generate data for all three tests, enabling MVM to use the results from one sample set run to complete testing for accuracy, linearity and repeatability.

Based on the specifications defined in the validation protocol, the method was found to be accurate and linear within the range tested and the repeatability of the method was well within the established acceptance criteria (Table 1). Using the residual standard deviation from the linearity curve, LOD and LOQ were also calculated for ziprasidone API. The use of efficient sample sets and MVM allowed all of these tests to be completed in one sample set run, greatly minimizing sample preparation and instrument time. Automated data processing using Empower 2 with MVM allowed rapid determination of the status and results of each test, without the need to export data to a separate spreadsheet for manual analysis.

Test	Acceptance Criteria	Ziprasidone	Result
Accuracy	% Recovery 95- 105%	97.6-101.5	Pass
Linearity	R_2 > 0.99	0.993	Pass
	Residuals <2% RSD	1.6	
Repeatability	Ret Time <2% RSD	0.1	Pass
	% Area <2% RSD	1.1	Pass
LOD	-	0.004 mg/mL	
LOQ	-	0.013 mg/mL	

Table 1. Accuracy, linearity, repeatability, limit of detection (LOD), and limit of quantitation (LOQ) results for ziprasidone.

Robustness

Method robustness tests were configured in MVM using design of experiments (DoE). Based on the factors entered, MVM generates an efficient experimental design that will analyze multiple factors at a time, resulting in a significant time savings compared to a one-factor-at-a-time approach. The factors analyzed in this study included flow rate (0.7-0.9 mL/min), column temperature (30 to 60 °C) and injection volume (0.5-1.5 µL). Robustness testing was performed in eight runs using a full factorial experimental design.

Method robustness was evaluated for the ziprasidone API and the four ziprasidone degradation products based on peak retention time RSD and % area RSD (Table 2). The method is shown to be robust within the criteria defined in the validation protocol for all three factors evaluated. The use of experimental design and automated processing of multiple factors in MVM greatly facilitates robustness testing in validation studies.

Test	Acceptance Criteria	Ziprasidone	Zip-imp 1	Zip-imp 2	Zip-imp 3	Zip-imp 4	Result
Robustness	Ret Time <10% RSD	5.6	5.3	8.2	7.5	7.1	Pass
	% Area <2.5% RSD	1.2	0.7	2.1	1.6	1.5	

Table 2. Robustness results for ziprasidone and four impurities.

Intermediate Precision

The intermediate precision (or ruggedness) of the method was evaluated across column batches using a Method Validation Kit (MVK). MVKs consist of three different batches of the same column chemistry, hence maximizing the analytical variability that might be seen using different batches of columns. A comparison of the ziprasidone peroxide degradation separation on three different batches of ACQUITY UPLC CSH C_{18} columns in the kit is shown in Figure 2. MVKs provide easy accessibility to three different batches of the same column and their use in both method development and validation promotes analytical method ruggedness as columns are replaced over the lifetime of the method.

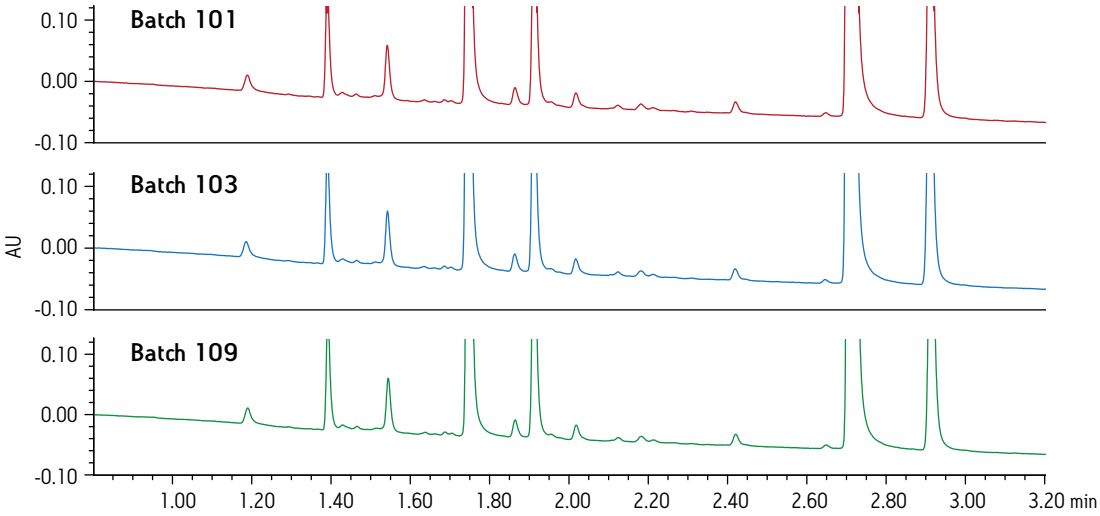

Figure 2. Zoomed-in comparison of the ziprasidone peroxide degradation separation on three different batches of CSH C_{18} using a Method Validation Kit (MVK).

Triplicate preparations of the ziprasidone peroxide degradation sample were analyzed on each of three batches of columns from the ACQUITY UPLC CSH C_{18} MVK. The intermediate precision criteria of retention time RSD and peak area RSD were evaluated for ziprasidone and each of the four impurities (Table 3). The results were found to be within defined acceptance criteria, demonstrating the ruggedness of the method across different batches of columns.

Test	Acceptance Criteria	Ziprasidone	Zip-imp 1	Zip-imp 2	Zip-imp 3	Zip-imp 4	Result
Intermediate Precision	Ret Time <2% RSD	1.1	1.1	1.1	0.9	0.8	Pass
	Peak Area <5% RSD	1.7	2.1	2.6	3.8	4.9	

Table 3. Intermediate precision results for ziprasidone and four impurities, testing three column batches using a Method Validation Kit.

CONCLUSIONS

- Software-based validation approaches such as Empower 2 with MVM use efficient sample sets and experimental designs, allowing validation testing to be performed much faster than setting up and running one experiment at a time. For the validation of the ziprasidone impurities separation, six validation tests were performed and results were processed all in one day.
- Intermediate precision evaluated using Method Validation Kits help ensure long-term ruggedness of the method across three different batches of columns, reducing the risk of reproducibility issues and downstream re-validation on a method developed using only one column or one batch of packing material.
- Method validation performed using Empower 2 with MVM requires no additional software to validate. MVM eliminates the need to transfer data to external spreadsheets for manual analysis, greatly reducing transcription error. All samples are run, processed and compiled in one location, facilitating data tracking, improving data security, and increasing audit confidence.

REFERENCES

1. Root, D.S., Aubin, A.J. *"Empower 2 Method Validation Manager Software: Tool for Rapid Method Validation,"* Waters Application Note [2007], Part Number 720002401EN.
2. Summers, M., Fountain, K.J. *"A Quality by Design (QbD) Based Method Development for the Determination of Impurities in a Peroxide Degraded Sample fo Ziprasidone*," Waters Application Note [2011], Part Number 720004072EN.

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September 2011 720004077EN LS-PDF

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Ensuring Data Quality by Benchmarking System Performance Using Waters Neutrals Quality Control Reference Material

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APPLICATION BENEFITS

- Reliably benchmark system performance using a strictly manufactured and certified reference standard
- Monitoring system performance can help prevent the collection of inaccurate data
- Detects system problems before they happen, potentially reducing instrument downtime

WATERS SOLUTIONS

Waters Neutrals QC Reference Material

ACQUITY UPLC® H-Class System

ACQUITY UPLC BEH C_{18} columns

Empower® 3 CDS

LCGC Certified Clear Qsert Vial

KEY WORDS

ACQUITY UPLC H-Class, Analytical Standards and Reagents, Neutrals QC Reference Material, ACQUITY UPLC BEH Columns

INTRODUCTION

Regardless of what industry a chromatographer works in, system performance and data reliability are of the utmost importance. If a system's performance starts to decline, the reliability and accuracy of the data could be in question. Furthermore, if a system's performance drops too much, system repairs might be in order, resulting in instrument downtime and reduction of productivity in the lab. By routinely monitoring system performance, however, a drop in performance can be observed earlier and corrective action can be taken, potentially reducing system downtime and preventing erroneous data from being collected. One way to monitor system performance is to benchmark the system using a system suitability standard, and then compare subsequent runs of the standard to the benchmarked data to ensure that the system is performing reliably.

Waters Neutrals Quality Control Reference Material (QCRM) is a mixture of three neutral compounds that are an ideal system reference standard. The use of neutral compounds allow the QCRM to be unaffected by mobile phase pH, making it compatible with buffered and non-buffered mobile phases at both high and low pH. Thus, the standard can be analyzed on many different HPLC and UHPLC systems, with different column chemistries, and different mobile phases. The highly controlled manufacturing process of the standard ensures a high quality and reliable standard that can be counted on to produce consistent results over time. This application note focuses on how the standard can be used to benchmark and monitor system performance over the life of the system. By using the reference standard to benchmark system performance, data integrity can be monitored and assured.

EXPERIMENTAL

ACQUITY UPLC H-Class Conditions

Mobile phase:	50:50 acetonitrile:water
Separation mode:	Isocratic
Detection:	UV 254 nm
Column:	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm 1.7 µm
Column temp.:	30 °C
Needle wash:	50:50 ACN:water
Sample purge:	50:50 ACN:water
Seal wash:	50:50 MeOH:water
Flow rate:	0.6 mL/min
Injection volume:	1 µL
Data management:	Empower 3 CDS
Sample preparation:	A vial of Neutrals QC Reference Material (PN: 186006360) was opened and transferred into an LCGC Certified Clear Qsert Vial (PN: 186001126C) for injection.

RESULTS AND DISCUSSION

System performance and data reliability are something that every chromatographer should be conscious of. A system should be monitored regularly to ensure that it is continually performing at an optimum level to generate quality data. The easiest way to evaluate system performance is to routinely use a QCRM standard to benchmark the system when it is performing optimally. At later dates, subsequent injections of standard can be compared to the original data to ensure that the system is still performing well. Waters Neutrals QC Reference Material (NQCRM) is a mixture of three neutral compounds: acetone, naphthalene, and acenaphthene. The separation of these compounds is achieved under common mobile phase conditions with sufficient organic content. Figure 1 shows the isocratic separation of the Neutrals QCRM on an ACQUITY UPLC BEH C_{18} 2.1 x 50 mm 1.7 µm Column with 50% acetonitrile in water as the mobile phase.

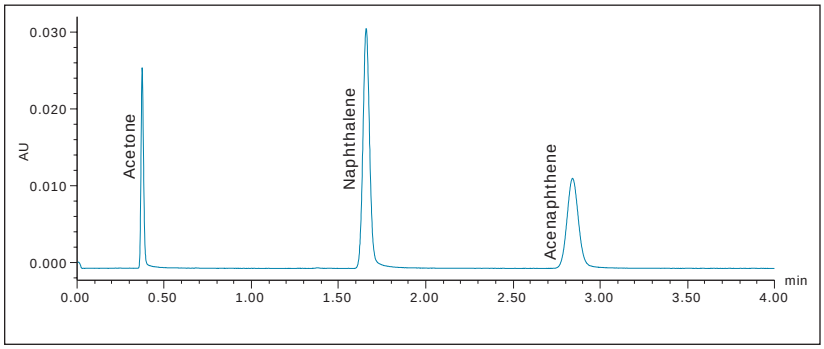

Figure 1. Sample chromatogram of the Waters Neutrals QCRM separation on an ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm 1.7 µm Column.

In this application, the standard was used to benchmark the system performance of an ACQUITY UPLC H-Class System equipped with a PDA detector for a period of five days (120 h). Prior to beginning the experiment, the system was calibrated and performance maintenance was performed to ensure proper operation of the system. Benchmarking a system that is not performing optimally could lead to irregular and unreliable benchmarking results. The Neutrals QCRM was injected in triplicate onto an ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm Column three times a day for five days. The first set of injections was performed in the morning, the second at mid-day, and the third in the late afternoon to simulate the standard being run before, during and after an eight-hour shift. A total of 45 injections were performed over five days (120 h). Retention time, USP tailing factor, and system pressure were monitored. These parameters were monitored since they are typically parameters that could indicate a serious system problem. If, for instance, the retention time of the peaks changed significantly, it could indicate a pump issue or an error in mobile phase preparation; while an increase in USP Tailing Factor could indicate a failing column or that the column outlet fitting is not seated properly.[1]

	Average Retention Time (min)	%RSD Retention Time
Acetone	0.323	0.69
Naphthalene	1.633	0.44
Acenaphthene	2.893	0.44

Table 1. System performance benchmarking data using the Waters Neutrals QCRM, showing highly reproducible retention times demonstrated by low %RSD (n=45) over five days (120 h).

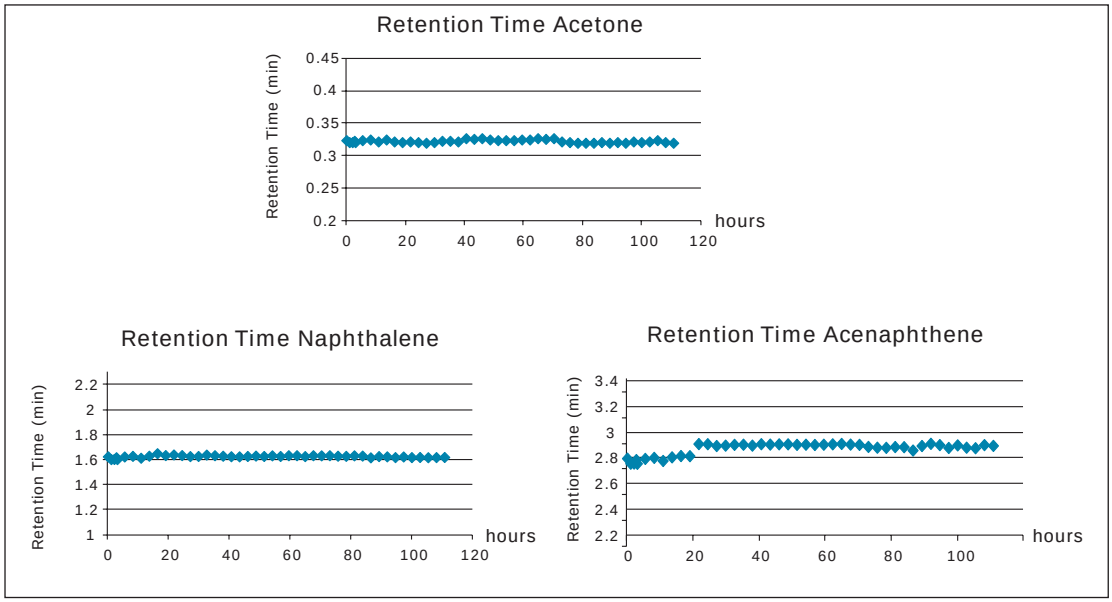

Figure 2. Retention time trending data for the Neutrals QCRM over five days (120 h).

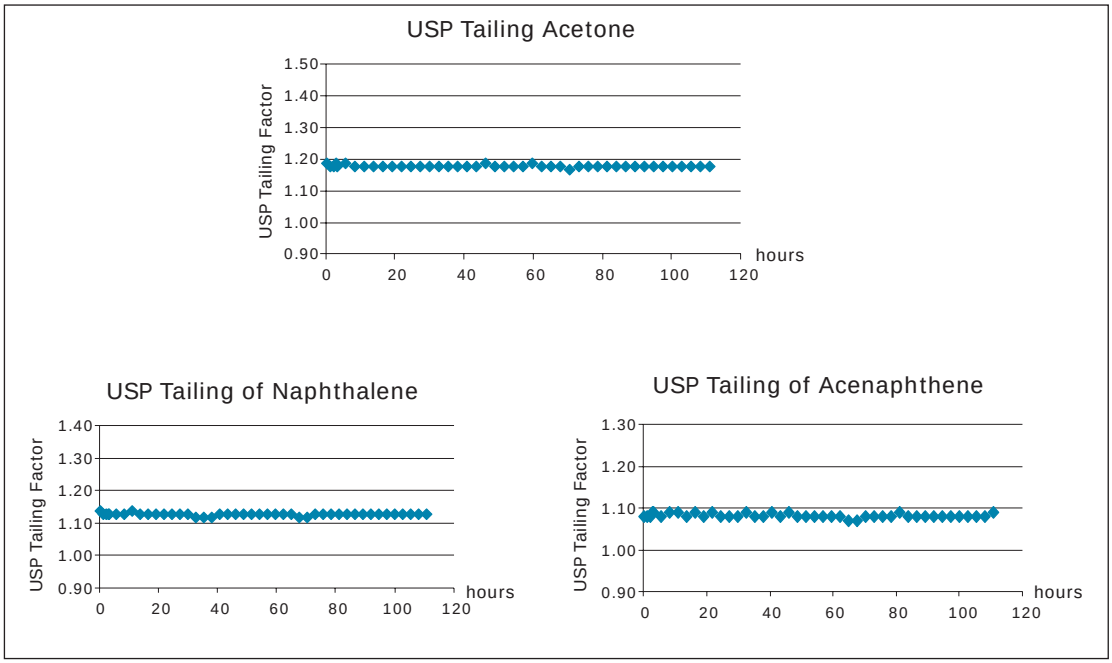

Figure 3. USP tailing factor trending data for the Neutrals QCRM over five days (120 h).

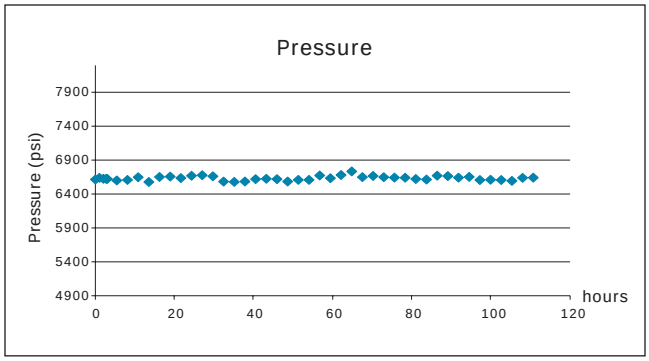

Figure 4. System pressure trend plot of the Neutrals QCRM indicating consistent system pressure over time.

As the trending data shows, the Neutrals QCRM is a valuable tool for benchmarking a system's performance. The data shows the high reproducibility of the system over time, with a retention time %RSD < 0.7 for all three peaks in the Neutrals QCRM standard, as shown in Table 1. The trending data for the USP tailing factor shows very little deviation over the course of the analysis, indicating that the peaks are not changing over time. The system pressure trending data shows very little variation as well, displaying a stable pressure over the course of the experiment. In this experiment, the monitoring of retention time, USP tailing factor, and system pressure was important, since any change in these parameters could indicate a system or column problem, and potential collection of erroneous data for experiments run on the system over these five days.

In addition to monitoring system performance, this data 'benchmarks' a starting point that future injections of the Neutrals QCRM can be compared to. After gathering the benchmark data, a set of specifications can be created to monitor the system.[2] After these specifications are created, the QCRM can be run periodically and checked against these specifications to determine if the system is still operating optimally. If the Neutrals QCRM falls out of specification, the system may need to be repaired. After these repairs are completed, the Neutrals QCRM can be run again and the data can be compared to the specifications to see if the system is working properly.[1]

CONCLUSIONS

Monitoring system performance is an important aspect of liquid chromatography that should be performed routinely to ensure the highest quality data generated. Routinely monitoring a system with a well-characterized and controlled standard can lead to early detection of system problems, potentially reducing system downtime. Using Waters Neutrals QC Reference Material (QCRM) is an ideal way to benchmark an optimally functioning system. Waters Neutrals QCRM is a mixture of three neutral compounds that can be separated using a wide variety of column chemistries and mobile phases, making it compatible with most methods and laboratory practices. Once the system has been benchmarked, the Neutrals QCRM can be run on a regular basis and compared to a set of specifications that the operator creates to ensure that the system is still working optimally. If the system is outside of specification, corrective action can be performed before erroneous data is collected or the system fails. Using the Neutrals QCRM to benchmark system performance can lead to reduced system downtime and the efficient acquisition of reliable data, saving considerable time and resources in the laboratory.

References:

1. Berthelette KD, Summers M, Fountain KJ. Troubleshooting Common System Problems using Waters Neutrals QC Reference Material. Waters Application Note
2. Quality Control Reference Material and Benchmarking Instrument Performance. Waters White Paper.

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Implementation of Methods Translation between Liquid Chromatography Instrumentation

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APPLICATION BENEFITS

- Future proof your laboratory
- "Equivalent" vs. "Equal" column selectivity
- Increase productivity while decreasing costs
- Maximize asset utilization
- Understand the importance of L/dp
- Discover software tools to facilitate method translation

WATERS SOLUTIONS

ACQUITY UPLC® and
ACQUITY UPLC H-Class

Waters Method Transfer Kits

ACQUITY UPLC Columns Calculator

Reversed Phase Selectivity Chart

KEY WORDS

Method Transfer, Compendial Methods, USP, Method Translation, UPLC, HPLC

INTRODUCTION

Pharmaceutical research and development (R&D) organizations were early adopters who recognized the many benefits of UltraPerformance LC® (UPLC®) Technology including resolution, sensitivity, throughput, and productivity as compared to HPLC. Today, the number of projects involving new drug entities are increasingly performed utilizing UPLC.

Adopting UPLC for R&D activities is less complex than for laboratories involved with routine analysis, where its use requires consideration about the need to re-file methods for existing products. Routine analysis areas such as Quality Control (QC) laboratories own a vast supply and variety of HPLC instrumentation. Asset procurement regarding new technologies within these groups often requires convincing financial as well as scientific justification.

Although information illustrating UPLC's return on investment (ROI) for solvent consumption and analysis per unit time can be convincing for R&D, the QC environment requires key practical-use considerations. Managers and end users within QC laboratories require new instrumentation to provide dual purposes: first, the ability to perform both legacy methods and, second, the ability to use sub-2-µm particle columns and methodology in a routine analytical environment without complications. UPLC's adoption must also strategically provide seamless integration within current laboratory practices and decrease learning curves of the end users.

In this application, various U.S. Pharmacopeia (USP) compendial methods are used as examples to highlight a new method translation strategy to facilitate the transfer of methods to and from any LC-based instrument with ease.

EXPERIMENTAL

United States Pharmacopeia reference standards

- USP Monograph Galantamine Hydrobromide
  - USP Galantamine Hydrobromide RS and USP Galantamine Hydrobromide Related Compounds Mixture RS
- USP Dietary Supplement: Powdered Soy Isoflavone Extract Method
  - USP Apigenin RS, USP Diadzein RS, USP Diadzin RS, USP Genistein RS, USP Genistin RS, USP Glycitein RS, USP Glycitin RS, and USP Defatted Powdered Soy RS
- USP Monograph Loratadine
  - USP Loratadine RS, USP Loratadine Related Compound A RS, and USP Loratadine Compound B RS, Claritin

METHOD CONDITIONS

LC conditions

References to LC conditions are addressed as per USP Monographs, whereas specific utilization of LC instrumentation for each application is discussed in the figure captions.

Data management

Empower™ 2 CDS

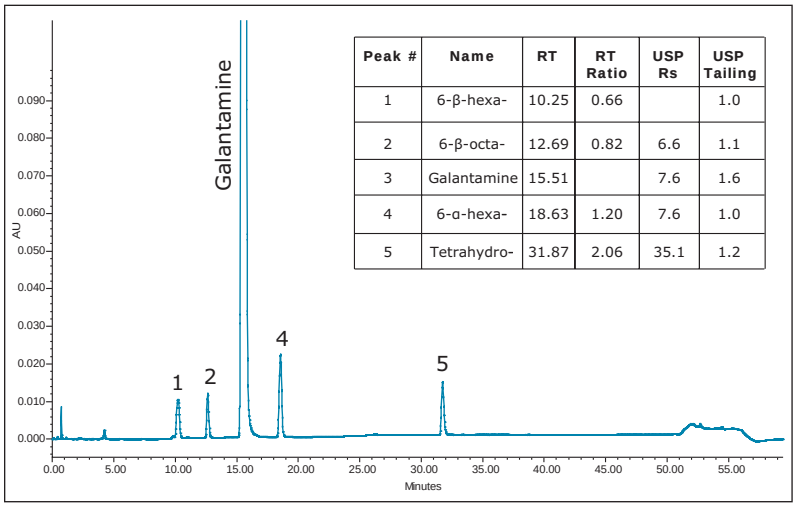
RESULTS AND DISCUSSION

Successful method translation requires understanding three key chromatographic attributes before implementation. The analyst must consider the differences between LC instrumentation, column selectivity, and the resolving capability of the original methodology versus the target methodology. By understanding these three essential aspects of method translation, the benefits of increasing productivity and decreasing costs while maximizing asset utilization of present and future instrumentation can be realized.

Future-proofing your laboratory: Translating HPLC methodology between LC instrumentation

The QC laboratory frequently utilizes a variety of LC instruments for API and drug product analysis. Therefore, instrumentation flexibility is essential. Direct transfer of methods to newer technology may result in retention time and selectivity differences that may be related to decreases in instrument dwell volume.

To illustrate the flexibility provided by the Waters ACQUITY UPLC H-Class System, the USP method for galantamine hydrobromide and related substances was performed on an HPLC instrument (Figure 1). USP system suitability requirements for the related substances assay specify USP tailing of galantamine NMT 2.0 and a resolution of galantamine and 6-alphagalantamine NLT 4.5. When utilizing the same HPLC column on each instrument, the ACQUITY UPLC Columns Calculator (Figure 2) can be used to calculate the differences in the instrument dwell volume. The resulting data yielded no compromise in chromatographic integrity during the translation of the method for use on a UPLC instrument of less dwell volume (Figure 3).



Peak #	Name	RT	RT Ratio	USP Rs	USP Tailing
1	6-β-hexa-	10.25	0.66		1.0
2	6-β-octa-	12.69	0.82	6.6	1.1
3	Galantamine	15.51		7.6	1.6
4	6-α-hexa-	18.63	1.20	7.6	1.0
5	Tetrahydro-	31.87	2.06	35.1	1.2

Figure 1. USP Method for galantamine and related substances performed on an Alliance® HPLC 2695 with measured dwell volume of 1.1 mL. An XBridge™ C18 (L1) column with dimensions 4.6 x 100 mm, 3.5 µm was used.

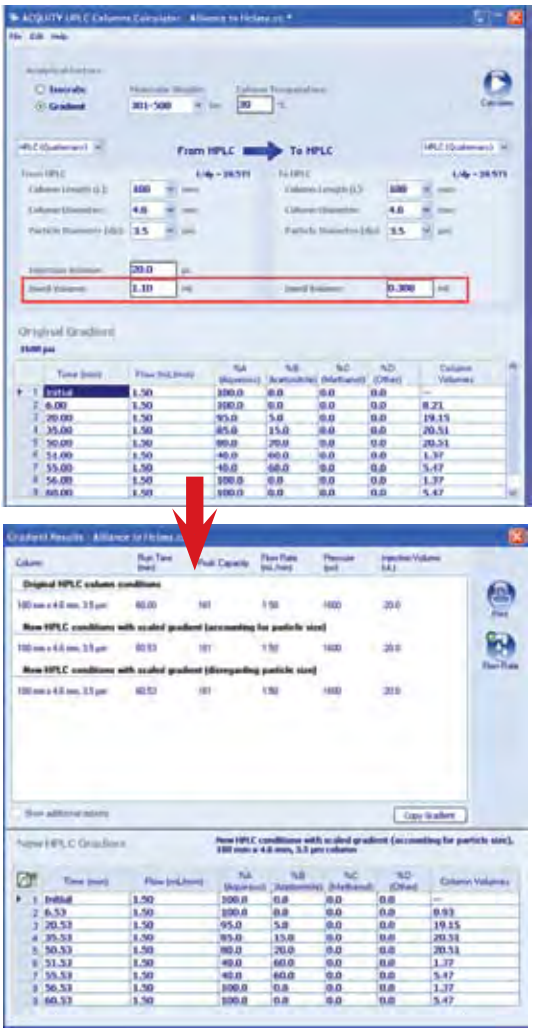
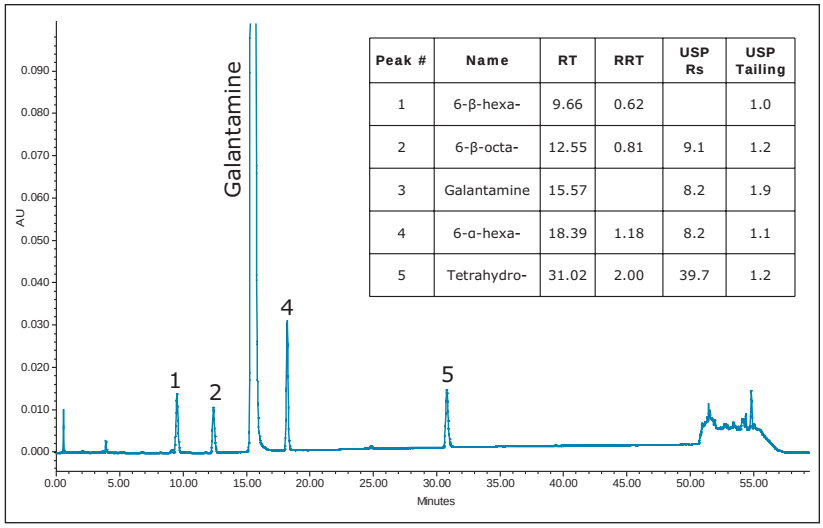


Figure 2. Example using the ACQUITY UPLC Columns Calculator for HPLC methodology translation, compensating for differences in dwell volume between two different LC system configurations. In red, the dwell volumes of the original and target instrumentation are entered. Once calculated, the gradient table is adjusted to compensate for the instrument differences. The same HPLC column was used for the new HPLC gradient performed on an ACQUITY UPLC H-Class System.



Peak #	Name	RT	RRT	USP Rs	USP Tailing
1	6-β-hexa-	9.66	0.62		1.0
2	6-β-octa-	12.55	0.81	9.1	1.2
3	Galantamine	15.57		8.2	1.9
4	6-α-hexa-	18.39	1.18	8.2	1.1
5	Tetrahydro-	31.02	2.00	39.7	1.2

Figure 3. USP method for galantamine related substances performed on the ACQUITY UPLC H-Class with a dwell volume of 280 µL. The relative retention times (RRT), USP Rs, and USP tailing compare to those reported in Figure 1. It should be noted that the decreased extra-column volume of ACQUITY UPLC family of instruments will sharpen the chromatographic peaks, hence slight increases in resolution can result in minimal variations of the calculated RRT when compared to the chromatography of the originating instrumentation.

Maintaining selectivity

Differences between instrumentation dwell volumes can be easily accounted for with calculated adjustments to the gradient table with the ACQUITY UPLC Columns Calculator. However, the challenging method translations of original methodology to a target methodology reside with differences in column stationary phase selectivity.

Ideally, when scaling from an HPLC column to a UPLC column, the stationary phase should remain constant (i.e., "equal" stationary phase) to maintain the selectivity of the separation. Unfortunately, many original HPLC columns are not available in the same chemistry in sub-2-µm particle sizes. Therefore, an equivalent stationary phase that is available in sub-2-µm particle size must be determined. This task is facilitated using the Waters Reversed Phase Column Selectivity Chart (www.waters.com/selectivitychart) (Figure 4).

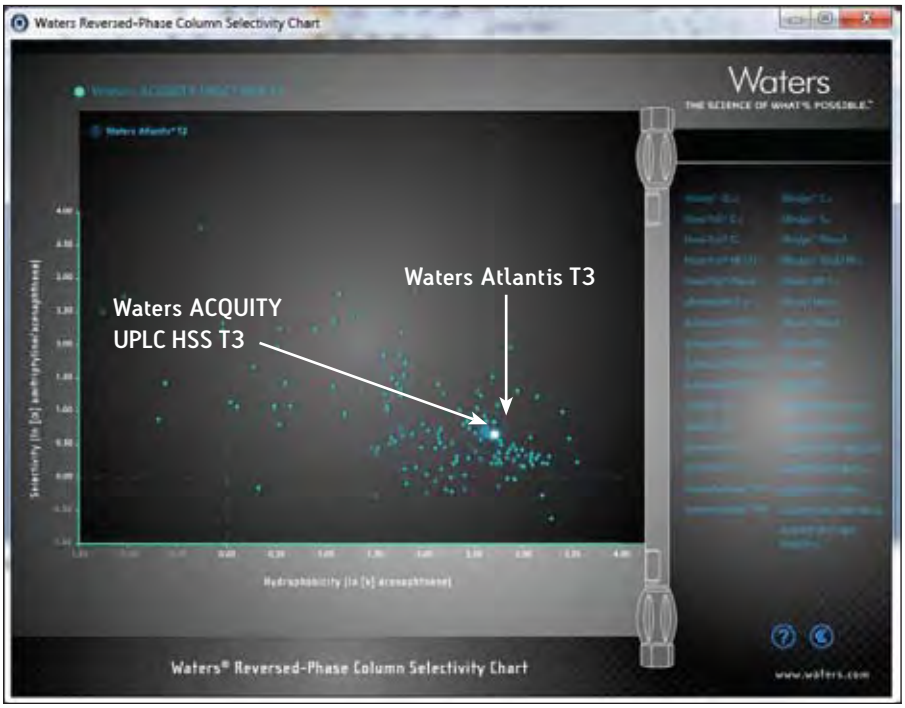


Figure 4. In this example, the original HPLC column used is an Atlantis® T3 column chemistry, however a sub-2-µm particle size utilizing this stationary phase is not available. Using the Reversed Phase Column Selectivity Chart, the ACQUITY UPLC HSS T3 column chemistry was identified as an equivalent sub-2-µm particle size column of similar selectivity.

Increasing productivity while decreasing costs: Translation between HPLC and UPLC methodology

New pressures in the pharmaceutical industry have created a need for QC laboratories to become more productive. It is important to reduce costs in QC, but not at the expense of R&D or any other part of the organization. Additionally, the reduction in cost cannot come at the expense of chromatographic accuracy, robustness, or reliability.

Dietary supplement manufacturers routinely use HPLC to analyze soy extracts for isoflavone content. The current USP compendial method uses a long, shallow gradient that takes 75 minutes per injection. This long run time limits the ability of manufactures to release products quickly. In addition, a sample set run consisting of a blank, five calibration standards, and two retention time check solutions requires more than 10 hours before running the analysis of the first sample. The benefits of analyzing isoflavones using a faster solution that maintains data quality are improved productivity, increased revenues, enhanced efficiency, faster sample turnover, and reduced labor and training costs.

Using the ACQUITY UPLC Columns Calculator, the HPLC USP method for soy isoflavones shown in Figure 5 was transferred to a UPLC method employing an ACQUITY UPLC HSS T3 Column, 2.1 x 100 mm, 1.8 µm. The geometrically scaled method had a runtime of 24.3 minutes. Because the scaled flow rate of 0.319 mL/min is below the optimum linear velocity for the sub-2-µm particle column, the columns calculator was used to recalculate the gradient at 0.60 mL/min, a flow rate that is closer to optimum (Figure 6).

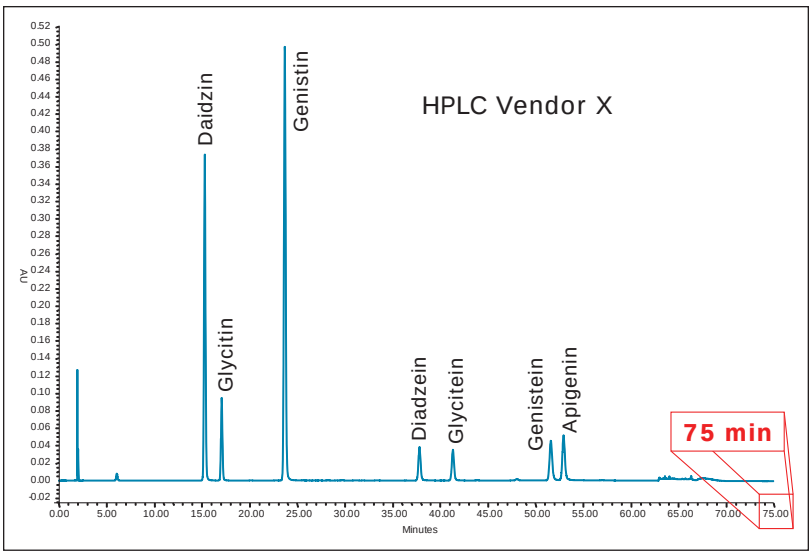


Figure 5. HPLC chromatogram using isoflavone USP methodology. Instrument system volume measured 1.3 mL. USP system suitability criteria were met. R2 for all compounds across five working standards; concentrations 0.999, Daidzin tailing = 1.1, and Genistin %RSD = 0.6.

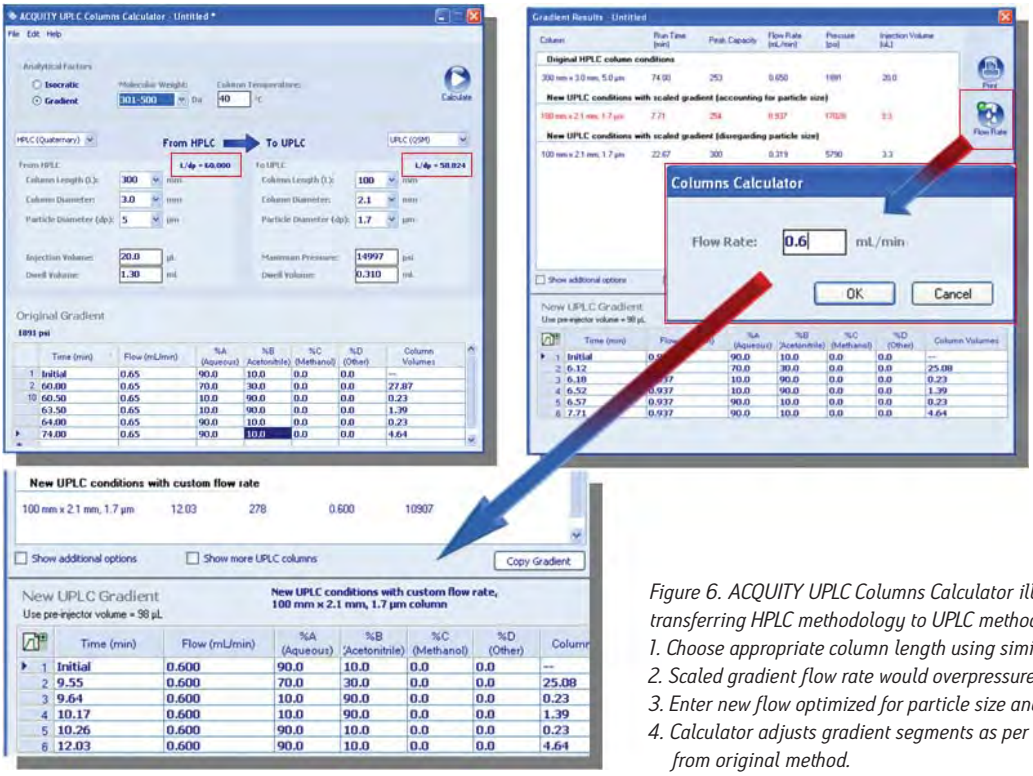


Figure 6. ACQUITY UPLC Columns Calculator illustrating the ease of transferring HPLC methodology to UPLC methodology.
1. Choose appropriate column length using similar L/dp value
2. Scaled gradient flow rate would overpressure as indicated in red.
3. Enter new flow optimized for particle size and system pressure limits.
4. Calculator adjusts gradient segments as per correct column volumes from original method.

Using the columns calculator, the resolving capabilities of the HPLC column was maintained by choosing a UPLC column dimension with the same column length to particle size (L/dp) ratio. The injection volumes and flow rate were scaled appropriately, and the gradient was corrected to keep the number of column volumes consistent for each time segment. The resulting chromatogram is displayed in Figure 7. We can see that the analysis time has been reduced to 16 minutes. Using this approach, the method was successfully transferred to UPLC with both improved throughput and assay performance. The quality of the analytical results using this new and significantly faster UPLC method were not compromised, and thus met the specified USP criteria.

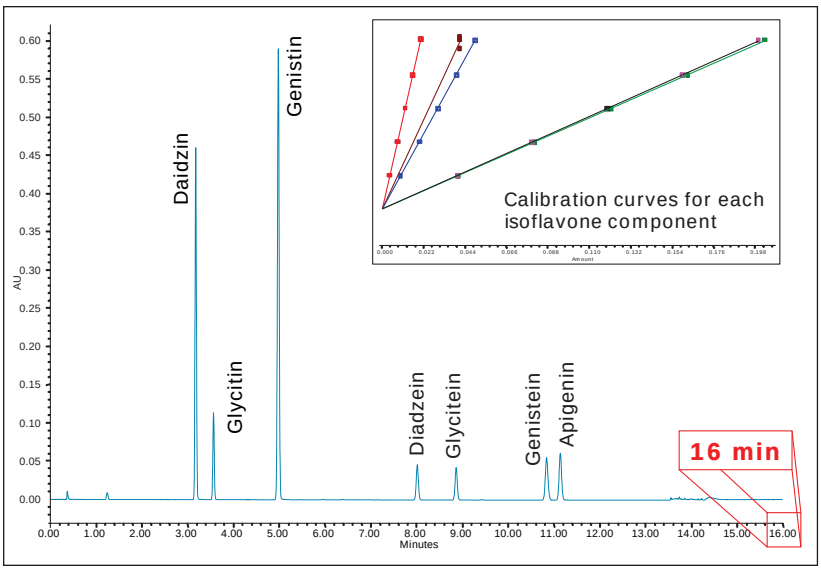


Figure 7. UPLC separation of isoflavones. ACQUITY UPLC instrument system volume measured 82 µL. USP system suitability criteria were met. R2 for all compounds across five working standards; concentrations > 0.999, Daidzin tailing = 0.99 and Genistin %RSD = 0.12

Maximizing asset utilization: Translating UPLC methodology to HPLC methodology

Analytical development organizations have decreased their method development time by implementing UPLC, however their customers in many situations across the globe have not yet implemented UPLC technology. Maximizing the utilization of the current instrumentation is key to their productivity until appropriate justifications and budgeting is available to adopt the new technology.

In such cases, the method innovator must adapt the UPLC methodology for HPLC use. Implementing the method translation strategy combining the ACQUITY UPLC Columns Calculator and the appropriate column Method Transfer Kit can facilitate the translation of UPLC methodology to HPLC methodology.

A UPLC method developed for loratadine and its related substances separated nine impurities and the API to meet a set of system suitability criteria as specified in the USP within 10 minutes (Figure 8). The methodology was translated utilizing the ACQUITY UPLC Columns Calculator and ACQUITY UPLC BEH Method Transfer Kit. The key aspects in allowing the transferability to HPLC from UPLC are similar to those stated in the previous example, such that the target column dimensions must have equivalent L/dp values and the column stationary phase selection is equivalent to the originating methodology. The resulting HPLC chromatogram (Figure 9) was compared to the UPLC chromatogram in terms of relative retention time ratios of the related substances.

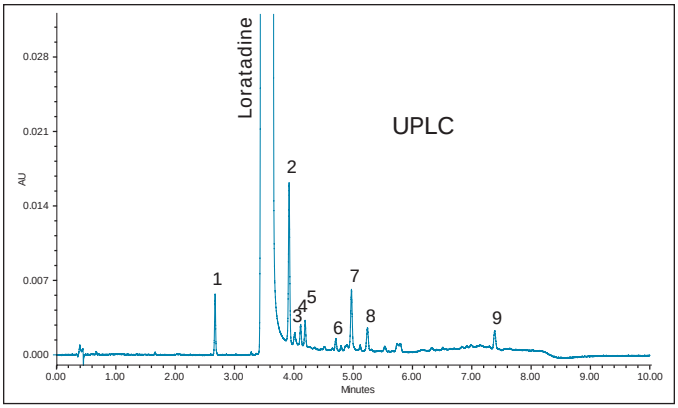


Figure 8. Example chromatogram of the ACQUITY UPLC method for loratadine related substances assay.

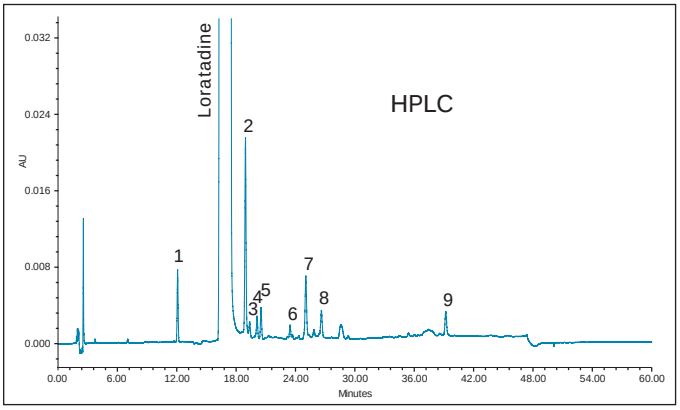


Figure 9. Example chromatogram showing the method translation strategy successfully converting the original UPLC methodology to the HPLC methodology for loratadine related substances analysis.

In addition to the UPLC to HPLC transfer of loratadine, the UPLC and HPLC methods were compared on three different instruments (Alliance HPLC 2695, ACQUITY UPLC, and ACQUITY UPLC H-Class) in order to evaluate the accuracy of the entire method transfer process (Table 1).

Peak	Relative retention time ratios			
	ACQUITY UPLC → (UPLC)	Alliance 2695 → (HPLC)	ACQUITY UPLC H-Class → (HPLC)	ACQUITY UPLC H-Class → (UPLC)
Imp. 1	0.71	0.72	0.70	0.74
Loratadine	-	-	-	-
Imp. 2	1.08	1.12	1.09	1.09
Imp. 3	1.11	1.15	1.12	1.11
Imp. 4	1.14	1.19	1.16	1.14
Imp. 5	1.16	1.22	1.18	1.16
Imp. 6	1.32	1.39	1.35	1.30
Imp. 7	1.41	1.49	1.44	1.36
Imp. 8	1.49	1.58	1.53	1.45
Imp. 9	2.16	2.32	2.24	2.05

Table 1. Relative retention time ratio comparisons of the loratadine related substances using HPLC and UPLC instrumentation, the ACQUITY UPLC Columns Calculator and the method transfer kit for XBridge C_{18}.

DISCUSSION

The compendia methods translation experiments were facilitated using a method translation strategy comprised of software tools, column Method Transfer Kits, and thorough knowledge of the instrumentation used. In each example, the chromatographic attributes and integrity of the original methodology were maintained.

Choosing a compatible column stationary phase exhibiting "equivalent" or "equal" selectivity and resolution characteristics was key when transferring from legacy HPLC methodology to UPLC methodology. The process of translating from HPLC to UPLC can be difficult due to the availability of a sub-2-µm particle size equivalent columns with the same originating HPLC stationary phase, especially if the originating HPLC stationary phase was introduced many years prior. The reversed-phase selectivity chart can facilitate proper stationary phase selection in many of these instances, however, some selectivity differences may be observed.

The process of translating methodology from UPLC to HPLC is made easier with columns that are available in both UPLC and HPLC particle sizes, as in the case of ACQUITY UPLC BEH and XBridge, ACQUITY UPLC CSH, and XSelect™ CSH, and HSS UPLC and HPLC columns.

In an effort to streamline method translation, QC organizations should open communications with R&D organizations presently implementing UPLC for methods development. Discussions should focus on the intricacies of maintaining column selectivity for UPLC and HPLC, as well as the importance of L/dp values for maintaining resolving capabilities of a column. These discussions would help devise a cohesive implementation strategy that can supplement the method translation strategic approach earlier within development.

CONCLUSIONS

- Successful methods translation is achievable with a strategy comprised of software tools, Method Transfer Kits, and an understanding of the basic characteristics of the instrumentation involved.
- Three USP compendial methods were successfully transferred to various LC configurations without compromising the integrity of the originating method.
- Techniques were demonstrated to maximize global asset utilization and maintain lab productivity.
- Methods were successfully translated to take benefit of sub-2-µm stationary phases.
- Software tools are available to facilitate the scaling and column selection
  - The ACQUITY UPLC Columns Calculator accounts for differences within system dwell volumes. Flow rates and injection volumes are scaled while compensating for appropriate column volumes per gradient time segment.
  - The Reversed Phase Column Selectivity Chart facilitates the selection of equivalent column selectivity when an equal selectivity column is unavailable.

References

1. Galatamine Hydrobromide: USP32-NF27 Supplement:No.2, page 4245.
2. Powdered Soy Isoflavones Extract: USP32-NF27, page 1074.
3. Loratadine: USP32-NF27, page 2805.

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September 2010 720003721EN LL-PDF

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PROTOCOL FOR ROUTINE CLEANING OF THE ACQUITY UPLC SYSTEM

Follow the steps in this procedure to clean the ACQUITY UPLC System when:

- increasing noise and/or baseline drift is seen in UV-based systems
- loss of sensitivity and/or poor signal-to-noise ratio is seen in MS-based systems
- low energy is observed when running ACQUITY UPLC console lamp energy diagnostics (refer to ACQUITY UPLC Console Online Help)
- distorted peaks and/or high injection counts observed in applications using complex biological sample matrices
- it is necessary to restore the system flow path to a near-new level of cleanliness

NOTE: For information on other ACQUITY UPLC cleaning methods, refer to the "Recommended Cleaning Mixtures for LC" table in the "Controlling Contamination in UltraPerformance LC®/MS and HPLC/MS Systems" document, P/N 715001307.

To Clean the System:

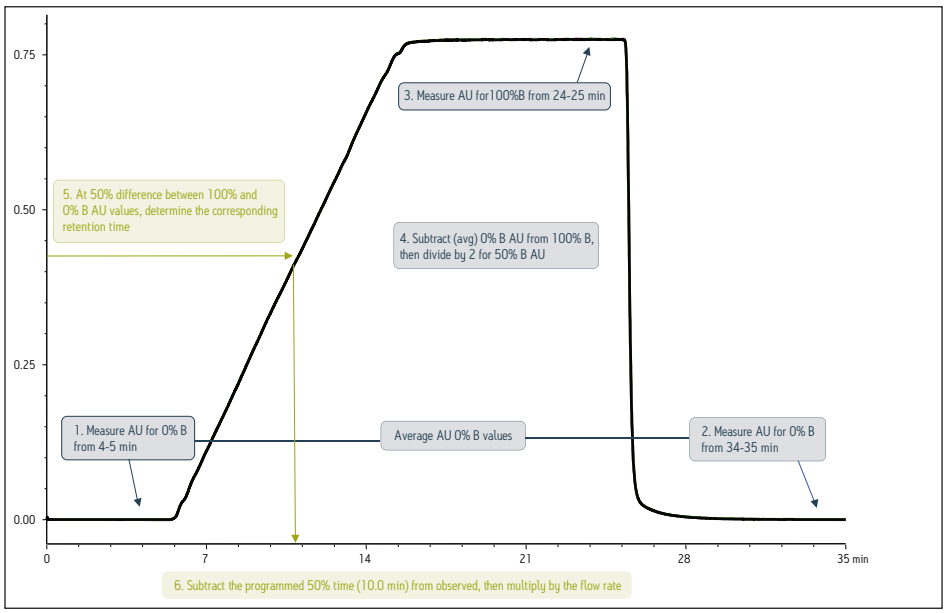
1. Remove all solvent filters.
2. Disconnect the column and attach a union (P/N 700002636) or flow restrictor (P/N 205000547) to the column inlet and outlet lines.
3. Place A1, A2, B1, B2, seal wash, weak needle wash, strong needle wash, and seal wash lines into a clean bottle containing **100% isopropanol.**

   NOTE: If there is an incompatibility between the mobile phase or solvents with the Isopropanol, flush first with the appropriate intermediate solutions to ensure compatibility.
4. Prime solvent lines A1, A2, B1, and B2 for **5 min** each.
5. Prime the seal wash.
6. Prime the wash and sample syringes for **5** cycles.
7. Purge the system at **0.2 mL/min** for **5.0 min** using **50% A1** and **50% B1**.
8. Repeat step 7 using **50% A2 and 50% B2**.
9. Perform **15 full loop** injections using **5X overfill** from a vial containing the cleaning solvent. **Run Time = 0.5 min / Flow Rate = 0.2 mL/min**.
10. Repeat step 3 through step 9 using **100% methanol**, except use a flow rate of 1**.0 mL/min** for step 7 through step 9.
11. Repeat step 3 through step 9 using **MilliQ water** (or equivalent), except use a flow rate of **1.0 mL/min** for step 7 through step 9. **Caution: If an MS detector is connected, take it off line before performing step 12. Direct the flow from the union or restrictor outlet to waste. If applicable, you can leave a TUV or PDA detector on line, however you may need to replace the back pressure device with large bore waste tubing to keep pressure lower than 1000 psi.**
12. Repeat step 3 through step 9 using **30% aqueous phosphoric acid**, except use a flow rate of **1.0 mL/min** for step 7 through step 9.

    Caution: To avoid damage, do not place the seal wash line in this solution. Place the line in Milliq water (or equivalent) through step 14.

13. Repeat step 3 through step 9 using **MilliQ water** (or equivalent), except use a flow rate of **1.0 mL/min** for step 7 through step 9. Flush until the pH is neutral (pH=7) before proceeding.
14. If applicable, reconnect the MS detector.
15. Repeat step 3 through step 9 using **100% methanol**, except use a flow rate of **1.0 mL/min** for step 7 through step 9.

PROTOCOL FOR GRADIENT DELAY (DWELL VOLUME) MEASUREMENT



System Volume Measurements

Line A: acetonitrile
Line B: acetonitrile with 7.5mg/L propylparaben
Needle Wash and Purge Solvents: 50:50 acetonitrile:water
Detection: UV at 254 nm
Sampling Rate: 5 Hz
Time Constant: no filter
Injection of Acetonitrile: 1 µL
50 µm ID fused capillary flow restrictor with 2 low dead volume unions installed in place of column.

Gradient Table:

Time (min)	Flow (mL/min)	% A	% B	% C	% D	Curve
Initial	0.75	100	0	0	0	*
5.0	0.75	100	0	0	0	6
15.0	0.75	0	100	0	0	6
25.0	0.75	0	100	0	0	1
35.0	0.75	100	0	0	0	1

Calculating System Volume Example

0% B Average = (-0.00018)+(0.00001)/2 = -0.00009 AU
Observed 100% B = 0.77479 AU
Adjusted 100% B = 0.77479 – (-0.00009) = 0.77488 AU
Absorbance at 50% = 0.77488 x 0.5 = 0.38744 AU
50% B Delivered at 10.527 min
System Volume: 10.527 – 10.00 min = 0.527 min
Flow Rate 0.75 mL/min: System Volume = 0.527 min x 0.75 mL/min = 395 µL

ADDITIONAL TOOLS FOR METHOD DEVELOPMENT

Waters Reversed-Phase Column Selectivity Chart

The Waters Selectivity Chart can be used to examine selectivity differences of comparative columns with different stationary-phase properties. Selection of the proper column in method transfer process is crucial to obtaining an optimal separation.

www.waters.com/selectivitychart

Waters ACQUITY UPLC Columns Calculator

The Console Calculator will geometrically scale an original HPLC method to a new UPLC method. The calculator allows the user to input the current HPLC conditions, including column length, mobile phase composition, and flow rate. The calculator takes into account changes in the gradient times, injection volume, and flow rate. When calculating the flow rate, it takes into account changes in column diameter and particle size. The calculator then generates three results: the conditions of Equal Efficiency; the conditions of Maximum Efficiency; and the conditions of Shortest Analysis Time. The ACQUITY UPLC Columns Calculator can be downloaded from the ACQUITY UPLC Online Community.

www.waters.com/myuplc

Waters Analytical Columns and Standards Wall Chart

Waters' state-of-the-art facilities manufacture HPLC and UPLC T echnology columns that maximize laboraotry performance. We are a primary manufacturer of silica and hybrid (BEH Technology) particles and are able to continually monitor and control the complete manufacturing process over the lifetime of a product.

Waters Wall Chart; 2014. 720002241en.

Waters Method Transfer Kits

Method Transfer Kits are designed to preserve the integrity of a separation as it is transferred between UPLC and HPLC platforms. Based on the concept of maintaining column length (L) to particle size (dp) ratio (L/dp), these kits provide an ACQUITY UPLC Column with an HPLC column of equivalent selectivity and resolving power. Using the ACQUITY UPLC Columns Calculator methods can be fully transferred from HPLC to UPLC or from UPLC to HPLC.

Waters Method Development Kits

With a seemingly endless number of method parameters to try, developing a new chromatographic method can be an overwhelming and time-consuming experience. Waters Method Development Kits consist of several UPLC Columns, encompassing a broad range in selectivity to accommodate your method development approach and enable more efficient and effective method development.

Waters Method Validation Kits

With exceptional batch-to-batch and column-to-column reproducibility, Waters well-established particle and column manufacturing process control provides confidence in the long-term reliability of your analytical method. ACQUITY UPLC Method Validation Kits include three batches of chromatographic media (derived from different base particles) to judge the quality, reliability, and consistency of your chromatographic method.

ADDITIONAL READING

Transferring Compendial HPLC Methods to UPLC Technology

The goal of this application notebook is to educate the reader on how to properly transfer HPLC methods found in the USP-NF (and other Pharmacopeias) to UPLC Technology in order to realize the full benefits of higher throughput, lower costs, and faster time-to-market for routine analysis of generic drugs.

Waters Application Notebook; 2013. 720004313en.

Advances in Chromatgraphic Method Development with UPLC and QbD

From development to manufacturing, the integration of UPLC Technology and QbD analytical approaches provides many benfits for pharmaceutical laboratories. Learn how UPLC combines with Fusion Method Development Software and Empower Software to facilitate the rapid development of robust QbD methods. The result is so much more than an excellent analytical method.

Waters Application Notebook; 2010. 720003705en.

Waters ACQUITY UPLC H-Class Method Development System

- Flow-Through-Needle (FTN) Sample Manager
- Quaternary Solvent Manager
- Solvent Select Valve (SSV)
- Auto•Blend Plus
- Column Manager and Column Manager-Aux
- PDA Detector
- ACQUITY QDa Detector

ACQUITY UPLC Columns

- BEH (Ethylene Bridged Hybrid)
- CSH (Charged Surface Hybrid)
- HSS (High Strength Silica)
- CORTECS

Empower 3 Software

- ApexTrack Integration
- Custom Calculations
- Scoring Reports
- Method Validation Manager

Quality Control Reference Materials (QCRM)

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Australia 61 2 9933 1777

Belgium and Luxembourg 32 2 726 1000

Brazil 55 11 4134 3788

Canada 1 800 252 4752

China 86 21 6156 2666

Czech Republic 420 2 617 11384

Denmark 45 46 59 8080

Finland 358 9 5659 6288

France 33 1 30 48 72 00

Germany 49 6196 400 600

Hong Kong 852 2964 1800

Hungary 36 1 350 5086

India 91 080 49292200 03

Ireland 353 1 448 1500

Israel 9723 3731391

Italy 39 02 265 0983

Japan 81 3 3471 7191

Korea 82 2 6300 4800

Mexico 52 55 52 00 1860

The Netherlands 31 76 508 7200

Norway 47 6 384 6050

Poland 48 22 101 5900

Portugal 351 21 893 61 77

Puerto Rico 1 787 747 8445

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Sweden 46 8 555 115 00

Switzerland 41 56 676 7000

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