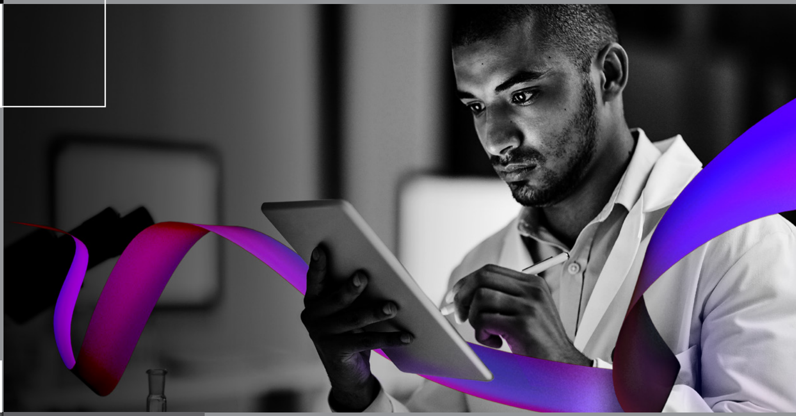
[PHARMACEUTICALS]

Method Lifecycle Management



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Method Lifecycle Management

Over their lifetime, analytical methods may have challenges meeting their original required criteria. These occurrences may be due to a number of parameters, including changes in instrumentation or columns, changes in other parameters that are not well understood, new technology, drug substance or drug product modifications, or a trend that occurred over the life of the method. To ensure methods are more robust and that there is improved control of method performance, Method Lifecycle Management (MLCM) can be considered.

MLCM is a strategy that is intended to improve the performance of analytical methods, and to assure those methods perform as originally intended over their entire lifetime. It uses a structured approach (Figure 1) that includes:

- **Analytical Target Profile (ATP) –** In the ATP, the requirements of the analytical procedure, such as accuracy and precision, are described. This document considers the uncertainty in the measurement when specifying these requirements. The ATP requirements are driven by the critical quality attributes (CQAs) of the pharmaceutical product.
- **Stage 1 – Method Design and Development –** Methods development, including understanding and proposed control strategy, for critical method parameters (CMPs) that may pose a risk to consistently achieving method requirements.
- **Stage 2 – Method Qualification –** The method, with the proposed control strategy, is qualified to ensure it meets its requirements consistently. This includes validation.
- **Stage 3 – Method Performance Verification –** Includes monitoring the performance after the method is put into routine/QC use, evaluation of any revisions to the method to verify that it continues to meet its original requirements, and the continuous improvement of methods.

MLCM is a fluid process, in that any modifications to a method in latter stages may require the previous stages be revisited, and a risk assessment to be carried out and some level of revalidation may be required. However, with this approach, a laboratory can: improve the performance of methods; reduce analytical uncertainties such as out-of-specification (OOS) and out-of-trend (OOT) results; minimize the need for post approval method changes; and improve the success of method transfers to other laboratories.

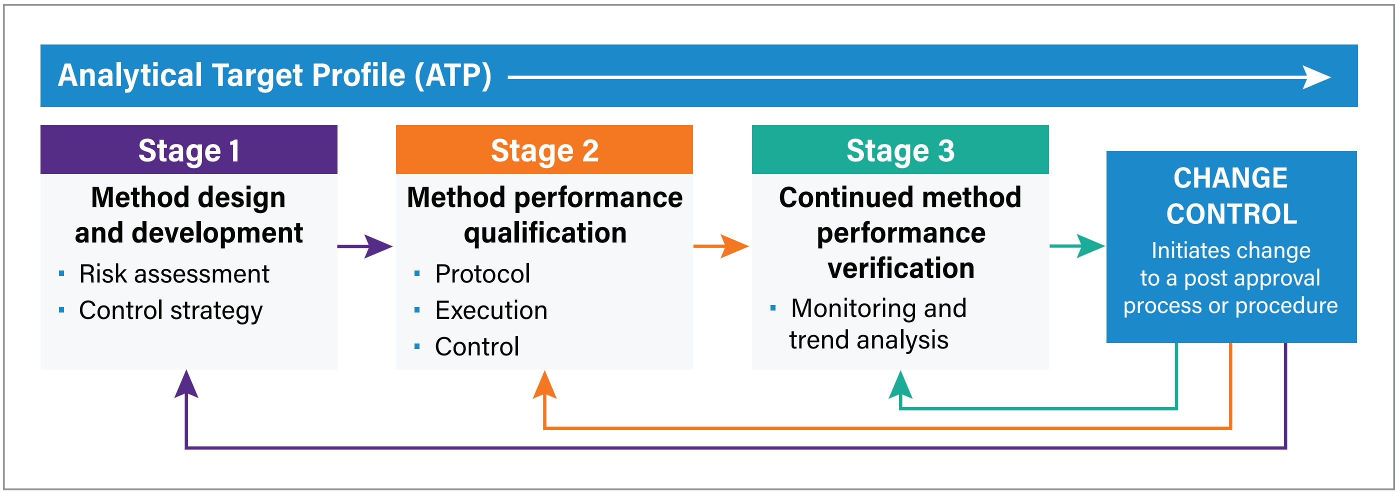


Figure 1. Method Lifecycle Process (adapted from Martin, et al, Proposed New USP General Chapter: The Analytical Procedure Lifecycle <1220>, Pharmacopoeial Forum, 43(1), Jan–Feb, 2017 and Schmidt, A. H., & Parr, M. K., 2018. Life cycle management of analytical methods. J. Pharm. Biomed. Anal. 2018, 147, 506–517.

Analytical Target Profile

For any analytical method, the criteria or objective should be clearly stated to ensure the method meets its intended use. In MLCM, this is part of the Analytical Target Profile (ATP), which is used to document the requirements that apply across the life of the method. Typically, the ATP will identify the goal of the method, the requirement criteria (such as accuracy and precision), and the performance criteria. It is also important to remember the ATP is driven by the product critical quality attributes (CQAs) and it should describe the purpose of the method independent of the analytical technique.

The ATP should also consider the allowable amount of uncertainty in the measurement when describing the reportable value. This is done with the target measurement uncertainty (TMU), which is the level of variability a measurement can have while still satisfying the original objective or intent. Traditionally this uncertainty has been stated in terms of accuracy and precision, although some statistical expressions are beginning to emerge. In the ATP example below (Figure 2), the specification for Impurity A would be not more than 0.5% and the specification for Impurity B would be not more than 0.10%.

Requirement	Method performance criteria
Accuracy	Impurity A: 80–120% Impurity B: 75–125%
Precision of reportable results	Impurity A: %RSD ≤10% Impurity B: %RSD ≤15%
Intended purpose	Quantification of manufacturing process related impurities (not a degradation product)
Range	Impurity A: at least 0.05%–0.6% Impurity B: at least 0.05%–0.12%
Specificity	Interference is not more than the quantification of the following specified impurities — other related substances C, D, and E — the salt forming agent with impurities A, B or the API
Operating conditions and environment	The analytical procedure must be applicable for use in a standard analytical QC laboratory environment used for routine analysis. The test samples must be stable for at least 24 hours.

Figure 2. An example of an ATP for an impurity method adapted from https://www.ema.europa.eu/documents/presentation/presentation-performance-context-based-established-conditions-analytical-procedures_en.pdf.

Stage 1: Method Design and Development

Once the method requirements are identified, method design and development can begin. This is Stage 1 of the MLCM process. This first stage typically begins with gathering of information, such as chemical and physical properties of the analyte(s) or sample, and knowledge about methods for similar analytes. For LC methods, initial scouting experiments might be performed to identify preliminary method conditions. During this process of method design and development it is important to identify risks that could lead to poor method performance. Risk assessment tools including process maps or Ishikawa (fishbone) diagrams can help to prioritize the variables to be evaluated.

However, as shown in Figure 3, it may be challenging to quantify the impact of all the parameters identified in this process. To make the evaluation more manageable, risk assessment tools (Figure 4) and scientific judgement are used to quantify the previously identified risks. Through this process we can narrow the list of parameters to be investigated by scoring the potential risks with a numerical value based on both the likelihood and the impact on the results. This allows the risks to be numerically ranked, which allows for identification of critical method parameters (CMPs). For example, based on the scoring in Table 1, it's appropriate to evaluate the impact of stirring, diluent, and mobile phase pH, gradient table changes and dwell volume, since they have the highest scores.

This process allows for the impact of varying these parameters to be determined experimentally, and the data from those experiments can then be used to create a control strategy. For instance, after evaluation of the stirring time and speed, the results may indicate that samples should be stirred for at least 15 minutes at a speed of 100 rpm. This could then be written as a control statement that can be added to the method. Another form of control strategy is a well-constructed system suitability test, to assure the desired separation, sensitivity, and injection precision are obtained.

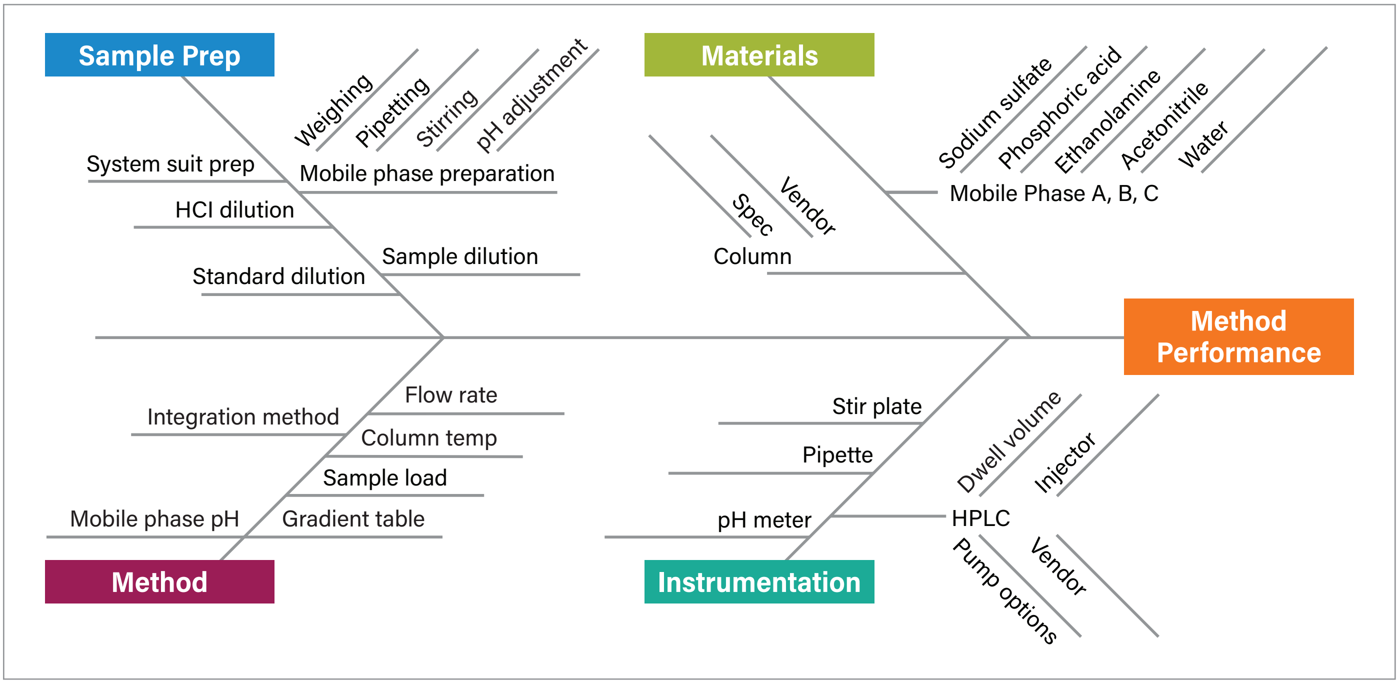
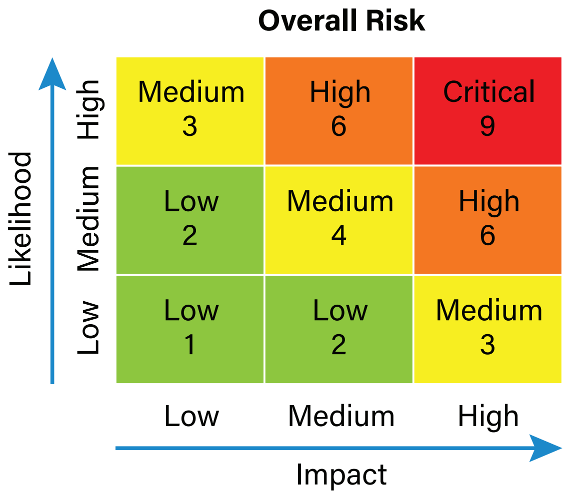


Figure 3. Fishbone diagram demonstrating risk identification.



Overall Risk

Likelihood \ Impact	Low	Medium	High
High	Medium 3	High 6	Critical 9
Medium	Low 2	Medium 4	High 6
Low	Low 1	Low 2	Medium 3

Figure 4. Risk assessment tool: impact and likelihood are assessed as low, medium, or high and assigned values of 1, 2, or 3 respectively. The values for impact and likelihood are multiplied to obtain the risk score.

Table 1. Risk scoring based on fishbone diagram, scientific judgement and risk assessment tool

Factor	Likelihood	Impact	Score (I x L)
Stirring speed and time	High	High	9
Diluent pH	High	Medium	6
Integration method	Medium	Medium	4
Mobile phase pH	High	Med	6
Flow rate	Med	Low	2
Column temperature	High	Low	3
Gradient table	High	Medium	6
Dwell volume	Medium	High	6

Additional steps of risk assessment, including evaluation of LC method conditions and sample preparation parameters, can be performed using a design of experiments (DOE) approach. This can be performed using internal knowledge and/or different software packages. As with the risk assessment scoring report above, DOE allows for factors to be evaluated for the impact of variables on the analytical method. This in turn helps develop a good control strategy.

Throughout this stage, knowledge gathering should be documented to help support any future change control strategy and prequalification exercises, including the assurance that there is no bias and that the ATP requirements are met.

Stage 2: Method Qualification

When method conditions have been well established and the impact of parameter changes is well understood, Stage 2 – Method Qualification can begin. In this step, regulators expect a protocol with analytical characteristics and acceptance criteria, an experimental execution, and a final report.

Today, we refer to this as method validation and follow USP <1225> or ICH Q2 guidelines. This step typically includes the evaluation of analytical performance characteristics such as accuracy, precision,and linearity. However, moving forward, in order to ensure that the method will perform as intended, the requirements expressed in the ATP should be used as the performance criteria. The risk assessments and control strategies performed and implemented in Stage 1 should help ensure these criteria are routinely met.

Table 2. Analytical performance characteristics ⟨1225⟩ Validation of Compendial Procedures, USP 41-NF36

Analytical performance characteristics	Category I (Assay)	Category II (Impurities)		Category III (Dissolution)	Category IV (ID)
		Quantitative	Limit Tests		
Accuracy	Yes	Yes	a	a	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	a	Yes
Detection limit	No	No	Yes	a	No
Quantitation limit	No	Yes	No	a	No
Linearity	Yes	Yes	No	a	No
Range	Yes	Yes	a	a	No

a *May be required, depending on the nature of the specific test.*

Perhaps in the future, as the MLCM approach gains acceptance, and regulators acknowledge that some of the evaluations are being done during Stage 1, there will be an opportunity to focus on the important characteristics of accuracy and precision, since specificity, sensitivity, linearity, and range will already have been evaluated in Stage 1.

Stage 3: Method Performance Verification

When a method is implemented and in routine use, it is important to continue to evaluate its performance. Stage 3 of MLCM addresses Continued Procedure Performance Verification. This step, which may include activities such as method transfer, has a goal to assure that the requirements identified in the ATP continue to be met. Monitoring of the method to check it is performing as intended and still adheres to the ATP performance over time can be accomplished in a number of ways, including:

- Trending system suitability test results and other instrument parameters
- Using control samples (sometimes used in other industries; not currently common in pharmaceutical laboratories)
- Trending of production or stability sample results (although these results also include variability related to production or stability)
- Demonstrating agreement of replicate sample preparations (indicates sample reproducibility)

In this stage, trending of system suitability test results can provide critical information that facilitates preventative actions, to ensure the ATP continues to be met. For example, if trending shows a dramatic decrease in plate counts, as seen in Figure 5, this could be an indication that the column needs to be replaced in order to continue to demonstrate good method performance.

Since it is unrealistic to expect a method to continue to perform well indefinitely, these procedures of constant monitoring allow for ongoing evaluation, and help quantify method changes.

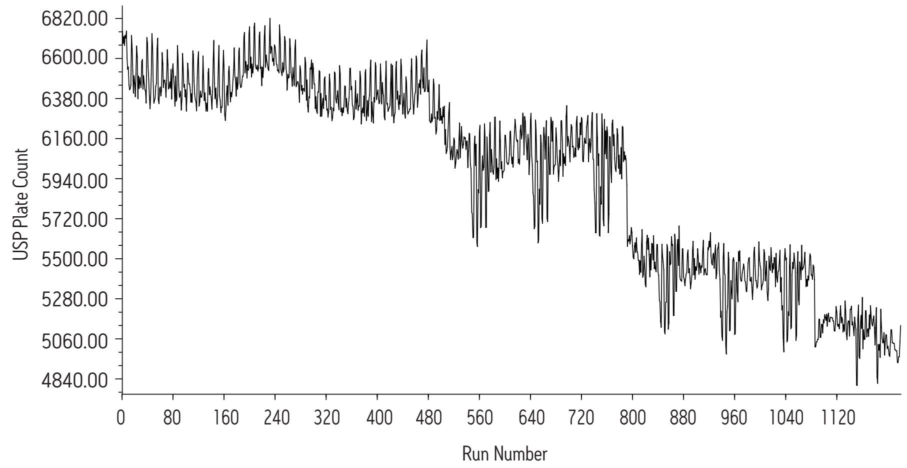


Figure 5. USP plate count trend plot from routine use study 1 on an ACQUITY UPLC BEH C_{18}, 1.7 µm Column.

In fact, method changes or revisions can be expected over the method lifetime. Perhaps there are instrument or column changes, such as acquiring new equipment and consumables, or the formulation of the drug product is modified requiring changes to the method. Whenever a change is being introduced, it is important to evaluate the change's impact on the performance of the method. A new risk assessment would be performed, looking at what is changing and how the change is likely to impact the method. Based on this step, it may be appropriate to return to Stage 2 repeating some or all of the qualification/validation experiments and consider implementing new control strategies.

As described earlier, method transfer can be a part of performance verification. In fact, one of the most difficult challenges is transfer of a method to another laboratory. This can introduce a variety of untested variables, such as different brands of instruments, different sources of reagents or analysts who are working under a completely different set of SOPs. If efforts to understand the sensitivity of the method in Stage 1 were performed well, and an appropriate control strategy was introduced into the method, it can ease the burdens associated with method transfer and increase the likelihood of a successful transfer. However, if there are challenges in this stage, a new risk assessment can be performed to determine the parameters that are impacting the performance of the transferred method.

Whatever the analysis, using an MLCM approach increases the potential to improve method performance, reduces deviations such as OOS and OOT, minimizes the need for post approval changes, and facilitates changes such as method transfer from one laboratory to another.

Stage 1: Method Design and Development (includes Understanding and Optimization)

Illustrates defining criteria (i.e., ATP), demonstrates building understanding using chromatography data system (CDS) tools for reviewing scouting results and screening (including column switching, scoring report).

Shows how using a systematic protocol for method development can simplify the process, highlights Empower scoring report, functionality.

Builds on systematic protocol with concrete example.

Shows how *forced degradation experiments, use of QbD software (Fusion) and mapping of Method Operable Design Region within Fusion, can foster method understanding.*

Addresses another facet of method understanding: evaporation from mobile phase bottles and from vials (factors to consider for risk assessment).

Stage 2: Procedure Performance Qualification

Demonstrates use of a protocol/execution/report approach and illustrates how compliant software, Waters Method Validation Module, can assist with execution and report writing.

Shows how specificity and precision can be evaluated during validation. Additionally, potential advantages of using two detectors, especially when dealing with unknown peaks, is demonstrated.

Shows how automation of the method validation process using CDS software can simplify both the experimentation and documentation required for validation.

Stage 3: Continued Procedure Performance Verification

[NOTES]

Stage 1

Method Design and Development (includes Understanding and Optimization)

A fundamental component of the lifecycle approach to methods is the analytical target profile (ATP), which needs to be defined prior to method development. Once the ATP has been defined, method design and development can be undertaken. This includes understanding and optimizing your method. In this section there are several documents that illustrate some of the considerations and strategies to evaluate risk. For example, "***Complete Solution to Performing a Systematic Screening Protocol***", page 13 discusses a systematic strategy to define your analysis criteria (or analytical target profile) and evaluates some variables within method development that can be identified during a risk assessment exercise (stationary phase, organic solvent, gradient, temperature, etc.). These method screening results can be reviewed using a compliant chromatography data system (CDS) with a means of evaluating pre-defined criteria, as presented in "***Improving Decision Making During Method Development***", page 27 For a view of how the systematic screening and scoring report can be used to evaluate a method for an API and related impurities, we can refer to "***Improving Effectiveness in Method Development***", page 29

Aside from a systematic protocol, an LC method can be developed using a statistical Quality by Design (QbD) software package, which may use design of experiments (DOE) or another algorithm. One such example, "***Developing Analytical Chromatographic Method for Pharma Stability***", page 39 illustrates this approach for a forced degradation sample. In this example, QbD software is used in combination with the chromatography data system (CDS) to test variables based on a risk assessment, while also allowing for visualization of robust design space using Fusion software.

In method design, other factors requiring control may be identified during risk assessment. These factors may include the sample, system, or method. For example, evaporation from mobile phase bottles and from vials are discussed in "***USP Method Case Study Part I: Understanding the Impact of Sample Preparation and Mobile Phase Stability***", page 51.

A Complete Solution to Perform a Systematic Screening Protocol for LC Method Development

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INTRODUCTION

In the analytical lab, a number of strategies can be used for reversed-phase liquid-chromatographic (RPLC) method development. Many labs perform method development by evaluating one factor at a time. While this approach often consists of a results-guided, step-by-step sequence, it can be time-consuming. It may be straightforward to implement, yet it often fails to provide a robust method because of the limited experimental space explored. Other labs approach method development using a statistics-based approach that aligns with quality-by-design (QbD) principles.[1,2] QbD is a more comprehensive approach in that experiments are statistically designed to explore multiple variables using the methodology known as design of experiments (DOE). QbD approaches can require specialized software platforms to evaluate data. These approaches can, in turn, require further investment and additional analyst training. A third option employed by many labs is systematic method development, which consists of a predefined experimental setup and an ordered protocol.[3-5] Systematic method development is a controlled process in which the screening data can be collected and analyzed within a single run.

The approaches to systematic method development have been well-documented and described,[6] and many of these approaches rely considerably on the efficacy of instrumentation and the chromatography data systems (CDS). Method development instrument manufacturers have invested in improving the ease-of-use, robustness, and versatility of LC instrumentation and detection. For example, current instrumentation enables the analyst to set up for unattended screening of multiple variables (columns and mobile phases). UltraPerformance LC™ (UPLC™), can be combined with low-dispersion systems and detectors, resulting in greater resolving capabilities. With advances in detection, including the ACQUITY™ QDa™ Detector, this system can be used with multi-detection for peak tracking and detection of co-elutions. In addition, the acquisition software can provide additional information through the use of customizable features such as reporting, processing, and interpretation of data.

This white paper describes a synergistic, systematic protocol that takes advantage of UPLC instrumentation, sub-2-µm column chemistries, and Empower™ 3 Software for a streamlined method development approach. The instrumentation used for this systematic approach consists of the ACQUITY UPLC H-Class PLUS System with a quaternary pump fitted with a solvent-select valve, column manager, low-dispersion detectors (including the ACQUITY UPLC PDA Detector), and the ACQUITY QDa Mass Detector. Combined with sub-2-µm columns and Empower 3 Software, this system provides the analyst the means to perform systematic method development for a range of compounds.

OVERVIEW

For the analytical chemist, chromatographic method development represents a considerable investment of time. Yet a workflow that considers the chemical and physical factors affecting chromatographic resolution increases the ability to develop a reliable separation. Incorporating these considerations into a clear, guided strategy minimizes any delays caused by repeat or circular testing, and it provides the analyst an organized approach for developing streamlined methods.

The workflow for systematic method development follows a series of steps, each with a clear objective. Figure 1 illustrates the steps for a systematic protocol. Also shown are the conditions, the columns, mobile phase, and other variables. Each step is designed to provide the analyst with the knowledge he or she needs to ensure that key parameters affecting selectivity and resolution of a separation are evaluated. The end goal is to develop a robust method by adopting a systematic approach.

To achieve these goals, we must consider the principles of chromatographic separations. As the fundamental resolution equation shows, efficiency, selectivity, and retention affect the resolution of two peaks, and they do so to varying degrees.

$$R_s = \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha-1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{k+1}}_{\text{Retention factor}}$$

Thus, to affect resolution, we must increase efficiency while taking advantage of chemical factors (retention and selectivity). The former requires optimized instrumentation and stationary phases for highest efficiency and reduced dispersion, whereas the latter can be explored more efficiently using a systematic protocol that prioritizes those parameters.

Step 1: Define Criteria

A method development exercise should include a set of predefined criteria and objectives. The objectives can be numerous and varied, including sensitivity and linearity, throughput and turnaround time, sample preparation, cost, transferability, required operator skill, etc. Additional goals can include assay-suitability criteria (USP resolution and USP tailing factors) or other factors such as the total number of peaks. Using a CDS capable of evaluating these objectives and criteria, as well as automatically generating the corresponding numerical values, reduces the time required to process and evaluate the data.

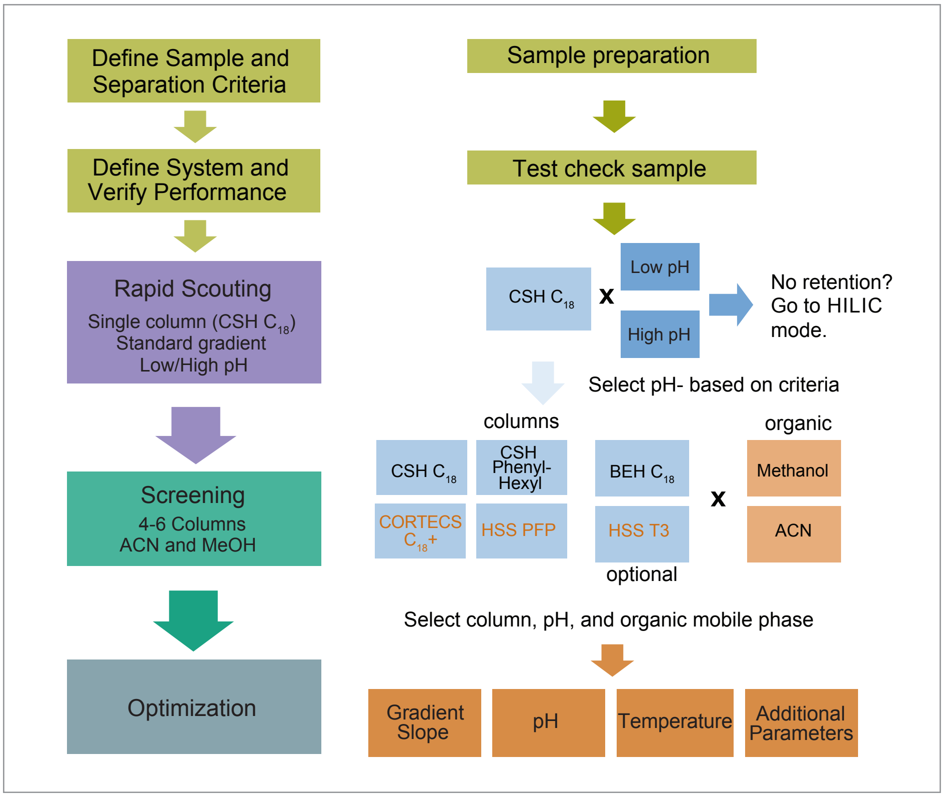


Figure 1. Steps and conditions for a systematic method development workflow.

Step 2: Define System and Verify Performance

SYSTEM SET-UP

Performing method development can be laborious. Often, the choice of instrument and CDS can impact the development process or subsequent transfer of the method. Instrumentation that is incompatible with the analyst's workflow can make method development even more cumbersome. The lack of appropriate maintenance can lead to instrument downtime, decreasing the laboratory's productivity. For these reasons, UPLC Systems, which include a quaternary solvent manager and offer the option to manage multiple columns, allow the greatest selectivity space to be explored. UPLC Systems also provide the efficiency benefits of low-dispersion flow paths, detectors capable of rapid sampling of the column effluent, and the productivity benefits of Automated System Qualification Testing protocols and quality control reference material for system performance verification. The choice of injector can also affect method development. With flow-through-needle injectors, such as the Sample Manager FTN-H, the entire gradient passes through the needle and sample loop. Thus, all of the sample transfers to the column, reducing the potential for carryover phenomena. The flow-through-needle design allows for injection-volume flexibility without the need to reconfigure the injector. Upper injection volume on the ACQUITY UPLC H-Class PLUS System is listed in the specification sheet as 1.0 mL with optional extension loop and syringes. This enables the method developer to quickly change the amount of sample on column when needed to explore sensitivity or changes in column configuration.

In addition to the solvent and sample delivery system, careful consideration must be given to the detection technique. A single detection technique may fail to provide enough information to ensure the detection of all sample components. Nor may it identify non-homogeneous or co-eluting peaks, provide information that helps identify a novel or unknown component, or track peaks. The routine use of orthogonal detectors, such as UV and mass-based detectors, can provide greater assurance that all of a sample's components are detected and resolved. Incorporation of a mass detector, such as the ACQUITY QDa Detector, facilitates this process and reduces the need to verify analyte retention times using individual standards. The availability of other detection techniques (evaporative light scattering, fluorescence) provides additional means for detecting a wide range of compounds.

The CDS is also an important consideration. A CDS such as Empower 3 Software provides multiple tools that are important for method development including instrument control, injection sequencing, and function coordination. In addition, Empower 3 Software utilizes a relational database to catalog, store, retrieve, and protect data in a compliant environment. Custom or user-created calculations and input fields offer additional functionality, as do view filters, for organizing data, and batch processing and reporting, for streamlined data analysis. Empower 3 Software combines these attributes to provide simple measurements of method-suitability criteria to evaluate each separation. Furthermore, by providing a single window for reviewing mass and UV information simultaneously, it simplifies data evaluation.

Combining the features and capabilities of versatile, newer instruments, columns, and software provides the analyst the means to utilize an optimally defined system to facilitate higher-quality, more efficient method development (Table 1). The method development process is streamlined using software and hardware that facilitate automation.

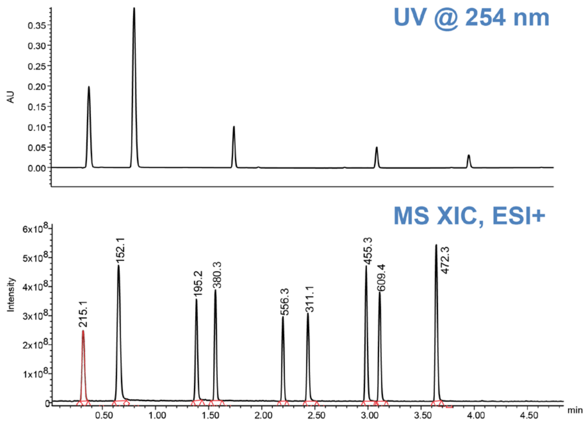
LC conditions	
System:	ACQUITY UPLC H-Class PLUS with PDA with Column Manager
Seal wash and purge lines:	90:10 water:acetonitrile
Wash line:	50:50 water:acetonitrile
Column temperature:	40 °C
Flow rate	0.6 mL/min
Injection volume:	1–5 µL
Sample diluent:	Methanol
Wavelength range:	210–500 nm
Resolution:	1.2 nm
Sampling rate:	10 pts/s
Filter constant:	Normal
Columns:	ACQUITY UPLC CSH C_{18}, 1.7 µm, 2.1 x 50 mm CORTECS UPLC C_{18}+, 1.6 µm, 2.1 x 50 mm ACQUITY UPLC CSH Phenyl-Hexyl, 1.7 µm, 2.1 x 50 mm ACQUITY UPLC HSS PFP, 1.7 µm, 2.1 x 50 mm
Solvents:	Line A: 125 mM formic acid in water Line B: 125 mM ammonium hydroxide in water Line C: MilliQ water Line D1: Acetonitrile Line D2: Methanol
Gradient:	Low pH: 10% A, 85% C, 5% D to 10% A, 0% C, 90% D in 5 minutes High pH: 10% B, 85% C, 5% D to 10% B, 0% C, 90% D in 5 minutes
Chromatography Data Software:	Empower 3 FR2
MS conditions	
MS system:	ACQUITY QDa Detector
Ionization mode:	ESI+, ESI-
Acquisition range:	100–1250 *m/z*
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temperature:	600 °C
Data collection:	Centroid
Sampling rate:	5 points/sec

Table 1. System and conditions for systematic method development.

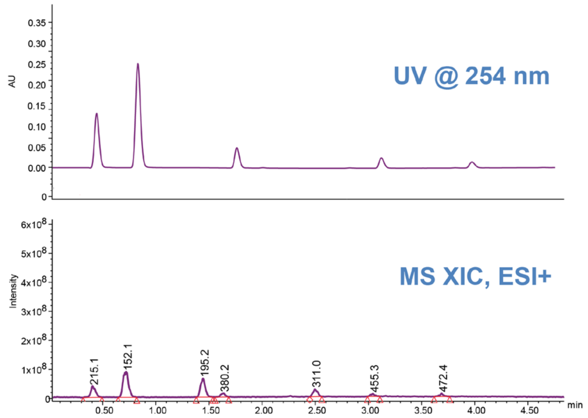
SYSTEM CHECK

The investment in time and resources for method development can be significant. Testing should be performed on a system that was evaluated to ensure it is operating to specifications. The evaluation can be accomplished by running a known set of components or a quality-control reference material (QC Reference Material), preferably that comprises a set of analytes that elute across the gradient and that can verify the performance of the column and system. Thus when changes in retention time or peak-shape occur, the chromatography can be reviewed for evidence of physical or chemical abnormalities in either the system or column.

As illustrated in Figure 2, an ACQUITY QDa QC Reference Material standard (p/n 186007345) was tested on an ACQUITY UPLC H-Class PLUS System that includes an ACQUITY QDa Detector, to evaluate the performance of the system and column. As compared with earlier analyses, the resultant UV and extracted-ion chromatogram (XIC), met the expected retention times and peak shapes. Yet a later check, or benchmark, of the system produced broadened peaks, lower sensitivity, and a drop in system pressure (Figure 2, bottom chromatogram). Troubleshooting revealed an improper or leaking column connection. Check samples that serve as reference standards provide a tool to benchmark a system's performance before developing methods.



Normal operation: system pressure ≈ 8100 psi



System with slight leak: system pressure ≈ 7300 psi

Figure 2. Verification of system performance using a check standard ensures a properly working instrument and provides the analytical chemist confidence in the results. In this example, poor or loss of sensitivity and increased peak widths of the check standard were caused by a leak in the system.

Step 3: Rapid Scouting

An efficient method development protocol reduces duplication. It is structured to obtain informative and meaningful data at each step of the process. For the method development protocol we have outlined an initial rapid-scouting step, at high and low pH. This provides key information that can streamline the method development process. The use of mobile phases of high pH to dramatically affect selectivity in reverse phase LC (RP-LC) has been documented.[8] Operating at high pH is made possible by column packings that are stable at high pH.[7] With this added tool, a single sample can be analyzed at high and low pH using the same column.

IMPACT OF MOBILE PHASE pH

In RPLC, retention is driven by the interaction between the analyte, stationary phase, and mobile phase. The separation is determined by partitioning of the sample between the hydrophobic (nonpolar) stationary phase and the hydrophilic (more polar) mobile phase. When the ionization state of the compound changes, the retention time is affected by differences in the partitioning of the compound between the nonpolar stationary phase and the polar mobile phase. For example, at low pH (<3) the weak acid is fully un-ionized, resulting in greater interaction with the nonpolar stationary phase and greater retention (Figure 3). At high pH (>8), the same effect occurs for bases, resulting in greater retention. In the middle pH range, many acids and bases undergo fractional charging, with less pronounced changes in retention. Thus, analysis at high and low pH can determine if the sample contains acids and/or bases that would preclude retention at the low or high pH.

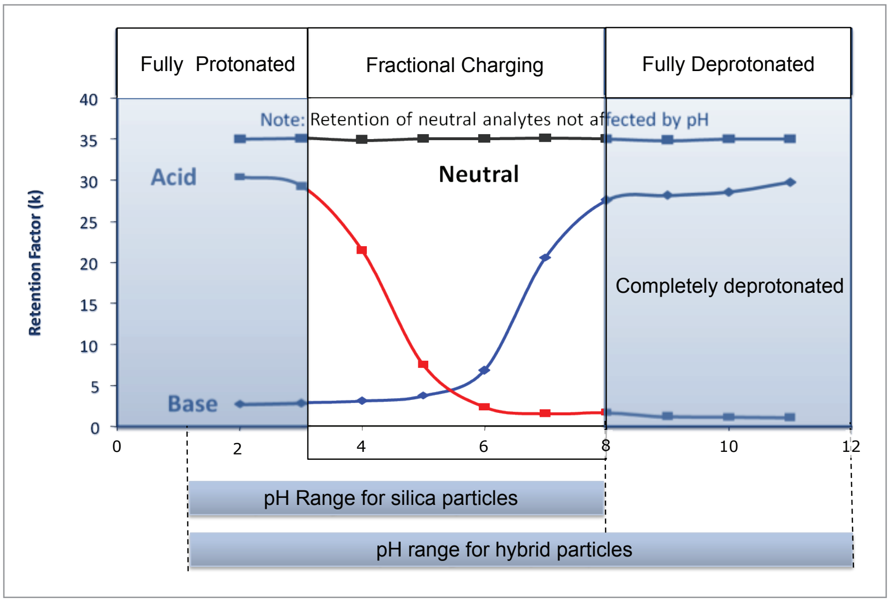


Figure 3. Reversed-phase retention map illustrating the effect of pH. Ionizable analytes are most retained in their un-ionized state. Neutrals do not have an ionizable functionality and are, therefore, unaffected by pH.

This set of experiments also allows the classification of samples into five categories (strong acids, weak acids, strong bases, weak bases, and neutrals), which will define subsequent experiments. If no retention is observed at either high or low pH, the analytes are, most likely, strong acids or bases, making retention by reversed-phase chromatography more challenging and likely requiring additional considerations. While the use of ion-pairing reagents in the mobile phase or sample diluent can be considered, hydrophilic-interaction chromatography (HILIC) may also provide greater retention and the desired separation for the polar analytes.[9, 10] If both low and high pH give adequate retention with different selectivity, then both should be subsequently evaluated. If both give adequate retention with no change in selectivity, the sample may be devoid of weak acids and bases and additional development might omit pH control.

COLUMN SELECTION FOR pH RAPID SCOUTING

To screen at both low and high pH, the stationary phase must be compatible with the mobile-phase conditions. Silica-based stationary phases are not chemically stable at high pH (>8), owing to dissolution of the silica particles.[11] Hybrid particle technologies are more inert to these effects and can be used with high-pH mobile phases.[7] The lower surface-silanol concentrations of these particles can result in differences, compared with silica, in selectivity and peak shape. To further improve peak shape of basic compounds, a positive surface charge can be applied to the surface of the BEH base particle.[12] The positive charge applied to the particle (CSH™) provides improved peak shape for basic analytes under low-ionic-strength acidic conditions (0.1% formic acid), a suitable choice for rapid scouting at both low and high pH.

INSTRUMENTATION TO FACILITATE RAPID SCOUTING

In addition to the column, the instrument configuration can be selected to facilitate the rapid-scouting step. The quaternary pump of the ACQUITY UPLC H-Class PLUS System allows the unattended screening of both low-and high-pH mobile phases. Referred to as Auto•Blend, this ternary or quaternary mixing approach enables blending of stock solutions to produce the desired mobile phase. For example, pH can be altered simply by varying the proportion or amount of acid or base in the mobile phase. For the rapid-scouting step, both low-and high-pH mobile phases can be screened with solvents from four bottles: a stock acid (125 mM formic acid), a stock base (125 mM ammonium hydroxide), water, and acetonitrile (Figure 4), facilitating rapid scouting.

RAPID SCOUTING FOR AN API AND RELATED COMPOUNDS

Combining these various components with the protocol, rapid scouting was conducted for the active pharmaceutical ingredient (API) ondansetron and its related compounds (Figure 5). Analysis at low and high pH reveals differences in retention and selectivity. Separation of all of the analytes is achieved at low pH, while at high pH, the bases (1, 2, and 3) have shifted retention significantly but moved as a block. While the elution order of the bases is different at high pH, the spacing among the bases is approximately the same, indicating that these analytes respond differently to the large pH shift. For these analytes, therefore, exploring the pH range in smaller increments might lead to differences in selectivities.

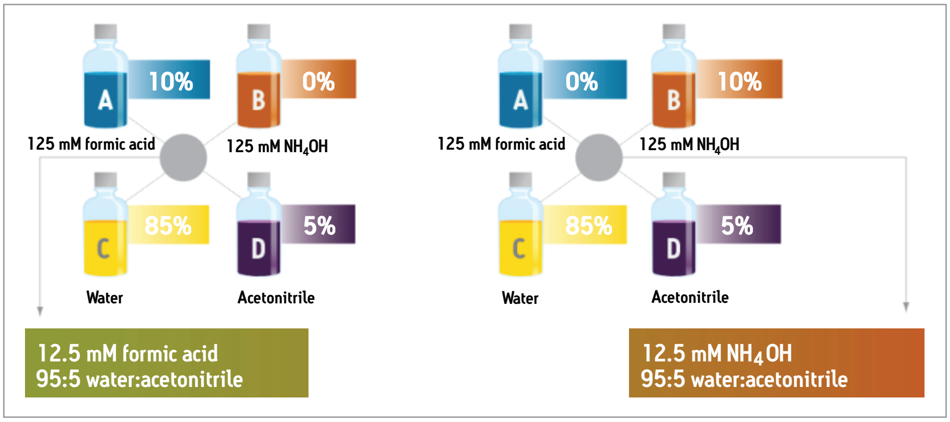
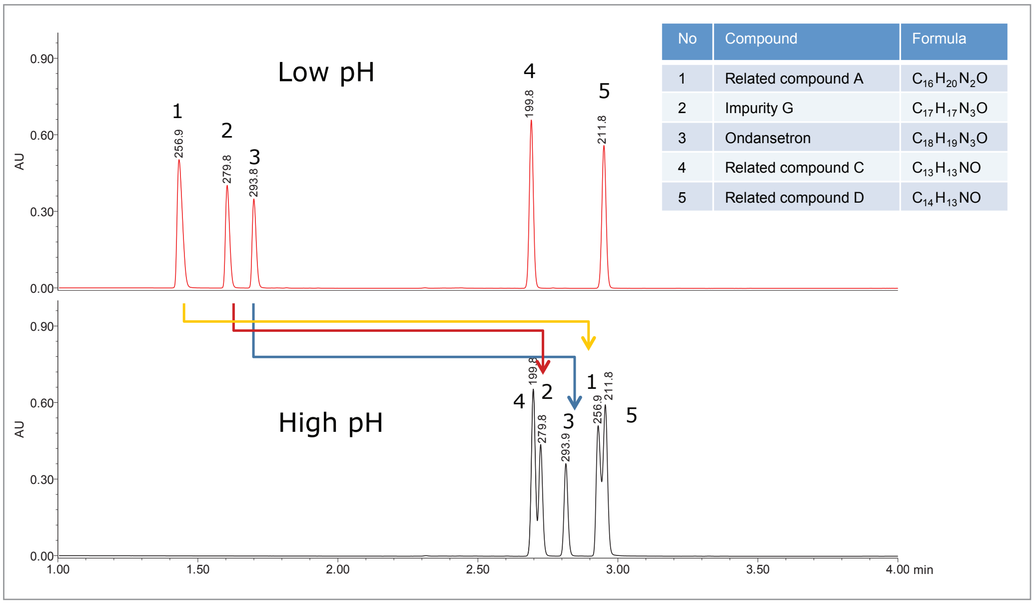


Figure 4. Quaternary mixing using Auto•Blend™ enables automated formulation of mobile phase by combining stock buffers and pure solvents in varying ratios.



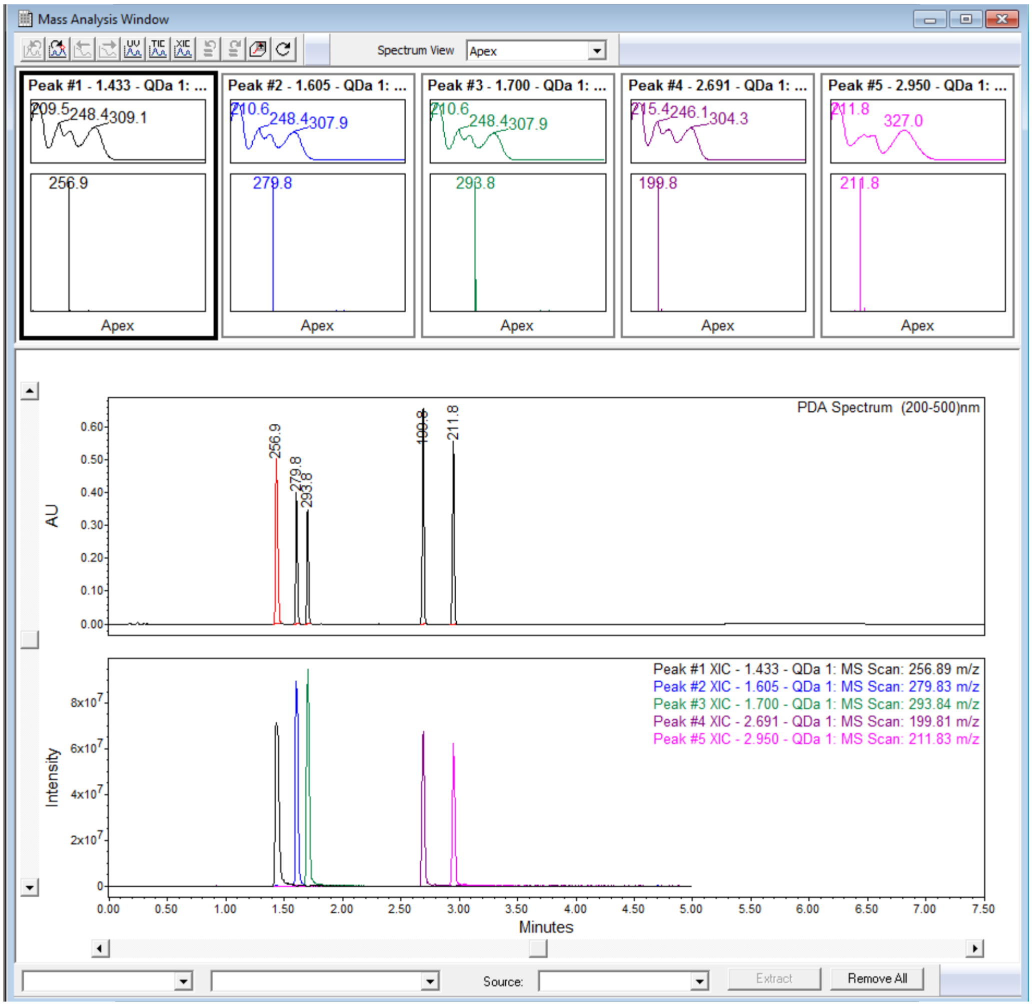
No	Compound	Formula
1	Related compound A	$C_{16}H_{20}N_2O$
2	Impurity G	$C_{17}H_{17}N_3O$
3	Ondansetron	$C_{18}H_{19}N_3O$
4	Related compound C	$C_{13}H_{13}NO$
5	Related compound D	$C_{14}H_{13}NO$

Figure 5. Rapid scouting of ondanestron and related impurities allows for determination of appropriate conditions for further testing. Analysis at low-mobile-phase pH provides greater resolution. Streamlined software allows for scoring of runs based on suitable criteria.

USING A CDS TO REVIEW SCOUTING RESULTS

To help the analyst evaluate the data, the CDS can provide numerous tools. In the above-mentioned example, peak tracking can be performed more easily using the mass label of each peak, which represents the most intense ion at the peak apex (Figure 5). In addition, the analysis of multiple streams of data is simplified by a single window that includes both chromatographic and spectral views (Figure 6). The chromatographic display (lower portion of window) includes the UV chromatogram, the total-ion chromatogram (TIC), and the extracted-ion chromatogram (XIC) for the separation. The XIC, which is automatically generated, is composed of the extracted base peak ion from the TIC for each of the integrated UV peaks. The spectral data for each peak are displayed at the upper portion of the screen and show both the UV and mass-spectral information. This display provides a combined view of mass and UV chromatograms and spectral information, providing the analyst ways to track peaks and determine co-elutions.

The CDS can also provide the means to assist the analyst in determining the presence of co-eluting peaks. As shown in the rapid scouting of orange extract at high pH (Figure 7), the asymmetrical peak shape of peak 3 indicates two partially resolved analytes. The analyst can investigate further by evaluating the spectral information at the leading, apex, and trailing segments of the peak (inset). Specifically, the UV and mass spectra of different segments of a single peak help the analyst determine homogeneity of the peak and the presence of co-elutions. Evaluation of the spectra of peak 3 show different ratios of the most prominent ions (*m/z* 403.1 and 343.0) at the peak's apex, leading, and trailing segments. This view – the purity view – suggests the presence of two analytes instead of a single component, an indication that would prompt further investigation. The comprehensive view enables the analyst to perform peak tracking and obtain information on peak purity.


Mass Analysis Window

Spectrum View: Apex

Figure 6. CDS displays combined view of mass and UV chromatograms and spectra, providing analysts information for peak tracking and determining co-elutions.

Step 4: Screening

After evaluation of the scouting results, additional method development may be necessary for greater resolution, for alternative selectivity, to correct poor peak shape, or to address any other critical attribute in need of rectifying. Therefore, a more thorough screening can be performed with columns and strong solvent that can provide alternative selectivity. As described in the resolution equation, the factors of selectivity, efficiency, and retention all influence resolution. The choice of column, too, can affect those parameters. For example, separations with increased efficiencies can be achieved with sub-2-µm particles. Nevertheless, one of the most powerful means of improving resolution in a separation is to alter selectivity, achievable by using stationary phases with various ligands and base particles, which cover a wide range of selectivity. For example, selectivity differences can be observed using Ethylene Bridged Hybrid (BEH Technology™) particles or High Strength Silica (HSS) particles, the latter having higher mechanical stability than typical high-pore-volume silica columns.[13]

Apart from selectivity differences caused by the base particle, the stationary phase also can affect selectivity. Extensive studies have documented differences in selectivity through π-π interactions with phenyl columns or through the use of embedded polar phases, among others.[8] The stationary phases selected must also be compatible with the selected mobile phase, pH, and temperature. Given these considerations, the columns used for the screening step should include those that provide a wide range of selectivity both at low and high pH (Figure 1).

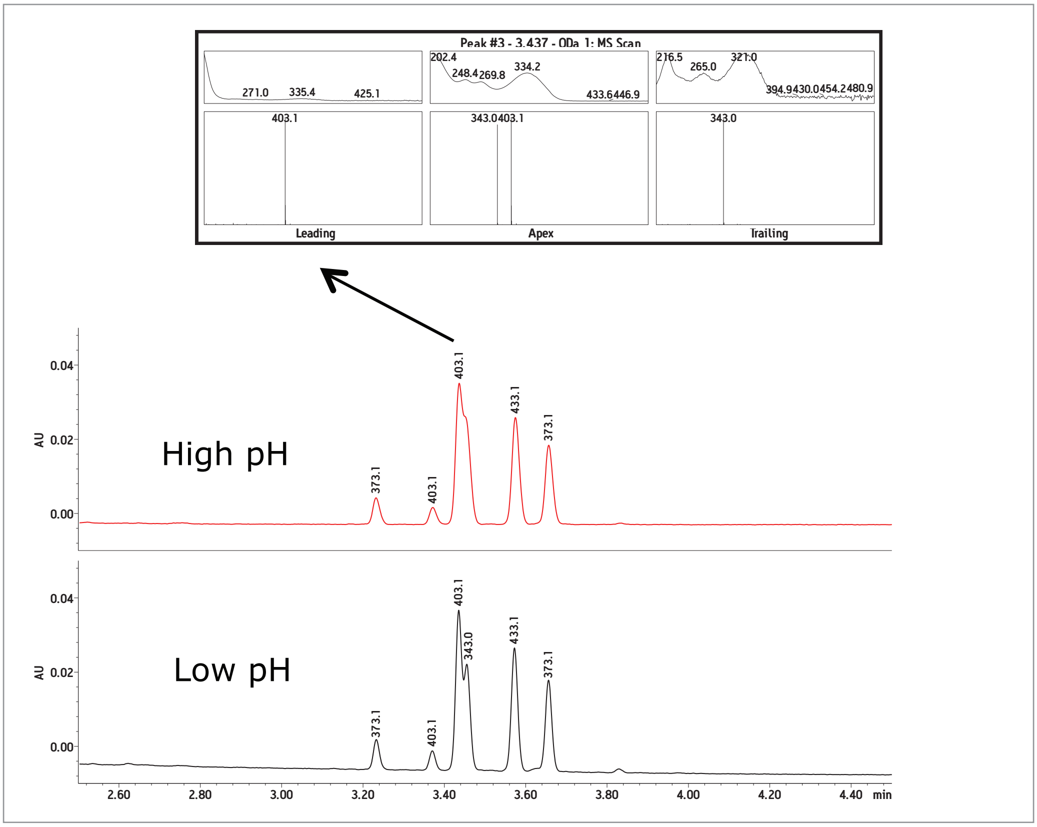


Figure 7. Rapid scouting of orange extract. Peak purity view allows the analyst to gather information about peak purity and possible co-elutions. In this example, two partially resolved analytes are observed in the rapid scouting of orange extract at high pH. The peak purity view in the mass analysis window reveals varying ion ratios of 403.1 and 343.0 at the leading, apex, and trailing segments of the peak, indicating the presence of two unresolved analytes

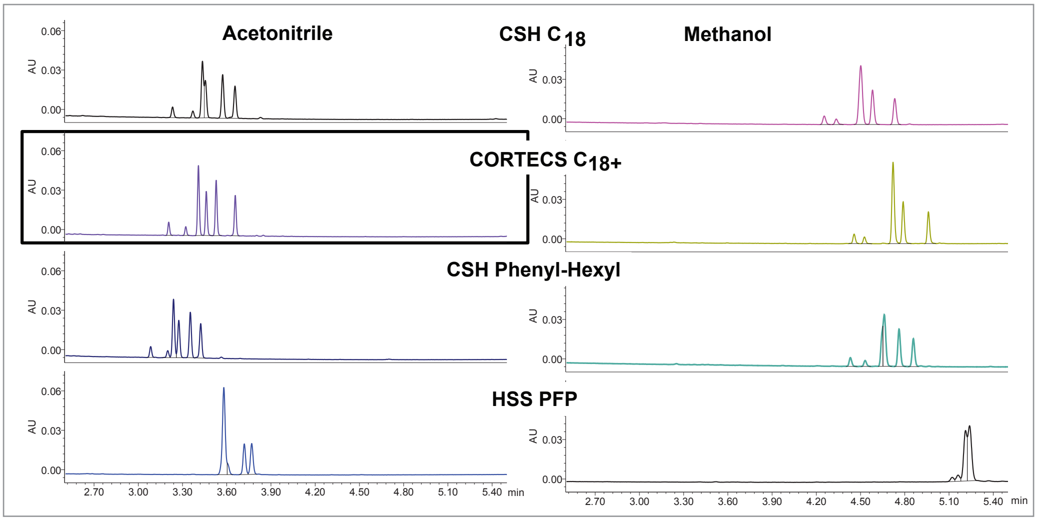


Figure 8. Following the initial scouting of an orange extract sample (Figure 7), Step 2 or Screening was performed. This step was carried out using four columns and two strong solvents at low pH. The highlighted chromatogram represents the conditions chosen for optimization (see Figure 9).

INSTRUMENTATION TO FACILITATE SCREENING

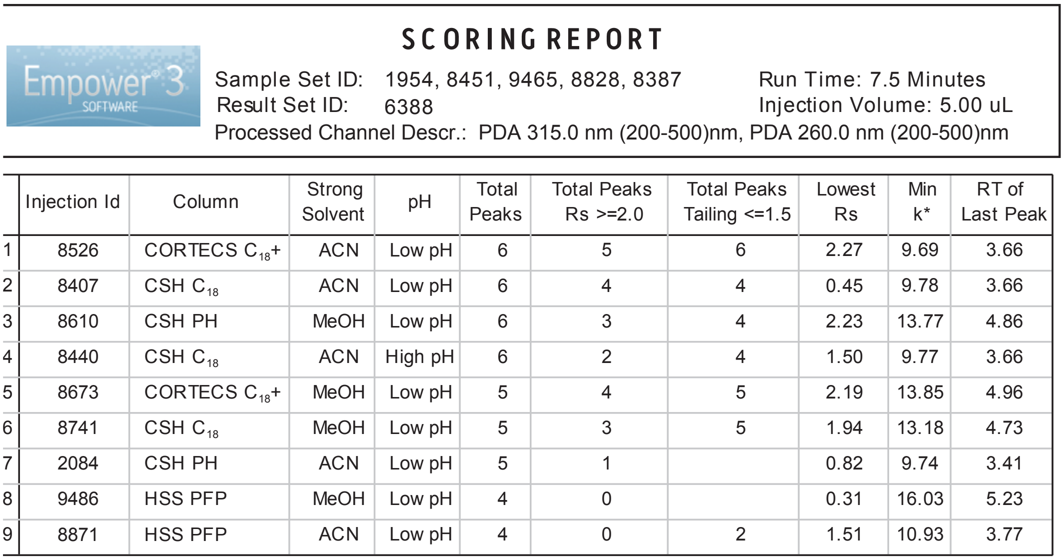
To screen these multiple columns in an unattended fashion, the analyst must consider column management. A multi-column compartment, as available on the ACQUITY UPLC H-Class PLUS System, is capable of independently switching columns using integrated valves. In addition, each column can be independently temperature-controlled, giving users the flexibility to select a variety of column chemistries regardless of their operational specifications. Column compartments capable of holding multiple columns provide flexibility and eliminate the need for manually changing columns between analyses.

In the screening step, adjustments to selectivity can also be made by altering the strong solvent. A quaternary pump fitted with a solvent-select valve allows the unattended screening of both low-and high-pH and strong solvent combinations. By combining multiple columns and both protic and aprotic strong solvents (methanol and acetonitrile, respectively), a wide selectivity space can be evaluated.

SCREENING OF COLUMNS AND STRONG SOLVENTS

To illustrate this screening approach, flavonoids present in orange extract were evaluated. It is important to note that, because the screening step is an extension of the rapid-scouting step, to eliminate duplication, the CSH C_{18} Column requires evaluation only with methanol, as a strong solvent. The scouting step at low and high pH for this set of compounds (Figure 7) produced minimal changes in selectivity and retention, suggesting the class of compounds is neutral or, possibly, slightly acidic. Given the improved resolution of the critical pair, screening was performed under low-pH conditions.

The various columns and strong solvents produced very different retention and selectivity (Figure 8). Greater retention on all columns was observed with methanol, as compared with acetonitrile. This is expected since methanol, which can undergo polar-polar interactions with solutes, is a weaker solvent in reversed-phase systems. The separations on both C_{18} stationary phases were similar. Nevertheless, the superficially-porous particle (CORTECS C_{18}+ Column) provided slightly greater efficiencies and separation of the critical pair. The phenyl-hexyl stationary phase provides interaction through both phenyl pi pi interactions and the alkyl chain. Therefore, for this set of analytes the CSH phenyl-hexyl column produced lower retention with selectivity similar to that of the BEH C_{18} stationary phase. Lastly, the pentafluorophenyl stationary phase (HSS PFP) column, which undergoes pi-pi interactions with Lewis bases, is often used for basic compounds that are difficult to retain. In this example, the PFP interacted with the neutral and acidic solutes for greater retention; however, selectivity differences resulted in less overall resolution.



SCORING REPORT

Sample Set ID: 1954, 8451, 9465, 8828, 8387
Result Set ID: 6388
Run Time: 7.5 Minutes
Injection Volume: 5.00 uL
Processed Channel Descr.: PDA 315.0 nm (200-500)nm, PDA 260.0 nm (200-500)nm

	Injection Id	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	8526	CORTECS C_{18}+	ACN	Low pH	6	5	6	2.27	9.69	3.66
2	8407	CSH C_{18}	ACN	Low pH	6	4	4	0.45	9.78	3.66
3	8610	CSH PH	MeOH	Low pH	6	3	4	2.23	13.77	4.86
4	8440	CSH C_{18}	ACN	High pH	6	2	4	1.50	9.77	3.66
5	8673	CORTECS C_{18}+	MeOH	Low pH	5	4	5	2.19	13.85	4.96
6	8741	CSH C_{18}	MeOH	Low pH	5	3	5	1.94	13.18	4.73
7	2084	CSH PH	ACN	Low pH	5	1		0.82	9.74	3.41
8	9486	HSS PFP	MeOH	Low pH	4	0		0.31	16.03	5.23
9	8871	HSS PFP	ACN	Low pH	4	0	2	1.51	10.93	3.77

Figure 9. Automated CDS reporting allows the analyst to determine appropriate column and strong solvent, for continued method development, in an unbiased fashion.

USING A CDS TO REVIEW AND REPORT SCREENING RESULTS

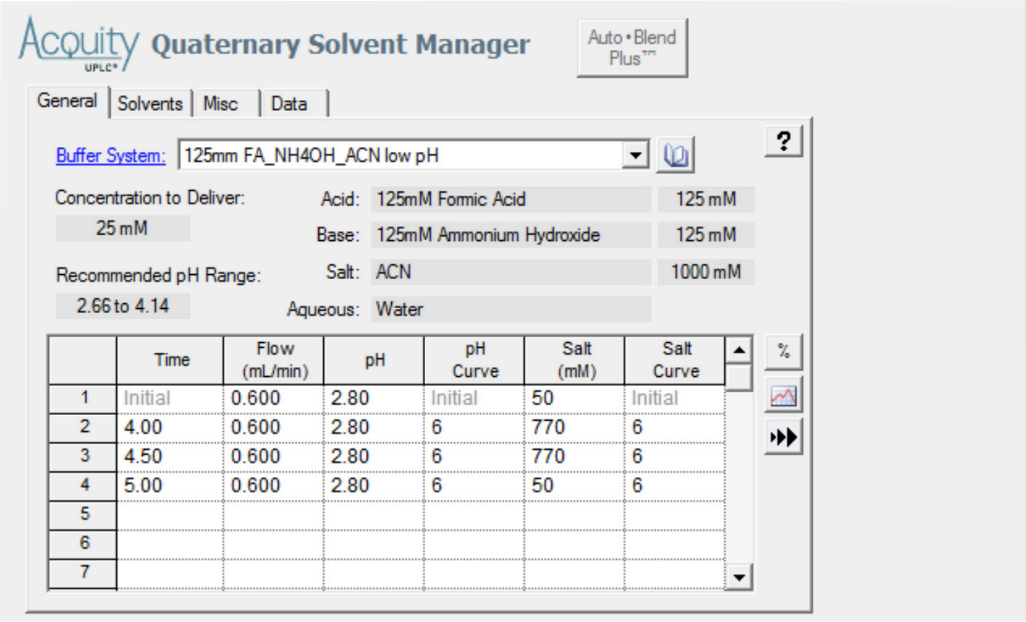
To evaluate the data, a CDS such as Empower 3 Software can enable automated measurement of method suitability criteria. The linkages in the relational database provide the ability to rank and display any combination of fields according to system-suitability criteria, as determined by the analyst's needs.[14,15]

Some examples of the types of custom fields that can be created include peak-result and sample-level fields. Furthermore, with a CDS platform that contains customizable processing and reporting, the analytical chemist can evaluate the data in an automated fashion (Figure 9).[14] This flexibility allows the results or report for an analysis to be automatically generated and tailored to place priority on the attributes most critical to the analyst. In this report separation-criteria values are automatically calculated from values in the predefined custom fields. Ranking is sequential and is based on the order of fields selected in the table view. Multiple parameters are evaluated only if there are ties in those evaluated earlier. The analyst can then review the results to ensure the appropriate selection criteria are chosen.

To illustrate these features, the screening results of orange extract were processed and ranked by pre-established criteria (Figure 9) including the greatest total number of peaks, total number of peaks with USP Resolution greater than 2.0, and total number of peaks with USP tailing less than 1.5. For this particular example, multiple separations produced the same number of peaks – that is, six. However, the CORTECS C_{18}+ Column, with acetonitrile as the strong solvent, produced the greatest number of peaks, five in all, in which USP resolution was greater than 2.0. According to the criteria we selected, these conditions sufficed for the next step, optimization.

STEP 5: OPTIMIZATION

When necessary, optimization of the chromatographic separation typically requires additional analyses beyond pH, column, and solvent screening. In such cases, however, the experimental design varies from analysis to analysis, depending on the method requirements. Goals of method development might include completeness of the separation, certainty of peak identification, sensitivity, and quantitative properties. Additional requirements might include separation criteria such as resolution, peak purity, analysis time, or tailing factors. To achieve these goals, numerous means are available for optimization. They include column temperature, column dimensions, flow rate, pH, gradient slope, and gradient time.



Buffer System: 125mm FA_NH4OH_ACN low pH

Concentration to Deliver: 25 mM

Acid: 125mM Formic Acid 125 mM

Base: 125mM Ammonium Hydroxide 125 mM

Recommended pH Range: 2.66 to 4.14

Salt: ACN 1000 mM

Aqueous: Water

	Time	Flow (mL/min)	pH	pH Curve	Salt (mM)	Salt Curve
1	Initial	0.600	2.80	Initial	50	Initial
2	4.00	0.600	2.80	6	770	6
3	4.50	0.600	2.80	6	770	6
4	5.00	0.600	2.80	6	50	6
5						
6						
7						

Figure 10. Automation of instrument control software allows for pH of mobile phase to be programmed directly into the gradient table using Auto•Blend Plus. The proportion of acid and base for a specific pH are based on empirical table or pKa of acid/base combination. The salt column is used for the organic or strong solvent and reflects the percent delivered multiplied by a factor of 10 (50 mM = 5%).

USING AUTO▪BLEND PLUS FOR OPTIMIZATION OF MOBILE PHASE pH

Manipulating many of these variables is fairly straightforward, but evaluating the effect of slight changes in mobile-phase pH can be laborious. Preparing multiple mobile phases at different pH levels can be tedious and time-consuming. Nevertheless, slight changes in mobile-phase pH can significantly affect selectivity, retention, and sensitivity, particularly for fractionally charged analytes (Figure 3). Thus, to develop a robust method, mobile-phase pH should be evaluated.

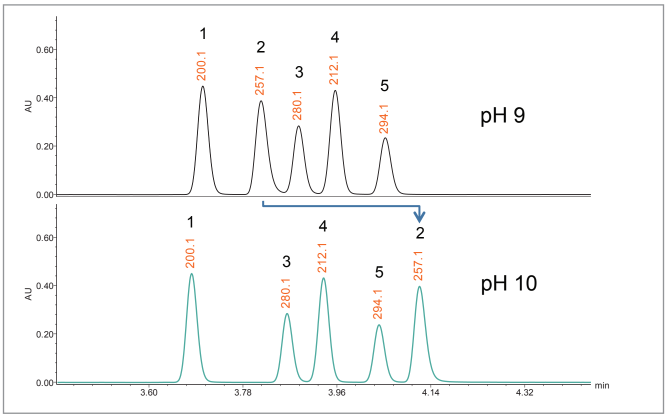


Figure 11. Effect of mobile phase pH on separation of ondansetron (peak 5) and related impurities. Changes in mobile phase pH produce changes in retentivity and selectivity for this class of compounds. In this example a change of 1 pH unit resulted in peak 2, shifting retention 0.4 minutes to latest eluting peak.

Using the Auto▪Blend Plus™ feature of the ACQUITY UPLC H-Class PLUS System, analysts can use specifically designed software to program methods to control pH (Figure 10). This approach to controlling pH provides flexibility. It allows the analyst to use any buffer to meet the needs of the sample and method and to create appropriate mixtures of buffers for an extended pH range. According to the programmed value, the ratio of acid and base are determined, either from an empirical table or the pKa of the acid/base combination. This determination enables the analyst to evaluate small changes in pH in an unattended fashion, a useful tool for method development, particularly in the case of ionizable compounds.

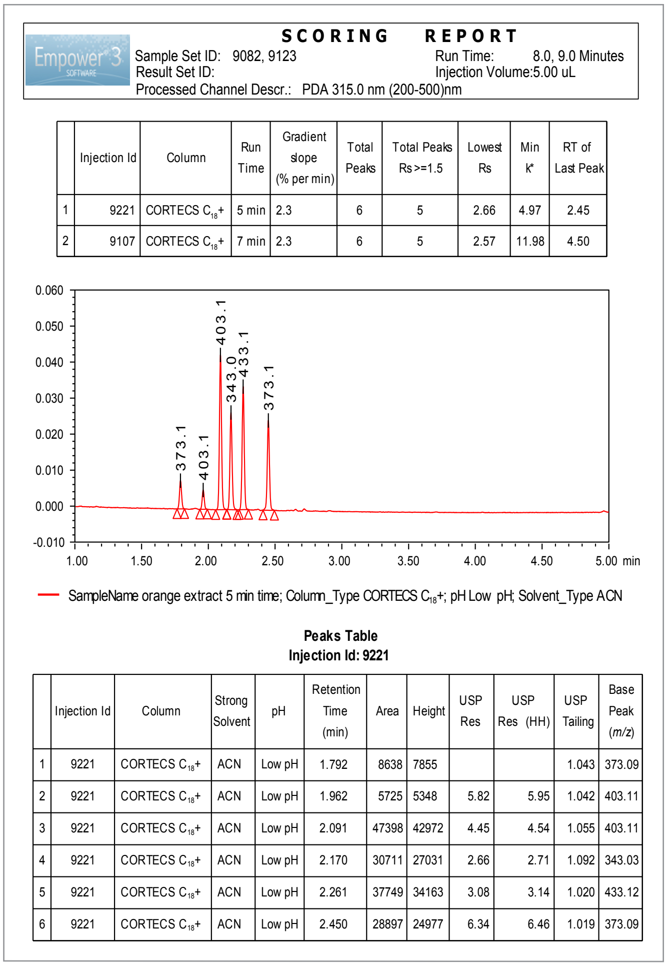
In Figure 11, Auto▪Blend Plus is used to evaluate the separation of ondansetron and related impurities. As discussed above (see “rapid scouting”), the bases shifted retention and changed selectivity with a large, pH shift. Exploring the pH range in smaller increments was postulated to lead to a wide range of differences in selectivities. In fact, using Auto▪Blend Plus, a change of one pH unit (9 to 10) was found to dramatically effect selectivity. Peak 2 (*m/z* 257.1) switched selectivity from the second eluting peak to the last eluting peak. This example illustrates the effect that a change of one pH unit can have on the retention and electivity. In addition, the online generation of mobile phase from pure solvents and concentrated stocks means fewer measurements in preparation.

EVALUATING OPTIMZATION RESULTS FOR FINAL SEPARATION

In addition to pH, a wide range of variables (temperature, gradient, flow rate, etc.) may be explored. The analyst can evaluate the effect of each of these variables with the aid of tools in the CDS. As described earlier, after the data has been processed, the automated report method may be customized, placing greater weight on key attributes. Figure 12 shows the report for optimizing the separation of flavonoids in an orange extract. In this example, our goals included complete separation of the flavonoids, USP resolution greater than 2.0 for all peaks, and a retention time of less than 3.0 minutes for all analytes.

The sample was evaluated under varying column temperatures and gradient slopes (not shown). The final adjustments were made to the run time (Figure 12 report). The final method conditions were specified as 35 °C, a gradient slope of 2.3% B change per column volume, and a run time of 5 minutes.

To provide assurance that all the flavonoids were fully resolved, each peak within the separation was evaluated by both UV and mass spectra. Comparison of the UV and mass spectra (Figure 13a) at the leading, apex, and tailing portions of the peak indicate its homogeneity. Each portion of the peak contained the same ions as well as the same UV spectra. Peak purity was also confirmed by the Empower 3 Software’s PDA Peak Purity algorithm (Figure 13b). As shown for peak 3 in the final method, the PDA peak-purity analysis shows the purity angle (green line) is lower than the purity threshold (blue line), indicating homogeneity of the peak. Therefore, method development for this sample is complete.



SCORING REPORT

Sample Set ID: 9082, 9123
Result Set ID:
Processed Channel Descr.: PDA 315.0 nm (200-500)nm
Run Time: 8.0, 9.0 Minutes
Injection Volume:5.00 uL

	Injection Id	Column	Run Time	Gradient slope (% per min)	Total Peaks	Total Peaks Rs>=1.5	Lowest Rs	Min k*	RT of Last Peak
1	9221	CORTECS C_{18}+	5 min	2.3	6	5	2.66	4.97	2.45
2	9107	CORTECS C_{18}+	7 min	2.3	6	5	2.57	11.98	4.50

SampleName orange extract 5 min time; Column_Type CORTECS C_{18}+; pH Low pH; Solvent_Type ACN

Peaks Table
Injection Id: 9221

	Injection Id	Column	Strong Solvent	pH	Retention Time (min)	Area	Height	USP Res	USP Res (HH)	USP Tailing	Base Peak (*m/z*)
1	9221	CORTECS C_{18}+	ACN	Low pH	1.792	8638	7855			1.043	373.09
2	9221	CORTECS C_{18}+	ACN	Low pH	1.962	5725	5348	5.82	5.95	1.042	403.11
3	9221	CORTECS C_{18}+	ACN	Low pH	2.091	47398	42972	4.45	4.54	1.055	403.11
4	9221	CORTECS C_{18}+	ACN	Low pH	2.170	30711	27031	2.66	2.71	1.092	343.03
5	9221	CORTECS C_{18}+	ACN	Low pH	2.261	37749	34163	3.08	3.14	1.020	433.12
6	9221	CORTECS C_{18}+	ACN	Low pH	2.450	28897	24977	6.34	6.46	1.019	373.09

Figure 12. Optimization report for separation of flavonoids in orange extract. Flexible reporting allows the analyst to select variables and criteria for desired separation conditions.

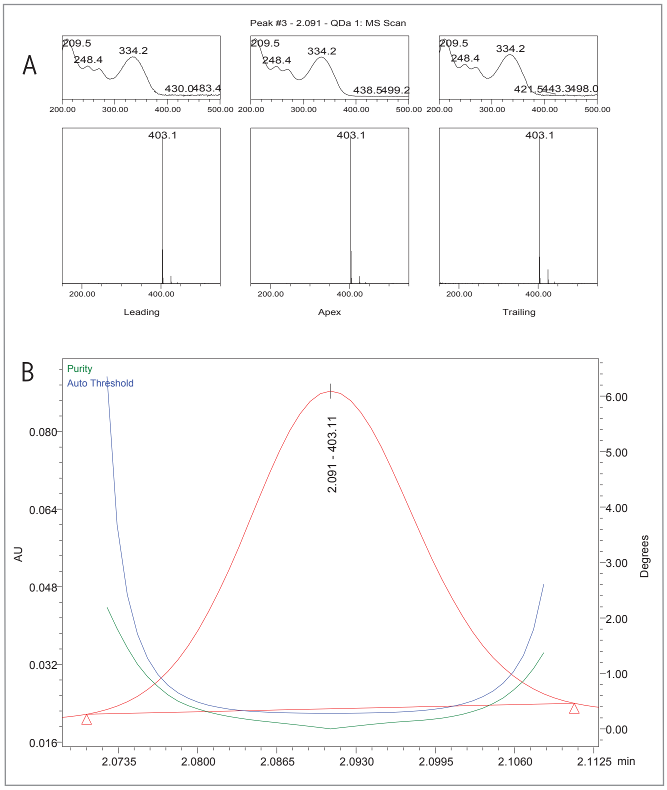


Figure 13. Peak purity view in Empower 3 software. Optimized separation of orange extract, peak 2. (a) Combined mass and UV spectral-data view of peak 2 in mass analysis window. Comparison of leading, apex, and trailing portions of peak indicate peak homogeneity. (b) PDA peak purity view of max plot indicates homogeneity of peak by UV.

CONCLUSIONS

Method development often requires a significant amount of resources both in labor and instrumentation. A thoughtfully chosen, synergistic system of columns, instrumentation, software, and mobile phases enables a streamlined, automated process for method development and the following tools for the method developer:

- A low-dispersion system, for improved throughput and higher resolution and sensitivity
- An automated system for screening multiple columns, mobile phases, and temperatures
- A quaternary mixing system to enable blending of stock buffers as well as the ability to program directly in units of pH
- A flow-through needle auto sampler to reduce carryover and ensure the entire sample reaches the column
- Column stationary phases that enable performing both high- and low-pH screening on a single column
- A wide range of available detectors to ensure complete sample characterization and detection of co-elutions
- An acquisition and processing-software platform that provides automated peak detection, data analysis, and reporting, all of which provide an analyst the means to readily evaluate data quality
- Customizable CDS calculations and reports, to aid in evaluating sample and separation criteria

The complete system solution equips the analytical chemist with the tools to develop a more robust, reliable, and reproducible separation. In doing so, it instills greater confidence that a method will provide consistent, accurate results and an increased opportunity for success in method validation.[16]

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[NOTES]

[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.

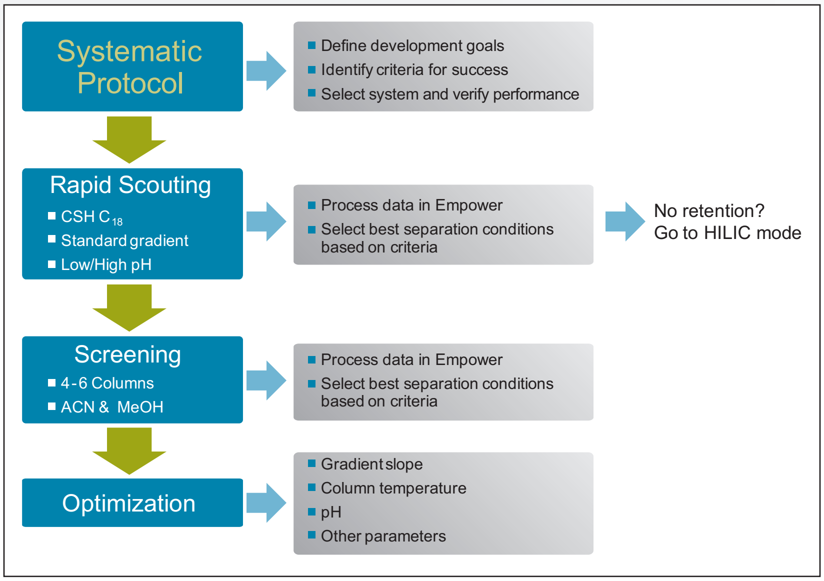

Figure 1. Systematic screening protocol for chromatographic method development.

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.

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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.

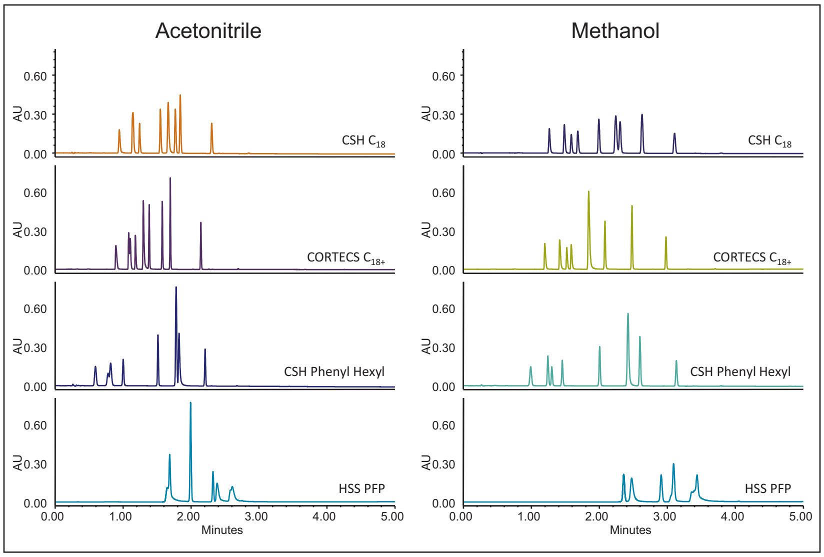
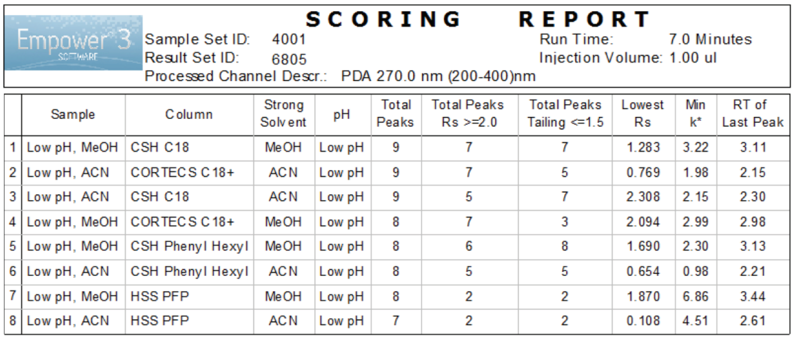


Figure 2. Separation of metoclopramide and USP-defined related substances. Both column chemistry and organic modifier were screened using a generic gradient.



SCORING REPORT

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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[APPLICATION NOTE]

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Improving Effectiveness in Method Development by Using a Systematic Screening Protocol for a USP Method (Metoclopramide HCl and Related Substances)

Margaret Maziarz, Sean M. McCarthy, and Mark Wrona
Waters Corporation, Milford, MA, USA

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 the USP-defined assay 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
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

System control, data acquisition, and analysis:

Empower 3 FR2 CDS Software

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]

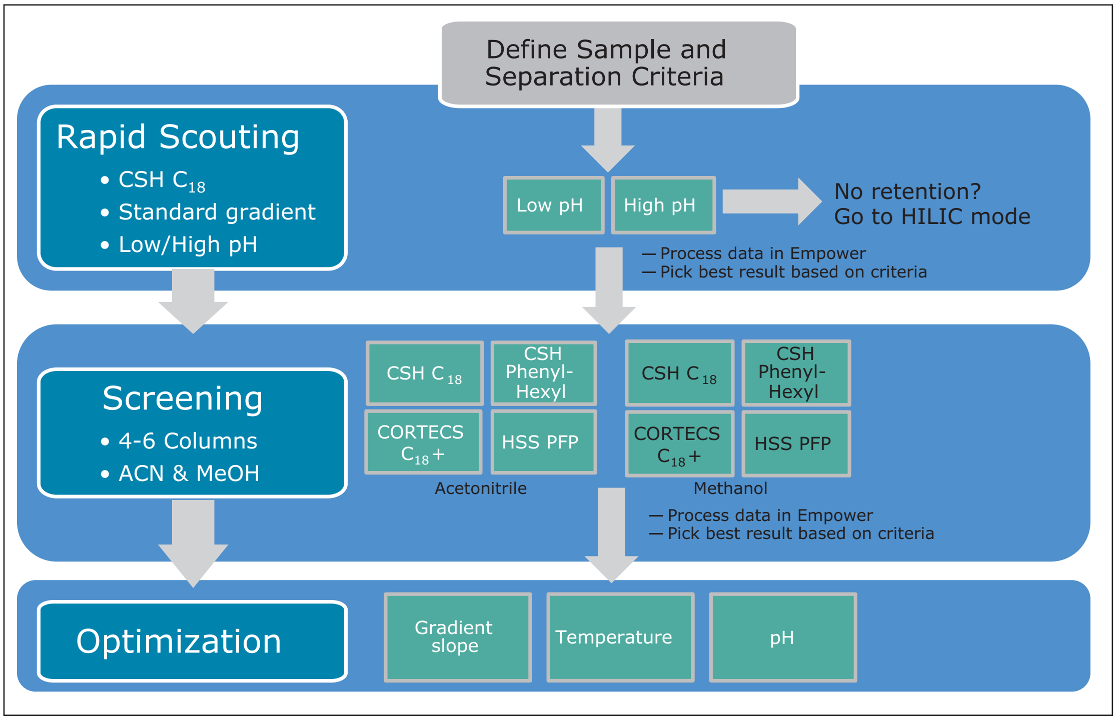


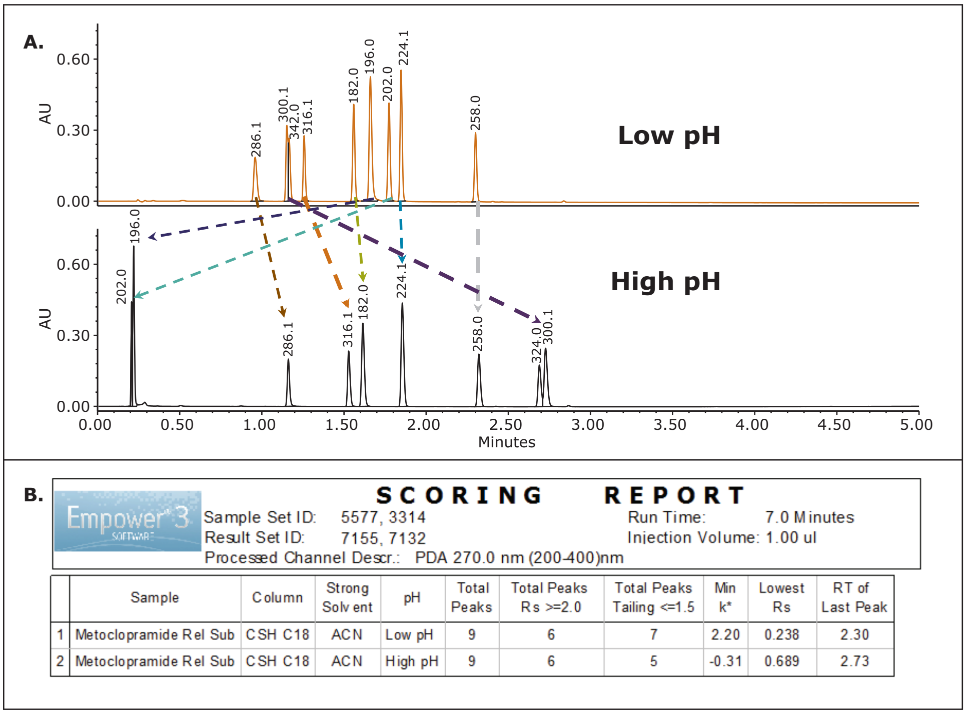
Figure 1. Systematic protocol for development of chromatographic methods.

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.



SCORING REPORT

Sample Set ID: 5577, 3314
Result Set ID: 7155, 7132
Processed Channel Descr.: PDA 270.0 nm (200-400)nm
Run Time: 7.0 Minutes
Injection Volume: 1.00 ul

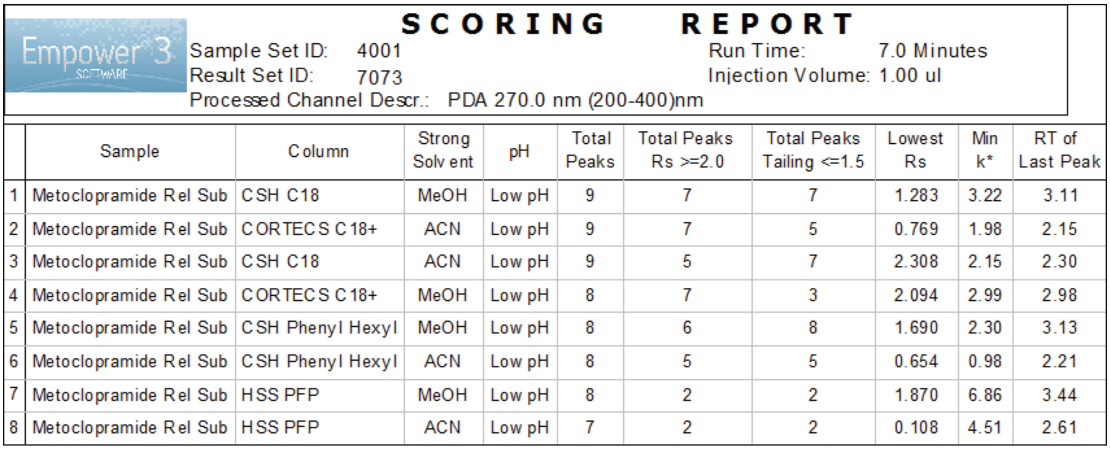
	Sample	Column	Strong Solv ent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Min k*	Lowest Rs	RT of Last Peak
1	Metoclopramide Rel Sub	CSH C18	ACN	Low pH	9	6	7	2.20	0.238	2.30
2	Metoclopramide Rel Sub	CSH C18	ACN	High pH	9	6	5	-0.31	0.689	2.73

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

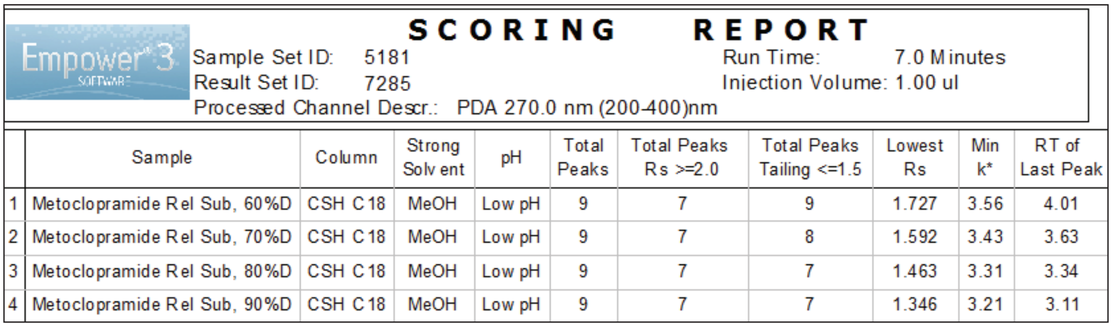
	Sample	Column	Strong Solv ent	pH	Total Peaks	Total Peaks Rs >=2.0	Total Peaks Tailing <=1.5	Lowest Rs	Min k*	RT of Last Peak
1	Metoclopramide Rel Sub	CSH C18	MeOH	Low pH	9	7	7	1.283	3.22	3.11
2	Metoclopramide Rel Sub	CORTECS C18+	ACN	Low pH	9	7	5	0.769	1.98	2.15
3	Metoclopramide Rel Sub	CSH C18	ACN	Low 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.

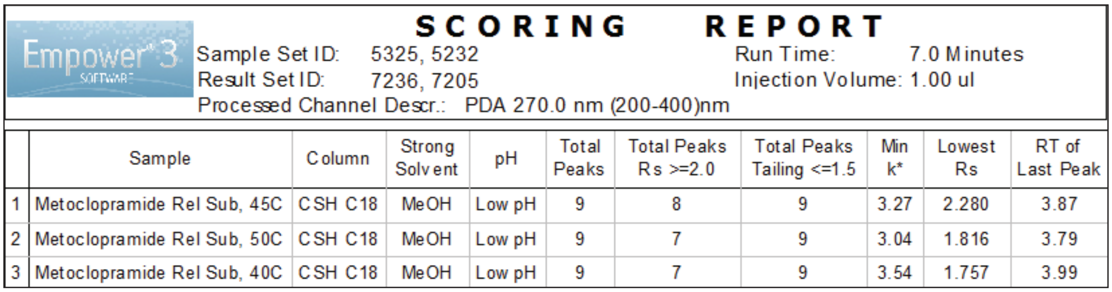


SCORING REPORT

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

	Sample	Column	Strong Solv ent	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

Empower 3 Software

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.

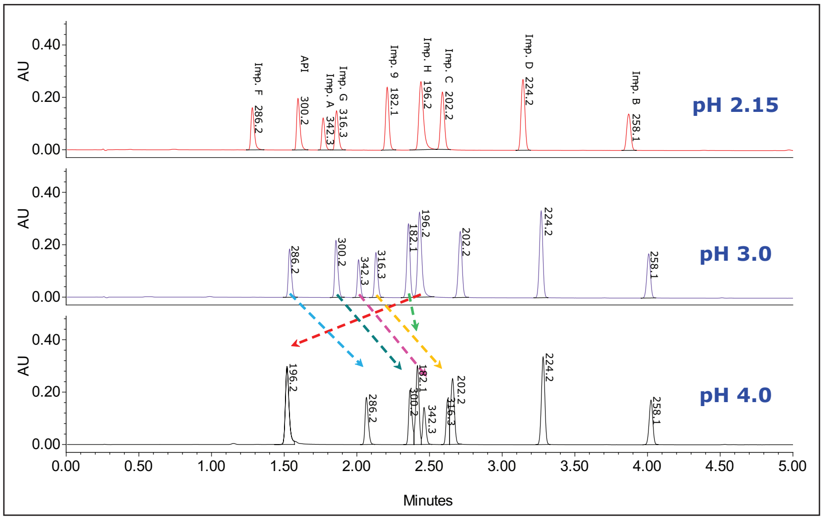
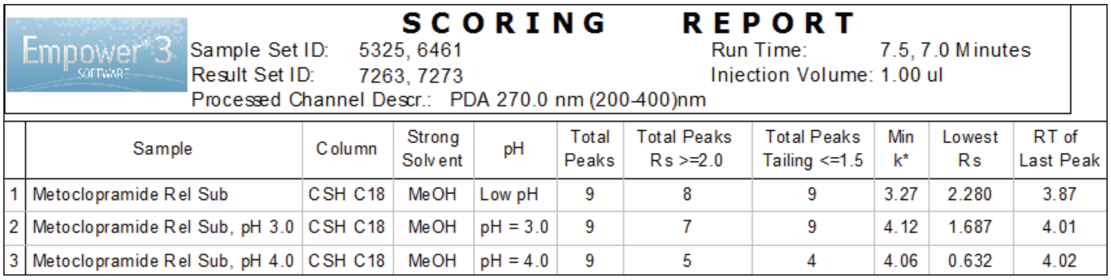


Figure 6. pH optimization to study the impact of pH on the separation of metoclopramide and related compounds. Peaks were tracked by mass detection using an ACQUITY QDa Detector. The best separation conditions were found to be at a pH of 2.15.



SCORING REPORT

Empower 3 Software

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

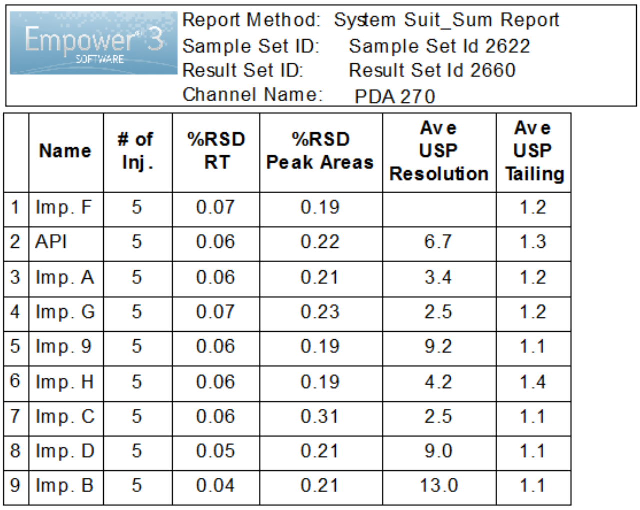
System control, data acquisition, and analysis:

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.



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. USP 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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[NOTES]

Developing Analytical Chromatographic Methods for Pharmaceutical Stability Investigations

Thomas Swann
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Understand factors, responses, response criteria, and strategies to consider when doing chromatographic method development.
- Learn the benefits and limitations of Quality by Design software assisted chromatographic method development.

WATERS SOLUTIONS

Empower® 3 CDS with S-Matrix Fusion QbD Software

ACQUITY Arc™

XBridge® BEH ***XP*** Column

XSelect® HSS ***XP*** Column

PDA Detector

ACQUITY® QDa® Mass Detector

KEYWORDS

Empower 3 CDS, Fusion QbD, Quality by Design (QbD), ACQUITY Arc, Method Validation Kit (MVK)

INTRODUCTION

An important aspect of pharmaceutical development is the determination of stability and shelf life for new pharmaceutical products. The ICH Quality Guidelines[1] specify the type and duration of stability investigations expected prior to drug registration in the European Union, Japan, and the United States. These are long term, accelerated, and stress studies where pharmaceutical companies use different exposure environments to evaluate drug quality over time.

The first two types of stability investigations assess shelf life. The temperatures and humidities used are, respectively, similar to and somewhat above that typically experienced by the drug product after manufacture. These studies require an analytical stability indicating method (SIM). This is a method capable of measuring drug and degradants in the presence of excipients and additives expected to be in the drug product formulation. Typically, SIMs are chromatographic methods. They are used to regularly monitor the time course appearance of impurities during long term and accelerated stability examinations.

The third type of study, stress, explores stability under harsher settings. Also called forced degradation studies, these investigations use reactive conditions (thermal, oxidative, photolytic, high/low pH, etc.) to produce "worst case" mixtures of drug and degradants. Method development with such difficult samples helps ensure the resulting analytical SIM has sufficient resolving power for the simpler mixtures from long term and accelerated stability investigations.

This application describes a case study where a Quality by Design (QbD) approach was used to develop an analytical stability indicating method for monitoring degradation of amoxicillin powder for oral suspension.

EXPERIMENTAL

Sample preparation

Amoxicillin Powder for Oral Suspension (APOS)

Amoxicillin Powder for Oral Suspension consists of the active pharmaceutical ingredient (API), amoxicillin, plus inactive excipients, and flavorants. The drug product dosage level is expressed as a target number of milligrams of amoxicillin per milliliter of aqueous suspension. This dosage level concentration is achieved when the powder is suspended in the requisite amount of water.

In this study, we used APOS manufactured at the 400 mg/mL dosage level. To prepare the stressed (forced degradation) sample required for this study, we suspended 40 mg APOS in 960 µL HPLC grade water. This mixture, in a sealed glass vial, was heated with magnetic stirring for 2 h at 90 °C. The resulting homogeneous solution was cooled to room temperature and then filtered (0.45 µm PDVF syringe filter). The filtered stock solution of stressed sample was stored frozen at -30 °C. As needed, the stock solution was thawed to remove a 40 µL aliquot. This aliquot was then diluted with 160 µL HPLC grade water to produce the working stressed sample solution of APOS for analysis.

Data management

Empower 3 Chromatographic Data System (Empower CDS) and Fusion QbD

Method conditions

System: ACQUITY Arc with Quaternary Solvent Manager (QSM-R), Sample Manager (FTN-R), Column Manager (CHC with 3 column select valve), PDA Detector, QDa Mass Detector, Isocratic Solvent Manager (ISM for QDa makeup flow)

Columns[2]: XBridge BEH C_{18} ***XP***, 2.5 µm, 3.0 x 50 mm (p/n: 186006033) (CV = 233 µL)
XBridge BEH Shield RP18 ***XP***, 2.5 µm, 3.0 x 50 mm (p/n: 186006057) (CV = 233 µL)
XSelect HSS T3 ***XP***, 2.5 µm, 3.0 x 50 mm (p/n: 186006153) (CV = 233 µL)

Mobile phase A: 20 mM aq formic acid (Weak Solvent 1, WS1)

Mobile phase B: Acetonitrile (Strong Solvent 1, SS1)

Mobile phase C: Methanol (Strong Solvent 2, SS2)

Mobile phase D: 20 mM aq ammonium formate (Weak Solvent 2, WS2)

On-line pH blending: Mobile phases A and D were blended in the following ratios to achieve set pH values

A/D = 100/0 gives pH 2.75
A/D = 80/20 gives pH 3.16
A/D = 40/60 gives pH 3.88
A/D = 5/95 gives pH 5.05
A/D = 0/100 gives pH 6.95

QSM-R flow rate: 1.10 mL/min

Profile: Equilibrate at 0% SS for 2.60 min (12.3 CV)
Isocratic at 0% SS for 0.46 min (2.2 CV).
Gradient from 0% to 25% SS for the following gradient times (normalized gradient slopes)
5.88 min (0.90% SS/CV)
8.82 min (0.60% SS/CV)
11.77 min (0.45% SS/CV)
14.71 min (0.36% SS/CV)
17.65 min (0.30% SS/CV)
Isocratic at 25% SS for 1.27 min (6.0 CV)
Gradient from 25% to 50% SS for 0.5 min (10.5 % SS/CV)
Isocratic at 50% SS for 1.27 min (6.0 CV).
Note: The % Strong Solvent plus the % Weak Solvents sum to 100%.

Column temp.: 30 °C

Detection (UV): 214 nm

Injection volume: 4.0 µL working solution

ISM flow rate: 0.1 mL/min (using 0.1% formic acid in 1:1 methanol/water)

ACQUITY QDa split ratio: 1:5

ACQUITY QDa settings: Ionization mode: positive and negative ESI
Cone voltage: 15 V
Capillary voltage: 1.5 kV (positive) and 0.8 kV (negative)
Mass range: 50–1000 *m/z*

BACKGROUND

Amoxicillin is a bactericidal antibiotic that functions by irreversibly binding to the protein that catalyzes bacterial cell wall cross-linking, via opening of the strained beta-lactam ring. This capability makes a potent and selective drug but the resulting lability presents challenges to manufacturers who need to maintain (and confirm) good drug product storage stability. An analytical SIM capable of measuring the amoxicillin and its breakdown impurities is therefore imperative.

Figure 1 depicts amoxicillin degradation pathways. Prominent breakdown modes are hydrolysis of the four membered ring and of the central amide linkage. Subsequent self-reaction, decarboxylation and/or cyclization sequences lead to a variety of breakdown products. Amoxicillin is a polar amino acid and the typical degradants are also polar with many having both acidic and basic functionality. The polarity limits the mobile phase options to weaker solvent blends. The contrasting chargeable groups emphasize the need for optimal choice of mobile phase pH.

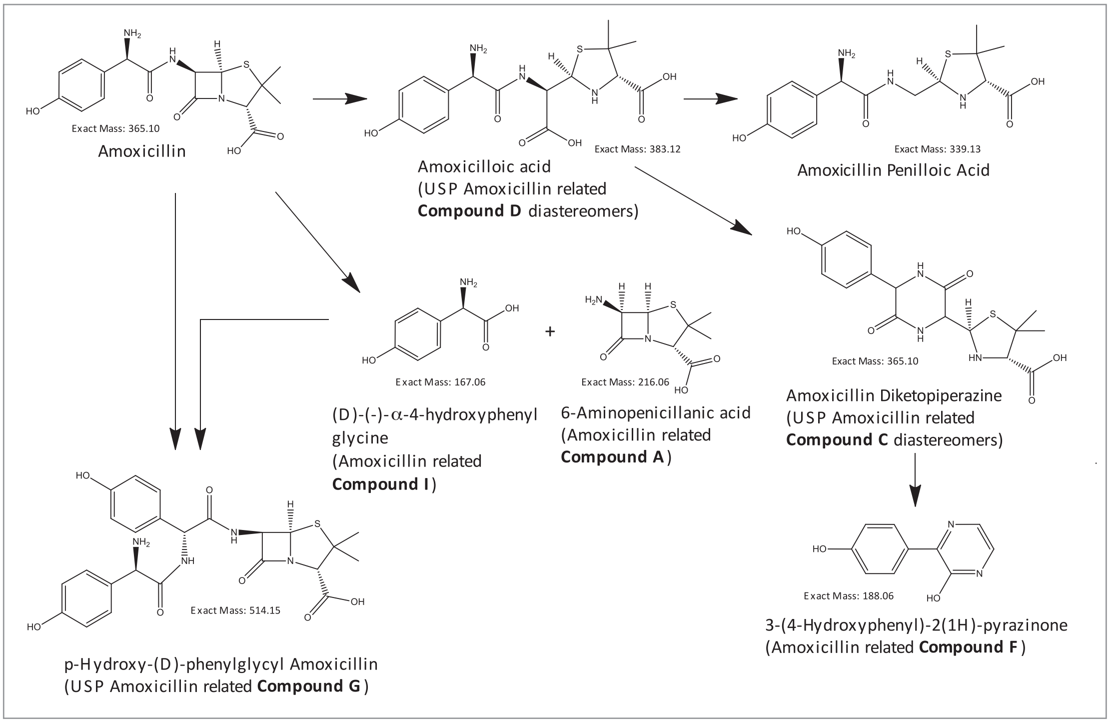


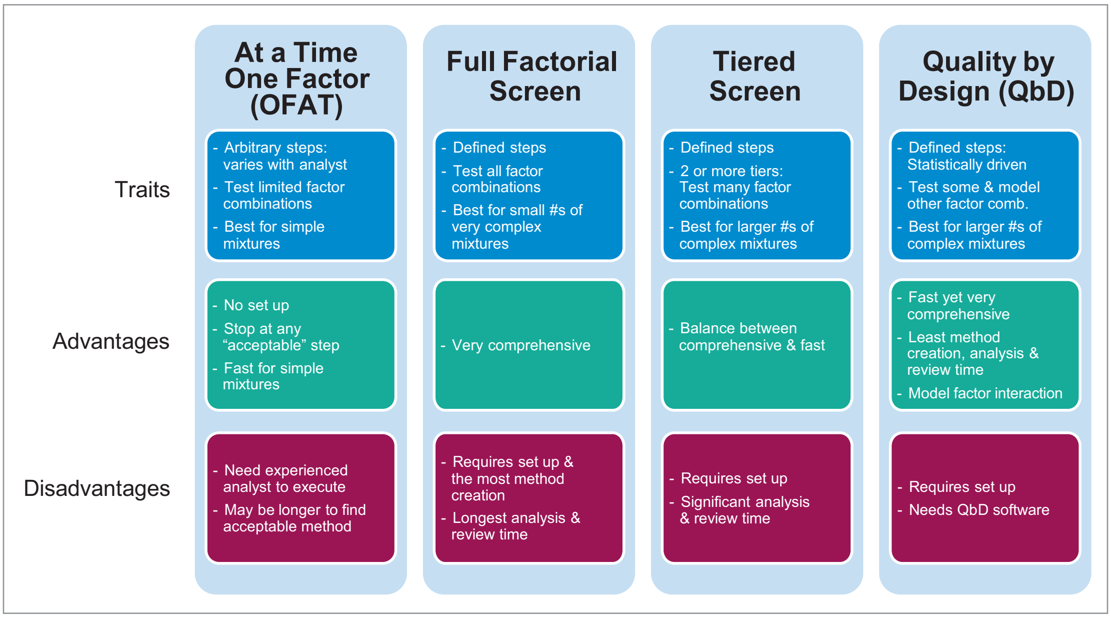
Figure 1. Amoxicillin and some degradation pathways.

The development of an analytical chromatographic method has three discreet stages: Set up, screen, and optimize. In the set up stage, the analyst selects the factors to vary, the responses to measure, the response criteria to assess progress, and the strategy to follow. Factors are the "inputs" to method development that cause analyte selectivity and resolution changes. Some factors, like the strong solvent, pH, and column stationary phase, have stronger effects whereas others, like gradient slope[3] and column temperature, have weaker effects. Furthermore, some factors are numeric and continuously variable, such as column temperature, pH, and gradient slope. Other factors are "categorical" or non-numeric, like column stationary phase. For each factor selected, the factor values are then set, such as which exact columns, solvents, and pH values to use.

Responses are the "outputs" to method development, as measured from chromatograms. Some responses are specific peak properties such as retention time, resolution, and tailing for specific components. Other responses are chromatogram properties from peak counting (aggregation), as in the total number of peaks in a chromatogram or the number of peaks with a specific desirable result.

Response criteria are used to compare the quality of different factor combinations. Examples of chromatogram property response criteria include "total # of peaks in the chromatogram = # of components known or believed to be present" or "maximize # of peaks found". For peak property response criteria, these include retention, tailing, and resolution in a specific range.

Figure 2 lists four method development strategies. The simplest strategy to execute but the least comprehensive is "One Factor At a Time" or OFAT, where the analyst alternates between picking one factor and value to test and assessing the responses from that test. The most comprehensive and labor/time intensive strategy uses a Full Factorial Screen where all combinations of factor values are tested to determine the best combination. A hybrid of these two approaches is the Tiered Screen. Here, all factors except one are locked to a specific value (e.g. a single specific column stationary phase, strong solvent, temperature, etc.). The one factor allowed to vary is changed to assess its affect on the responses. For example, the pH may be varied between a low and a high value. Based on how well response criteria are met, the one factor varied in the first tier is fixed to a single value. Moving to the second tier, some of the original locked factors are now systematically varied in full factorial fashion. This strategy is a balance between being comprehensive and being fast.



	At a Time One Factor (OFAT)	Full Factorial Screen	Tiered Screen	Quality by Design (QbD)
Traits	- Arbitrary steps: varies with analyst - Test limited factor combinations - Best for simple mixtures	- Defined steps - Test all factor combinations - Best for small #s of very complex mixtures	- Defined steps - 2 or more tiers: Test many factor combinations - Best for larger #s of complex mixtures	- Defined steps: Statistically driven - Test some & model other factor comb. - Best for larger #s of complex mixtures
Advantages	- No set up - Stop at any "acceptable" step - Fast for simple mixtures	- Very comprehensive	- Balance between comprehensive & fast	- Fast yet very comprehensive - Least method creation, analysis & review time - Model factor interaction
Disadvantages	- Need experienced analyst to execute - May be longer to find acceptable method	- Requires set up & the most method creation - Longest analysis & review time	- Requires set up - Significant analysis & review time	- Requires set up - Needs QbD software

Figure 2. Comparison of different method development strategies.

These three strategies are examples of "analyst only" method development strategies. More recently, "analyst with software assist" strategies have appeared. These use specialized software to model the relationships between the varied factors and the measured responses. Such software falls into one of three categories: Statistical Based Modeling, Structure Based Modeling, and Chromatographic Theory Based Modeling. Statistical Based Modeling uses the Design of Experiments (DoE) approach and Fractional Factorial design to model Full Factorial space. In the pharmaceutical industry, this is called Quality by Design or QbD. Structure Based Modeling uses knowledge of analyte structures and functional group properties to create a model of analyte, stationary phase and mobile phase interactions. Chromatographic Theory Based Modeling uses a framework such as solvophobic theory to also create a model of analyte, stationary phase, and mobile phase interactions.

The "analyst only" strategies could be applied to the development of a SIM but each has disadvantages for complex samples that a stability indicating method may have to deal with. Following an "analyst with software assist" course generally gives a more comprehensive understanding of the chromatographic space with fewer actual conditions run. It has the further benefit of specialized method development software to manage the investigation and to review/interpret the data. The structure based modeling software is not very appropriate for the stress samples used in SIM development since the structures of all components are not known. Either the statistical based or the chromatographic theory based modeling categories are suitable for the development of a SIM.

QbD has an advantage in that it excels at modeling the comprehensive understanding afforded by a Full Factorial approach at low "cost". The "cost" in this case is a smaller but representative number of factor values and combinations that are run. Figure 3 shows a generic schematic of runs (factor combination points) needed to perform a generic Full Factorial screen with four factors.

Full Factorial
(Measure all points)

- E.g. for 3 **gradient times (slopes)**, 3 **pHs**, 3 **columns**, 2 **solvents**
  - 3 x 3 x 3 x 2 = 54 Factor combinations points (all that are possible)

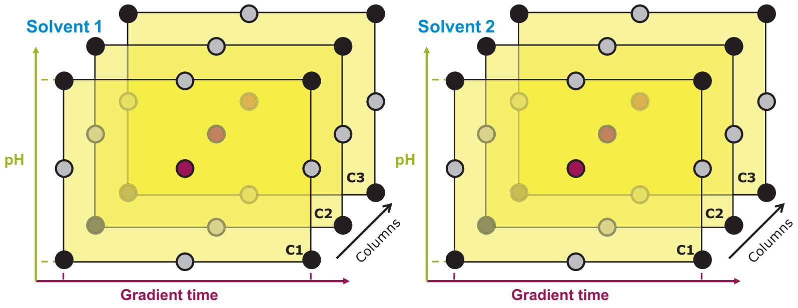


Figure 3. Generic schematic example for Full Factorial measurements at all factor combination points.

Figure 4 graphically depicts the same factor combination space but uses the Fractional Factorial screen of QbD wherein some factor combination points are not run but are still inferred due to appropriate sampling.

Fractional Factorial in QbD
(Measure some points & model remainder)

- E.g. for 3 **gradient times (slopes)**, 3 **pHs**, 3 **columns**, 2 **solvents**
  - Software selects 12 + 11 = 23 Factor combination points (ca. 57% reduction).
  - Uses measured responses to model & predict responses for untested factor combinations.

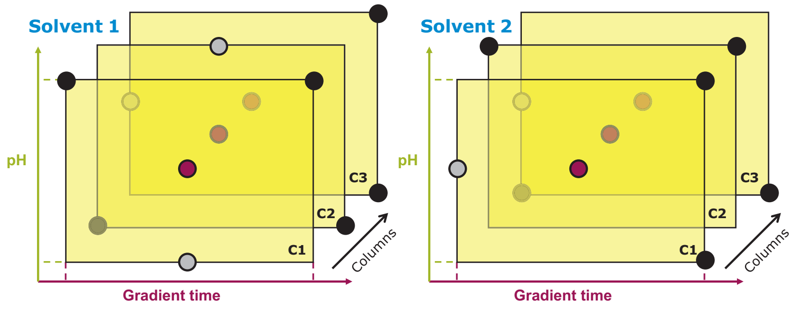


Figure 4. Generic schematic example for Fractional Factorial measurements at balanced and orthogonally selected factor combination points.

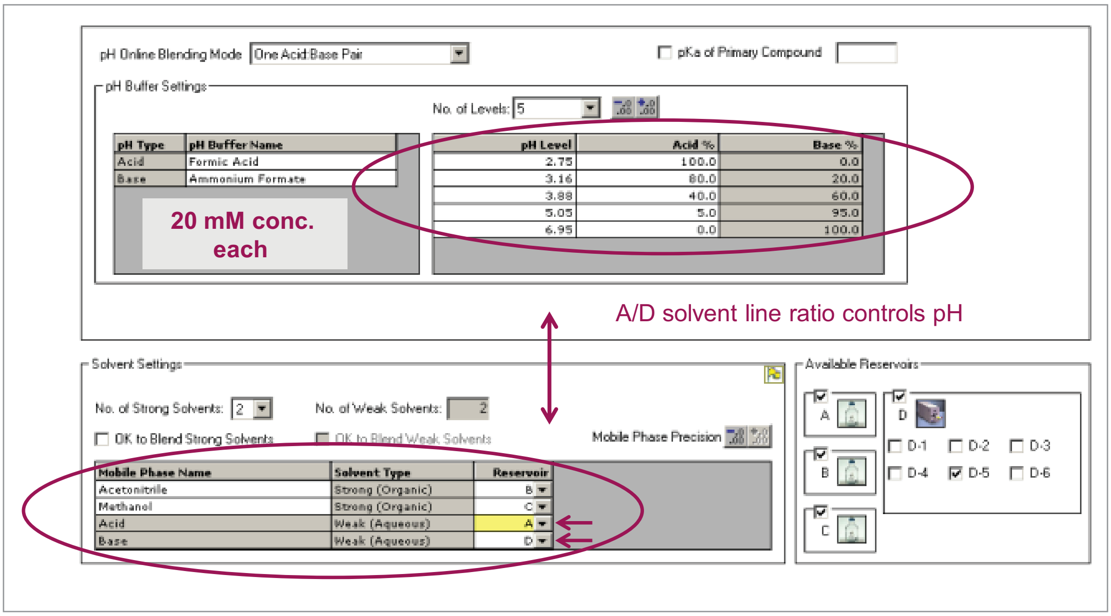
Where the statistical based modeling software is Fusion QbD by S-Matrix, there is the additional advantage of tight integration with the Empower CDS. The Fusion QbD Software manages all aspects of the method development. These include study design with balanced and orthogonal selection of factor values and combinations, automatic acquisition method creation in Empower, retrieval and processing of chromatographic data and responses from Empower, and scoring of results plus graphical display of factor values and combinations vs. responses. For these reasons, we used the Quality by Design approach in this application note.

RESULTS AND DISCUSSION

Our study set up began by selecting all categorical factors and their values to screen since such factors are modeled differently from numerical factors and need to be specified first. In this case, our categorical factors were column stationary phase and strong solvent. For the column stationary phase, we first picked XBridge BEH C_{18} for good all around reverse phase selectivity. We then chose XBridge BEH Shield RP18 which offers additional interactions from the embedded polar group and XSelect HSS T3 which provides additional silanol interactions. Both are potentially beneficial for the analysis of polar compounds. The strong solvents were acetonitrile and methanol. Since we used the ACQUITY Arc, a UHPLC class instrument, it was important to match the instrument dispersion and pressure capabilities to the appropriate column diameter and particle size. Also a short column length is desirable for the creation of rapid higher flow methods. These considerations led us to select a column geometry of 3.0 x 50 mm with 2.5 µm particles.

We also selected some numerical factors with specific values to screen. The mobile phase pH was picked since it is the numerical factor with the strongest effect on analyte selectivity and peak shape when acidic and basic functional groups are present. As stated above, this is especially important when developing an amoxicillin SIM. The five pH values in the Experimental section, for the pH range of 3 to 7, allowed use of all three columns. A second numerical factor of gradient slope was also selected (see the five specific values in the Experimental section). Examining this weaker effect was important to assess how this “method speed” factor[4] can impact the measured responses. The other “method speed” factor of flow rate was set to a “ballistic” value that achieves ca. 80% of the maximum instrument pressure (ca. 7600 out of 9500 psi) using the more viscous methanol strong solvent. This and other fixed factor values are in the Experimental section.

Using three columns, two solvents, five pHs and five gradient slopes, the Full Factorial number of combinations to run is 150 (3x2x5x5). To get the benefit of this rich data space at a fraction of the analysis (and analyst) time “cost”, we selected the “analyst with software assist” strategy of Statistical Based Modeling using Fusion QbD Software. Figure 5 contains a Fusion QbD screen image specifying the strong solvents and the on-line blending to produce the pH values. Figure 6 depicts the set up for the gradient slopes and the columns.



pH Online Blending Mode: One Acid:Base Pair

☐ pKa of Primary Compound

pH Buffer Settings

No. of Levels: 5

pH Type	pH Buffer Name
Acid	Formic Acid
Base	Ammonium Formate

pH Level	Acid %	Base %
2.75	100.0	0.0
3.16	80.0	20.0
3.88	40.0	60.0
5.05	5.0	95.0
6.95	0.0	100.0

20 mM conc. each

A/D solvent line ratio controls pH

Solvent Settings

No. of Strong Solvents: 2

No. of Weak Solvents: 2

☐ OK to Blend Strong Solvents

☐ OK to Blend Weak Solvents

Mobile Phase Name	Solvent Type	Reservoir
Acetonitrile	Strong (Organic)	B
Methanol	Strong (Organic)	C
Acid	Weak (Aqueous)	A
Base	Weak (Aqueous)	D

Available Reservoirs

☑ A

☑ B

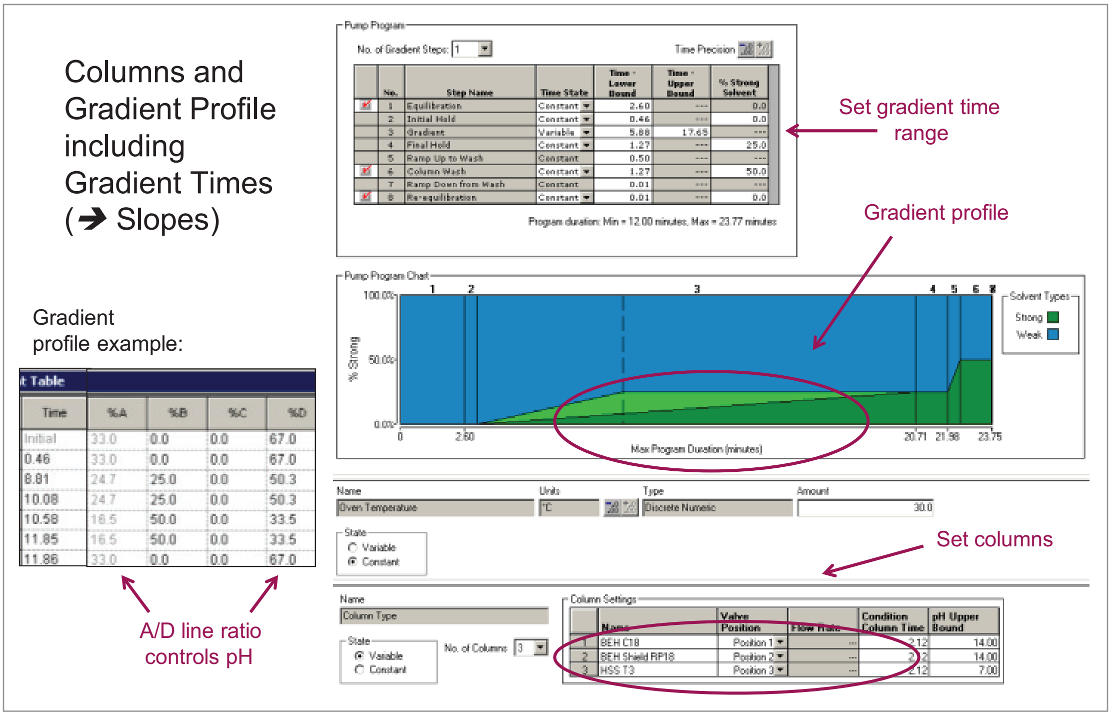
☑ C

☑ D

☐ D-1 ☐ D-2 ☐ D-3

☐ D-4 ☑ D-5 ☐ D-6

Figure 5. Fusion QbD set up of solvents and pH on-line blending.



Columns and Gradient Profile including Gradient Times (➔ Slopes)

Gradient profile example:

Time	%A	%B	%C	%D
Initial	33.0	0.0	0.0	67.0
0.46	33.0	0.0	0.0	67.0
8.81	24.7	25.0	0.0	50.3
10.08	24.7	25.0	0.0	50.3
10.58	16.5	50.0	0.0	33.5
11.85	16.5	50.0	0.0	33.5
11.86	33.0	0.0	0.0	67.0

A/D line ratio controls pH

Pump Program

No. of Gradient Steps: 1

	No.	Step Name	Time State	Time - Lower Bound	Time - Upper Bound	% Strong Solvent
☑	1	Equilibration	Constant	2.60	---	0.0
	2	Initial Hold	Constant	0.46	---	0.0
	3	Gradient	Variable	5.88	17.65	---
	4	Final Hold	Constant	1.27	---	25.0
	5	Ramp Up to Wash	Constant	0.50	---	---
☑	6	Column Wash	Constant	1.27	---	50.0
	7	Ramp Down from Wash	Constant	0.01	---	---
☑	8	Re-equilibration	Constant	0.01	---	0.0

Program duration: Min = 12.00 minutes, Max = 23.77 minutes

Set gradient time range

Gradient profile

Name: Oven Temperature
Units: °C
Type: Discrete Numeric
Amount: 30.0

State
○ Variable
● Constant

Set columns

Name: Column Type

State
● Variable
○ Constant

No. of Columns: 3

Column Settings

	Name	Valve Position	Flow Rate	Condition Column Time	pH Upper Bound
1	BEH C18	Position 1	---	2.12	14.00
2	BEH Shield RP18	Position 2	---	2.12	14.00
3	HSS T3	Position 3	---	2.12	7.00

Figure 6. Fusion QbD set up of gradient slopes and columns.

This established four factors and their values to screen. Next, we set the responses to measure and the response criteria to drive towards. For chromatogram property responses, we used the "Total Number Of Peaks Found" and the "Number Of Peaks With A USP Resolution of ≥1.50 and ≥2.00." The response criteria were to maximize these peak counts.

For peak property responses, we used "Max Peak #1 USP Resolution" and "Max Peak #1 USP Tailing." In Fusion QbD parlance, "Max Peak #1" is the largest peak observed in a chromatogram, in our case, the API. So here, the "Max Peak #1 USP Resolution" response measured specific API peak separation quality and the "Max Peak #1 USP Tailing" measured specific API peak shape quality. The response criterion for "Max Peak #1 USP Resolution" was ≥1.50 and for "Max Peak #1 USP Tailing" was = 1.00 ± 0.10.

From these factors and factor values, Fusion QbD selected 38 combinations to run. Of these, 29 factor combinations modeled the full factorial space and 9 provided replicates to assess the measurement uncertainties. This eliminated ca. 80% of the injections required for full factorial screening and greatly shortened the acquisition and data review time. Even this reduced set of work required 67 instrument methods and 67 method sets to handle column conditioning, equilibration, and the actual runs. Fortunately, Fusion QbD created all of these Empower methods plus the one sample set method needed to orchestrate these acquisitions.

Empower used the acquisition methods created by Fusion QbD to screen the APOS forced degradation sample under the Quality by Design selected conditions. The stressed conditions generated many degradants as Figure 7 illustrates in a representative chromatogram. Five Empower processing methods, one for each gradient slope/time, were used to objectively find and integrate peaks. All processing methods applied the ApexTrack™ integration algorithm and had the same peak integration parameters. The methods differed by having [1] increased apex detection peak width and [2] extended chromatogram end times as the gradient slope decreased (gradient time increased).

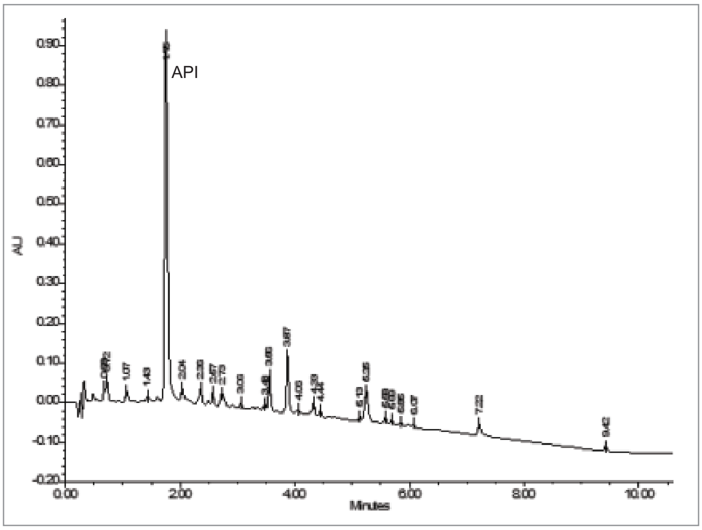


Figure 7. Representative APOS stress sample chromatogram.

This accounted for broadened peaks and longer acquisitions at shallower gradients. To focus only on the more abundant degradants, a minimum peak area was set equal to 1% of the average API peak area.

Fusion QbD fetched the processed data from Empower and used the measured responses to calculate models for each categorical factor combination. For each such model, a Cumulative Desirability Result score was calculated. This score (0 to 1 scale) assesses the likelihood that each combination will meet the response criteria. Table 1 summarizes these study results. The high score for the combination of acetonitrile as the strong solvent and the BEH C_{18} as the column stationary phase made clear each was the best choice for these categorical factors.

With the column and strong solvent categorical factor values selected, the Fusion QbD model provided a "performance map" for the numerical factors of gradient time and pH. A performance map segments numerical factor space into regions of predicted better response (closer to target criteria) and worse response (further from target criteria). It is built by sequentially plotting the effect of the numerical factors on each response.

Figure 8a shows a blank map where no responses are considered. The entire gradient time vs pH space is "white" because any such combination is acceptable when it doesn't matter what the responses are. Considering the chromatogram property response of "total number of peaks found", we set a threshold such that about a third of the factor space is colored and therefore rejected as lower performance (fewer peaks found), Figure 8b. In our case, this is somewhat arbitrary and up to the analyst's discretion since, in a stress sample, we have no way of knowing how many peaks "should" be present.

The chromatogram property responses "Number of Peaks With USP Rs ≥1.50 and ≥2.00" were considered next. Again, rather arbitrary thresholds were applied, Figure 8c and Figure 8d, to exclude underperforming factor combinations. The excluded regions had some overlap with the first chromatogram property but they mostly eliminated the higher pH region.

Table 1. Cumulative Desirability Result scores for strong solvent and column stationary phase categorical factor combinations.

Cumulative Desirability Result (scale of 0 to 1)	BEH C_{18}	BEH Shield RP18	HSS T3
Acetonitrile	0.9360	0.7476	0.3826
Methanol	0.7735	0.5279	0.7480

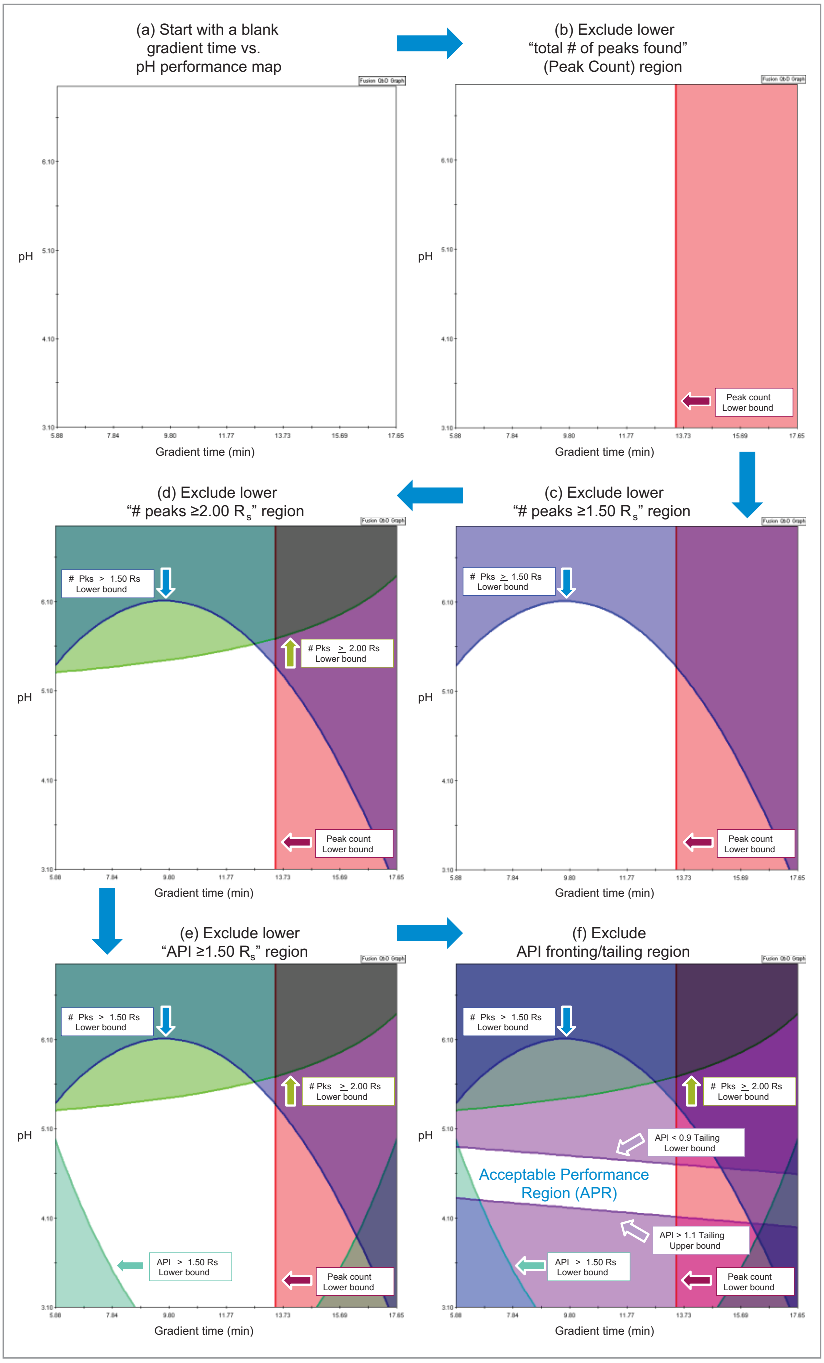


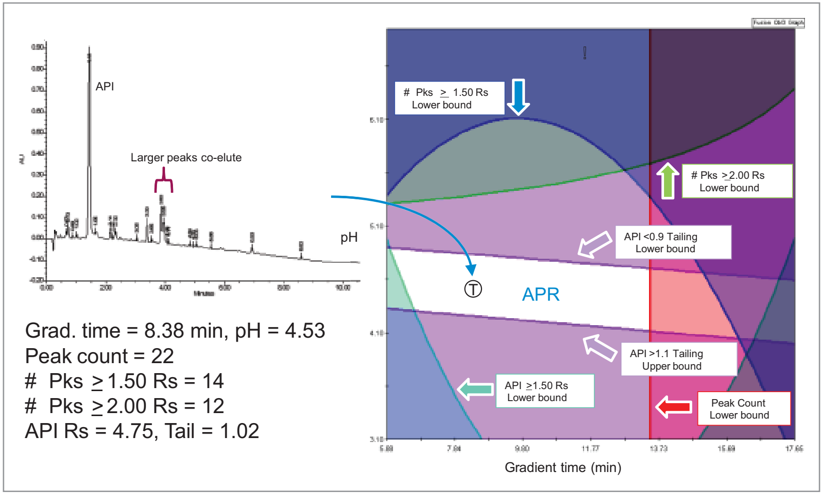
Figure 8. Construction of the Fusion QbD performance map.

Unlike the chromatogram property responses, which involved peak counting for an unknown number of degradants, specific peak property responses had well defined expectations. In Figure 8e, we applied the threshold “Max Peak #1 USP Resolution ≥1.50” response criterion. This rejected the lower left and right corners with low pH and both low and high gradient times. Finally, we used the threshold “Max Peak #1 USP Tailing = 1.00 ± 0.10” which eliminated all combinations except those in a relatively narrow pH range, Figure 8f. The unrejected white space is called the modeled Acceptable Performance Region (APR). This is the region in and around which verification test runs are next performed and where method optimization then occurs.

Figure 9 shows a verification chromatogram obtained at the lower end of the gradient time range and at the middle of the pH range defined by the APR. The measured API resolution was acceptable and consistent with the model. However, the resolution that is being optimized is a “before peak” resolution and it is obvious that this chromatogram has a poor “after peak” resolution from a component that closely follows the API. Also there are some larger peaks that coelute.

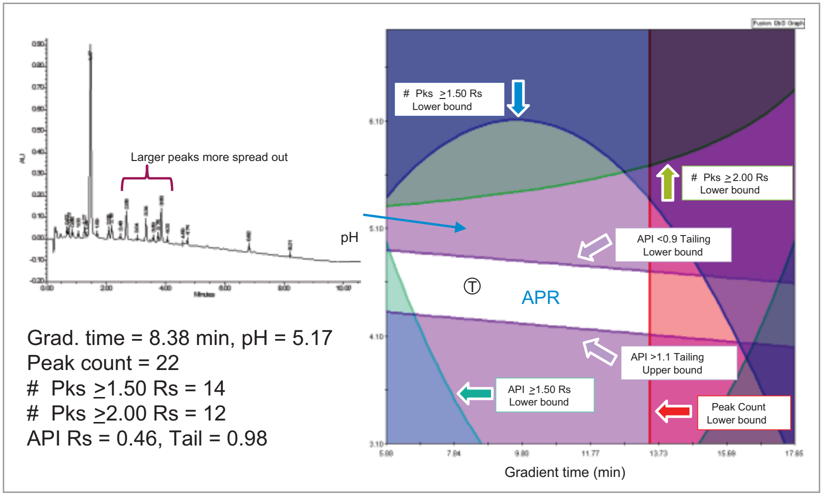
Verification testing above the APR, Figure 10, found the API resolution lower than the model predicted (observed USP Rs = 0.46; expected ≥1.50) but the larger peaks were more favorably spread out. This suggested that a pH somewhat outside of what the model indicated may provide useful separation for some peaks.

Testing below the APR, Figure 11, we found the resolution and tailing as expected (≥1.50 and >1.1, respectively), the peak counts were somewhat improved and the larger peaks were more spread out. These results illustrate the importance of doing verification runs in the vicinity of the APR, as software assistance provides only a guide on where to focus one’s efforts.



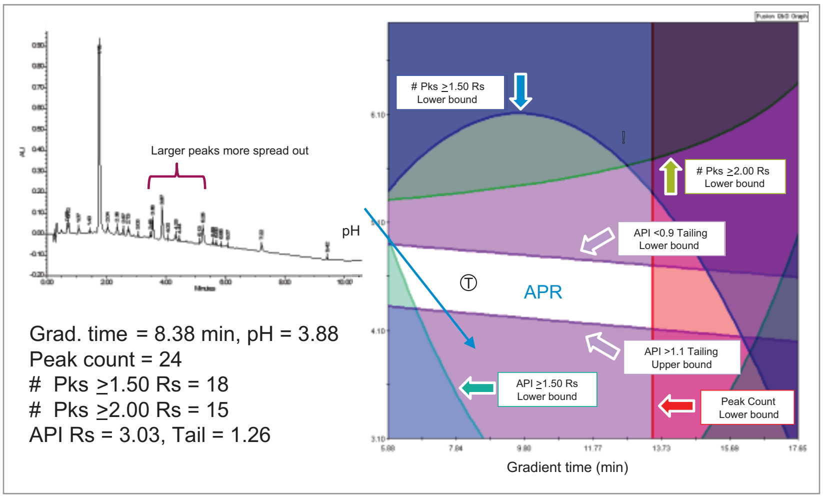
Grad. time = 8.38 min, pH = 4.53
Peak count = 22
Pks ≥1.50 Rs = 14
Pks ≥2.00 Rs = 12
API Rs = 4.75, Tail = 1.02

Figure 9. Example chromatogram within the modeled Acceptable Performance Region (APR).



Grad. time = 8.38 min, pH = 5.17
Peak count = 22
Pks ≥1.50 Rs = 14
Pks ≥2.00 Rs = 12
API Rs = 0.46, Tail = 0.98

Figure 10. Example chromatogram above the modeled Acceptable Performance Region (APR).



Grad. time = 8.38 min, pH = 3.88
Peak count = 24
Pks ≥1.50 Rs = 18
Pks ≥2.00 Rs = 15
API Rs = 3.03, Tail = 1.26

Figure 11. Example chromatogram below the modeled Acceptable Performance Region (APR).

The combination of the modeled APR plus the results from verification test runs allowed us to set an optimization region. In this study, the APR had a rather wide gradient time range but a relatively narrow pH range. The "width" of the optimization region was therefore set at the lower end of the APR gradient times to generate a faster final method. The "height" was set somewhat above and below the pH range defined by the APR for the reasons given above. Figure 12 depicts the optimization region.

For specific optimization factor values, we selected gradient times of 6.80, 8.35, and 9.90 min and pH values of 4.01, 4.49, and 5.04. Additionally, we added optimization factor values for column temperatures of 30, 35, and 40 °C. Some example chromatograms from the optimization are given in Figure 13. Coelution of degradants in the latter part of or just after the API peak was a common theme. Both situations are "invisible" to Fusion QbD.[5] The former condition can only be observed using the Empower Mass Analysis window tool (shown in the insets of these figures) whereas the latter can be found by the analyst on visual inspection of the chromatogram. Coelutions among degradants were also observed during optimization. This also presents a challenge, since Fusion QbD does not track individual degradant peaks. As a result, the optimization stage of method development may not benefit as much from the current generation of software assistance compared to the great advantages such help affords during screening.

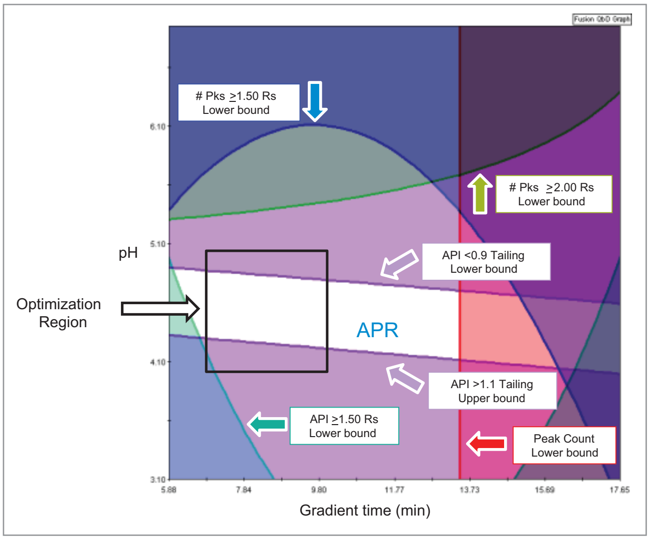


Figure 12. Fusion QbD Performance Map with the optimization region.

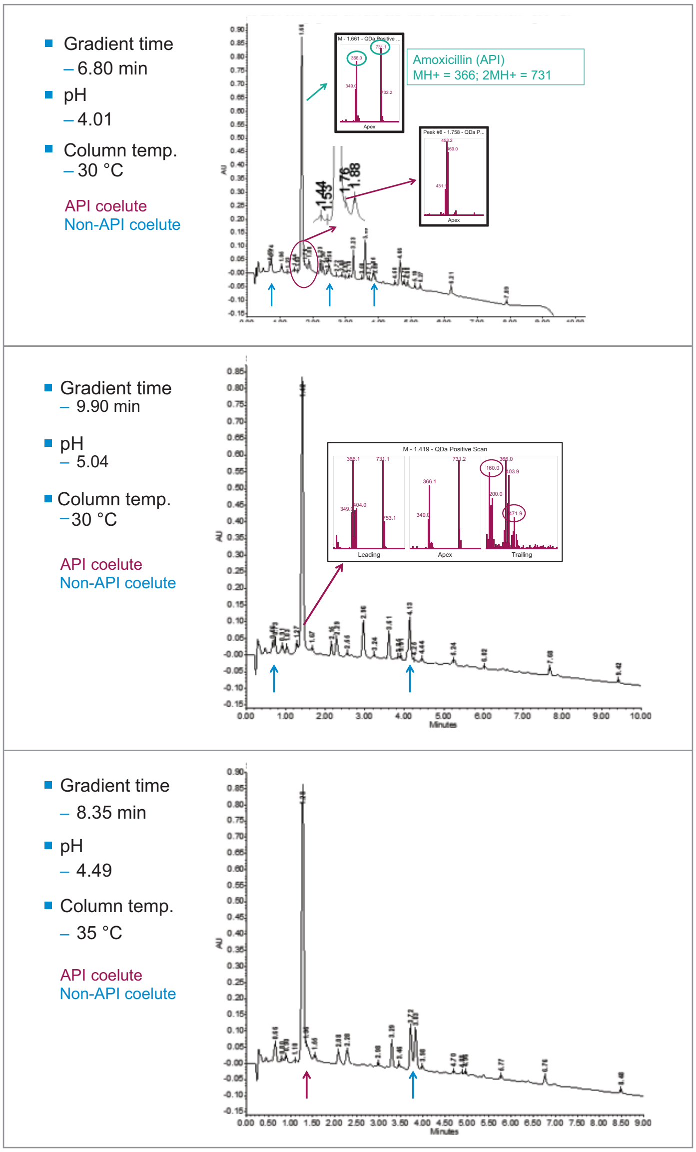


Figure 13. Example optimization chromatograms.

Figure 14 lists the optimized factor values for gradient time, pH, and column temperature and shows the resulting chromatogram. The Empower Mass Analysis window indicated good peak purity (homogeneity) for the API peak and there was acceptable resolution for the degradant peaks. Using these conditions, standard injections confirmed the identity of the indicated components.

The final chromatographic SIM after QbD software assisted screening and optimization is displayed in Figure 15. The best pH was outside of the APR and near the lower edge of the Optimization Region. This reinforces the need to treat the software assistance as a guide that must be verified. The best column temperature was found during the Optimization stage.

Once a final method is created, it is a good idea to validate that method on different column batches. This allows assessment of the method robustness to any small changes in different manufacturing lots of stationary phase particles or variations in column packing. After optimization, we therefore used an

XBridge BEH C_{18} ***XP***, 2.5 µm, 3.0 x 50 mm Method Validation Kit (MVK) (p/n: 186006199) and compared our method results from the different column batches with the results from the original column. As demonstrated in Figure 16, equivalent chromatograms were observed for all the columns.

- Gradient time
  - 8.35 min
- pH
  - 4.01
- Column temp.
  - 35 °C

- Good API peak purity
- Confirmed some components (std. inj.)
  - Related compounds C and D (as diastereomers)
  - APOS excipient component

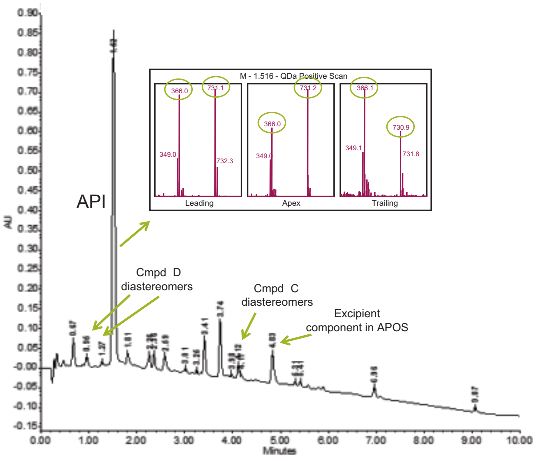


Figure 14. Chromatogram using best optimization conditions.

- System: ACQUITY Arc with PDA and QDa detection, 3 column positions
- Column: XBridge BEH C_{18} ***XP***, 2.5 µm, 3.0 x 50 mm
- Mobile phase A: 20 mM aq formic acid (for online pH blending)
- Mobile phase B: Acetonitrile
- Mobile phase D: 20 mM aq ammonium formate (for online pH blending)
- Flow rate: 1.10 mL/min
- Gradient: See Table
- Column temp.: 35 °C
- UV detection: 214 nm
- Injection volume: 4.0 µL

Time (min)	%A	%B	%D	#CV	Slope (%B/CV)
0.00	33.0	0.0	67.0	–	–
0.46	33.0	0.0	67.0	2.2	–
8.81	24.7	25.0	50.3	39.4	0.64
10.08	24.7	25.0	50.3	6.0	–

CV = Column Volume
(from the Waters Columns Calculator)

Listed Constant Mobile Phase A/D Ratio Sets pH = 4.0

Figure 15. Final Stability Indicating Method for analysis of APOS.

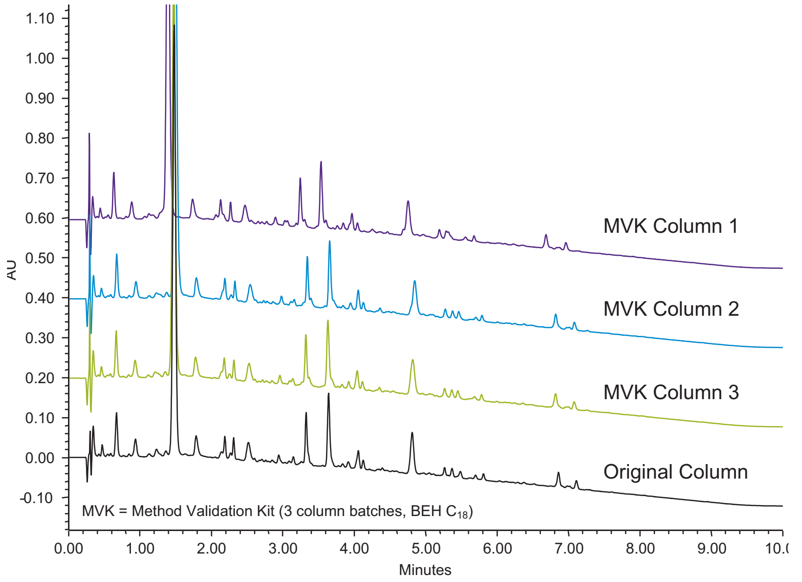


*Figure 16. Final SIM using a Method Validation Kit compared to the original XBridge BEH C_{18} **XP** Column.*

CONCLUSIONS

This application describes how factors affecting analyte separations can be examined in an orderly and efficient fashion to create an analytical chromatographic method. A Quality by Design approach was illustrated, leveraging the software assistance of Fusion QbD to select factor value combinations, create acquisition methods, and evaluate modeled responses. The resulting Stability Indicating Method has sufficient resolving power to separate the worst case mixture of analytes afforded by a forced degradation sample. This gives confidence that this SIM will be able to separate whatever degradants arise during long term and accelerated pharmaceutical stability investigations.

References

1. International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, Stability Sections Q1-A though Q1-E.
2. The packed column volume, CV (in µL), is calculated using the following relationship: $CV = \varepsilon_t L\pi(D/2)^2$. The terms L and D are the column length and diameter (in mm), respectively. The term ε_t is the total column porosity which is the fraction of the column taken up by the mobile phase in the space between particles and in the internal pores of the particles. A nominal value of total column porosity is generally applied in such calculations. In well packed columns, $\varepsilon_t = 0.66$ has been used for fully porous particles and $\varepsilon_t = 0.49$ has been used for solid-core particles. For example, see the Waters Columns Calculator at http://www.waters.com/waters/support.htm?lid=134891632&type=DWNL.
3. Gradient slope can be described in various terms. In this study, the % strong solvent change is a fixed parameter so the gradient time is sufficient to communicate the changing factor of gradient slope. However, it is useful to think of gradient slope in normalized terms, specifically the % strong solvent change per unit of column volume. In this way, the analyst can easily compare different gradient profiles independent of the column geometry and method parameters such as flow rate. Both descriptions of gradient slope are used herein.
4. "Method speed" factors are those that directly affect how quickly a method can be executed. The gradient slope and the flow rate are two such factors. A steeper gradient slope and/or a higher flow rate will provide a method that takes less time to perform and is hence "faster".
5. To help mitigate these challenging situations, Fusion QbD version 9.8.0 SR2 Build 858 and later have peak property responses and response criteria for resolutions before AND after a "Max Peak" such as the API. This makes it easier to find chromatographic conditions with adequate resolutions both before AND after large peaks such as the API.

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USP Method Case Study Part I: Understanding the Impact of Sample Preparation and Mobile Phase Stability

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APPLICATION BENEFITS

- ACQUITY™ Arc™ System, a 9500 psi system that can accommodate HPLC 4.6 × 250 mm columns
- Control of mobile phase components with low vapor evaporation bottle caps
- Sample preparation control using appropriate vials for sample composition

WATERS SOLUTIONS

ACQUITY Arc System

ACQUITY APC™ Reservoir Caps

Maximum recovery no-preslit screw caps vials

KEYWORDS

USP, atorvastatin, ACQUITY Arc, APC bottle caps

INTRODUCTION

Many established HPLC monographs have conditions or variables that can be sensitive to environmental factors, such as room temperature and humidity. While these contributors may vary from lab to lab and across continents, mitigating or reducing the impact often means that these methods require careful control of every aspect of an analysis, including mobile phase and sample preparation. For example, the USP monograph for the assay of atorvastatin[1] has a set of conditions that can pose a challenge to meeting the specified system suitability criteria. These include: a highly volatile sample diluent (1:1:2 acetonitrile: tetrahydrofuran (THF): water), a volatile component in the mobile phase (THF at 12%) and a long analysis time (2 hours). When these conditions are combined with system suitability requirements that include standard deviation of not more than (NMT) 0.6%, the method is particularly vulnerable and can fail system suitability with no relation to the column or instrument. Specifically, during the analysis of five replicate injections, which occurs over 10 hours, it is critical to ensure that no changes to the sample concentration or mobile phase occur as these impact the reproducibility of the method. To control these factors, sample preparation and mobile phase evaporation must be considered. In this example, we will review analysis of the method and steps taken to improve the reproducibility and therefore attain passing system suitability results for the USP assay.

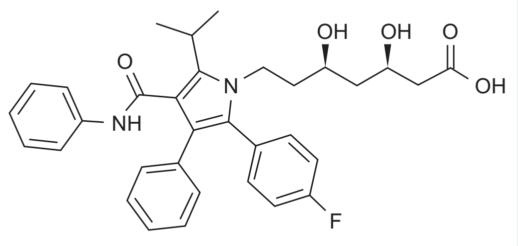


Figure 1. Atorvastatin.

EXPERIMENTAL

LC conditions

The monograph for the assay of atorvastatin calcium in the United States Pharmacopeia and National Formulary Compendium (USP39-NF34, 2016) was followed.

Sample description

Sample prep was performed following the USP monograph.[1] Reference standards were purchased from USP (Rockville, MS) including atorvastatin (p/n 1044516) and atorvastatin related compound B (p/n 1044535). The diluent consisted of 1:1:2 acetonitrile/stabilizer-free tetrahydrofuran/ water, which, as stated in the monograph, is permissible if significant fronting of the atorvastatin and atorvastatin related compound B is observed. All samples were prepared immediately prior to analysis. The system suitability standard was prepared from working stocks of atorvastatin calcium at 0.4 mg/mL and atorvastatin related compound B at 0.1 mg/mL in the diluent previously specified. The final concentration was 0.05 mg/mL in atorvastatin calcium and 0.06 mg/mL of atorvastatin related compound B. The standard solution was prepared at 0.4 mg/mL of atorvastatin calcium. A specific volume (300 µL) of standards and samples was aliquoted into each maximum recovery vials with both pre-slit and non pre-slit screw caps.

Method conditions

LC conditions

LC system:	ACQUITY Arc with 30-CHC and 2998 PDA Detector
Seal wash:	80:20 Water/methanol
Purge solvent:	Methanol
Wash solvent:	1:1:2 ACN/THF/H_2O
Wavelength:	244 nm
Vials:	Maximum recovery, screw cap non pre-slit septa – lectra bond (p/n 186000326C)
Column:	Zorbax RX C8, 4.6 × 250 mm, 5 µm, L7 packing material
Column temp.:	35 °C
Sample temp.:	20 °C
Injection volume:	20 µL
Flow rate:	1.5 mL/min
Buffer:	3.9 g/L Ammonium actetate in water, pH 5.0 adjusted with glacial acetic acid
Solution A:	21:12:67 Acetonitrile/stabilizer-free tetrahydrofuran/buffer
Solution B:	61:12:27 Acetonitrile/stabilizer-free tetrahydrofuran/buffer
Mobile phase bottle caps:	ACQUITY APC Reservoir Cap (p/n 205001152)

Time (min)	Solution A (%)	Solution B (%)
0	100	0
40	100	0
70	20	80
85	0	100
100	0	100
105	100	0
115	100	0

Table 1. Gradient.

System set up

Prior to analysis, each line (A, B, C, D) was primed for 5 min. The purge and wash lines were also purged for 20 cycles and 60 seconds, respectively. Buffer was prepared in a 3 L quantity. Mobile phase A was then prepared in a 3.5 L quantity and mobile phase B was prepared in 3 L. Prior to each test, a blank injection was performed.

Data management

Chromatography software:
Empower™ 3 FR3

USP Assay System Suitability Criteria

RESULTS AND DISCUSSION

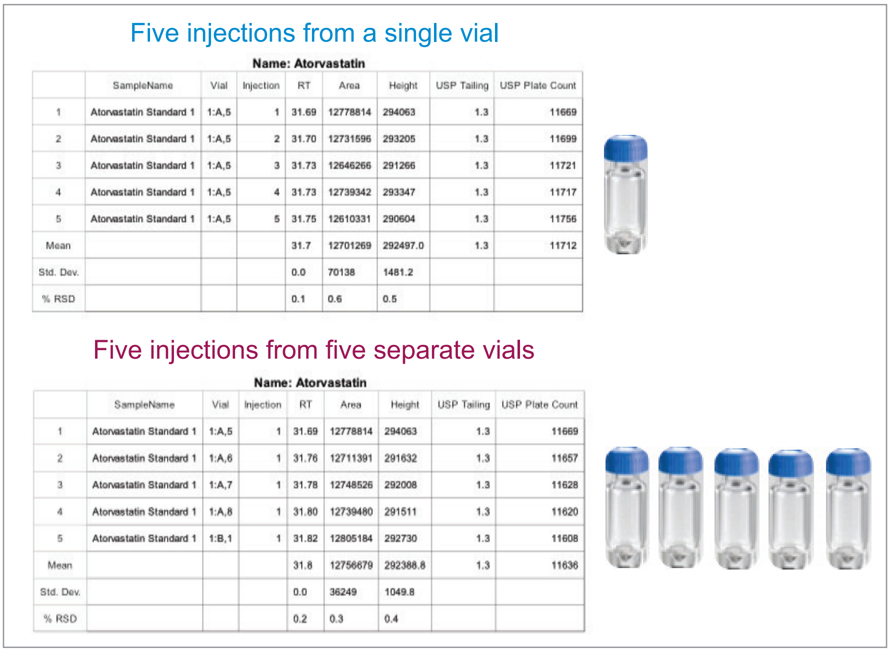
The assay for atorvastatin calcium, an HPLC analysis with a run time of 115 minutes was evaluated. The method, which uses the same conditions as the organic impurities method 1, elutes the drug substance and the related compound B in the initial 40 minute isocratic hold. As described earlier, the volatility of the mobile phase and sample diluent components can impact the repeatability of the analysis, in two separate ways. The differing rates of evaporation of the mobile phase components could impact the elution or retention time of the analytes. Organic evaporation from the sample diluent could impact concentration and solubility of the analytes. Both of which can impact the system suitability requirements (Table 2). However, per USP <621>, since the method is gradient, there are no options for scaling or shortening the run.[2] Thus, the goal of the experimental plan was to devise a strategy to reduce variability of the method and to examine tools to mitigate environmental factors.

	Criteria
Resolution	Not Less Than (NLT) 1.5 between peaks for atorvastatin related compound B and atorvastatin, System Suitability Solution
Tailing factor	Not More Than (NMT) 1.6%, Standard Solution
Relative standard deviation (n=5)	Not More Than (NMT) 0.6%, Standard Solution

Table 2. System suitability criteria for USP assay of atorvastatin calcium.

SAMPLE PREPARATION CONTROL

As mentioned previously, the presence of a highly volatile component in the sample diluent, can impact sample stability. To assess the impact of sample preparation on the assay, all samples were freshly prepared and sample aliquoting was tested. While the method did not specify a sample compartment temperature, 20 °C was used for minimal temperature control. In the first analysis, 500 µL of the standard was placed in a maximum recovery pre-slit septa vial. In the second set of analyses, a 300 µL aliquot of sample was placed into five separate vials, each vial capped with non-preslit septa. This ensured that there was no evaporation of the samples over the 10 hours required for the analysis. The results for atorvastatin are shown in Figure 2. Evaluating the area reproducibility of the two sets of injections, the repeatability of the atorvastatin injections from a single vial was 0.6%, which was at the system suitability limit for the assay of NMT 0.6%. For the analysis of standards in separate vials with non-preslit septa, the area RSD was 0.3% which met the system suitability requirements of the analysis. This approach is a viable option when handling samples with volatile components as per the USP.



Five injections from a single vial

Name: Atorvastatin

	SampleName	Vial	Injection	RT	Area	Height	USP Tailing	USP Plate Count
1	Atorvastatin Standard 1	1:A,5	1	31.69	12778814	294063	1.3	11669
2	Atorvastatin Standard 1	1:A,5	2	31.70	12731596	293205	1.3	11699
3	Atorvastatin Standard 1	1:A,5	3	31.73	12646266	291266	1.3	11721
4	Atorvastatin Standard 1	1:A,5	4	31.73	12739342	293347	1.3	11717
5	Atorvastatin Standard 1	1:A,5	5	31.75	12610331	290604	1.3	11756
Mean				31.7	12701269	292497.0	1.3	11712
Std. Dev.				0.0	70138	1481.2		
% RSD				0.1	0.6	0.5		

Five injections from five separate vials

Name: Atorvastatin

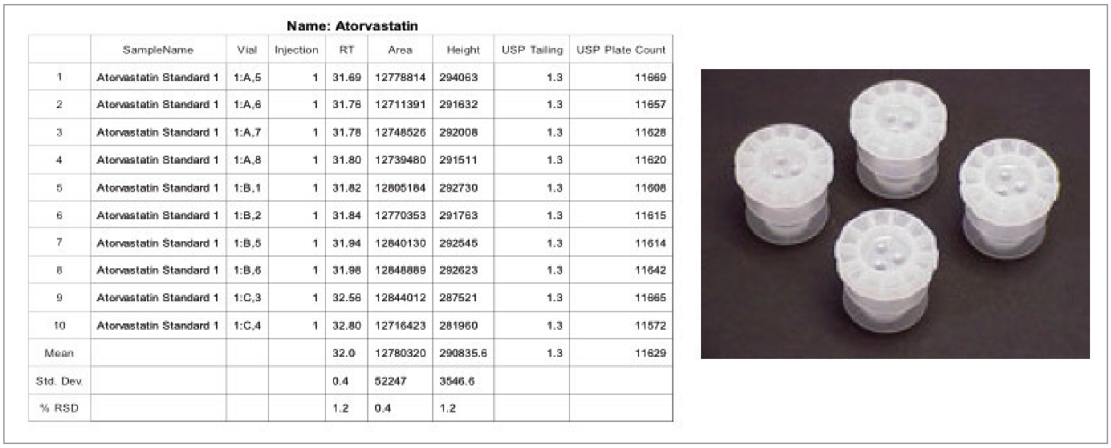
	SampleName	Vial	Injection	RT	Area	Height	USP Tailing	USP Plate Count
1	Atorvastatin Standard 1	1:A,5	1	31.69	12778814	294063	1.3	11669
2	Atorvastatin Standard 1	1:A,6	1	31.76	12711391	291632	1.3	11657
3	Atorvastatin Standard 1	1:A,7	1	31.78	12748526	292008	1.3	11628
4	Atorvastatin Standard 1	1:A,8	1	31.80	12739480	291511	1.3	11620
5	Atorvastatin Standard 1	1:B,1	1	31.82	12805184	292730	1.3	11608
Mean				31.8	12756679	292388.8	1.3	11636
Std. Dev.				0.0	36249	1049.8		
% RSD				0.2	0.3	0.4		

Figure 2. Data from five replicate injections of atorvastatin standard. The upper table is the data of five replicate injections of a sample from the same vial. The lower table is the data collected of five replicate injections from five separate vials with a non-preslit septa (p/n 186000326C), a single injection from each vial. Each run is 115 minutes; the lower table shows the stability of long term storage with proper sample placement.

CONTROL OF EVAPORATION OF MOBILE PHASE

The amount of time to complete testing of an assay can vary depending on number of samples, number of blanks among many variables. With regards to this, USP Chapter <621> on Chromatography describes the number of replicate injections required for a monograph. Specifically "Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation, %RSD, if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%."[2] Following these recommendations, the USP assay of atorvastatin calcium requires five replicate injections of the standards. However, this is in addition to any blanks, system suitability standards and samples that need to be analyzed. Thus based on the run time (115 minutes), testing of a drug substance requires a minimum of 8 injections (1 blank, 1 system suitability sample, 5 standards, and 1 sample injection) or approximately 16 hours.

Given the length of the analysis time and the mobile phase components (12% THF in A and B), control of the mobile phase composition is critical for retention time reproducibility. Therefore, the testing was evaluated for a longer period than was required to fully evaluate the mobile phase stability. Specifically the test was conducted over 38 hours and the data was compiled for 10 injections, (blanks and system suitability samples were injected along with standard injections). Standard reservoir or bottle caps were used in this experiment. The results show a significant shift in retention times as well as a retention time RSD of 1.2%, which is outside the system suitability requirement for this analysis (Figure 3). It is likely that evaporation of the organic portion of the mobile phase led to a change in mobile phase composition, a lower "strength" mobile phase with a higher aqueous content thus resulting in the retention time drift.

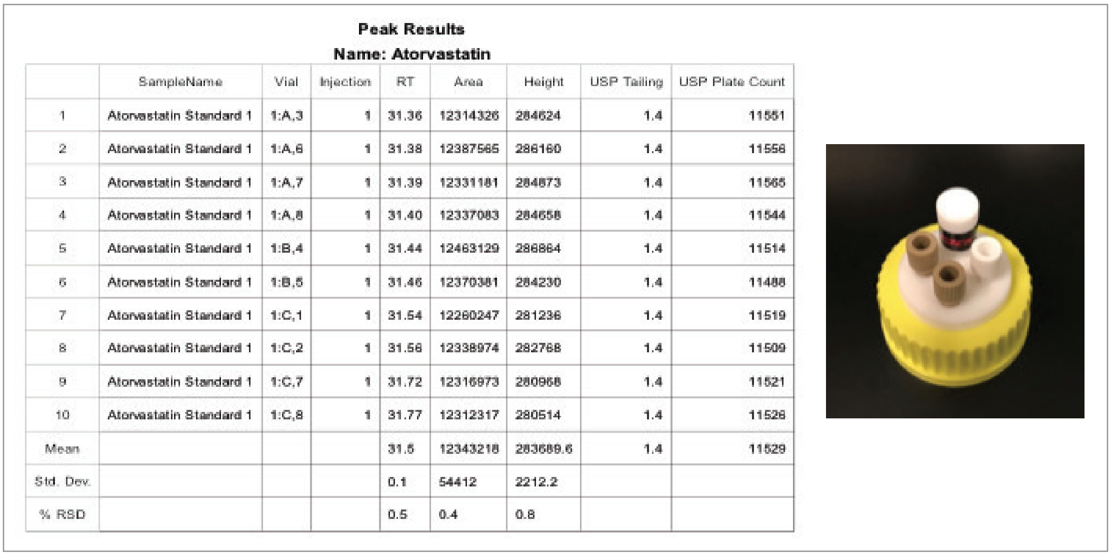


Name: Atorvastatin

	SampleName	Vial	Injection	RT	Area	Height	USP Tailing	USP Plate Count
1	Atorvastatin Standard 1	1:A,5	1	31.69	12778814	294063	1.3	11669
2	Atorvastatin Standard 1	1:A,6	1	31.76	12711391	291632	1.3	11657
3	Atorvastatin Standard 1	1:A,7	1	31.78	12748526	292008	1.3	11628
4	Atorvastatin Standard 1	1:A,8	1	31.80	12739480	291511	1.3	11620
5	Atorvastatin Standard 1	1:B,1	1	31.82	12805184	292730	1.3	11608
6	Atorvastatin Standard 1	1:B,2	1	31.84	12770353	291763	1.3	11615
7	Atorvastatin Standard 1	1:B,5	1	31.94	12840130	292545	1.3	11614
8	Atorvastatin Standard 1	1:B,6	1	31.98	12848889	292623	1.3	11642
9	Atorvastatin Standard 1	1:C,3	1	32.56	12844012	287521	1.3	11665
10	Atorvastatin Standard 1	1:C,4	1	32.80	12716423	281960	1.3	11572
Mean				32.0	12780320	290835.6	1.3	11629
Std. Dev.				0.4	52247	3546.6		
% RSD				1.2	0.4	1.2		

Figure 3. Retention time data from injections of atorvastatin standard for 10 injections which occurred over 30+ hours using standard reservoir caps. The retention time %RSD is 1.2% after 30+ hours, falling out of system suitability.

To mitigate the retention time drift, the standard bottle caps were replaced with low vapor ACQUITY APC reservoir caps shown in Figure 4. The ACQUITY APC reservoir caps are designed with an outlet relief valve that releases any pressure build-up in the mobile phase bottle while also minimizing evaporation. In addition, any cap outlet that is not in use was plugged. Analysis of this test shows minimal retention time drift: The shift in retention time over the same time period was significantly lower than with the previous analysis, with an RSD of 0.5%.



Peak Results
Name: Atorvastatin

	SampleName	Vial	Injection	RT	Area	Height	USP Tailing	USP Plate Count
1	Atorvastatin Standard 1	1:A,3	1	31.36	12314326	284624	1.4	11551
2	Atorvastatin Standard 1	1:A,6	1	31.38	12387565	286160	1.4	11556
3	Atorvastatin Standard 1	1:A,7	1	31.39	12331181	284873	1.4	11565
4	Atorvastatin Standard 1	1:A,8	1	31.40	12337083	284658	1.4	11544
5	Atorvastatin Standard 1	1:B,4	1	31.44	12463129	286864	1.4	11514
6	Atorvastatin Standard 1	1:B,5	1	31.46	12370381	284230	1.4	11488
7	Atorvastatin Standard 1	1:C,1	1	31.54	12260247	281236	1.4	11519
8	Atorvastatin Standard 1	1:C,2	1	31.56	12338974	282768	1.4	11509
9	Atorvastatin Standard 1	1:C,7	1	31.72	12316973	280968	1.4	11521
10	Atorvastatin Standard 1	1:C,8	1	31.77	12312317	280514	1.4	11526
Mean				31.5	12343218	283689.6	1.4	11529
Std. Dev.				0.1	54412	2212.2		
% RSD				0.5	0.4	0.8		

Figure 4. Retention time data from injections of atorvastatin standard for 10 injections which occurred over 30+ hours. The mobile phase bottles were capped with ACQUITY APC reservoir caps. With this approach, the retention time RSD is 0.4% over 30+ hours, meeting system suitability of the method.

The difference in retention time drift can be observed by comparing both the retention time and the %RSD for each set of analyses over time. The analysis included both a blank and a system suitability injection, therefore, the first injection of the standard occurred at 4 hours. As observed in the graph plot, the %RSD with the standard reservoir caps was approximately 1.2% at 38 hours. In contrast, analysis with the low vapor ACQUITY APC reservoir caps was <0.5% over the same time period, at which point the test was ended (Figure 5). This can also be verified with analysis of the retention time trend lines.

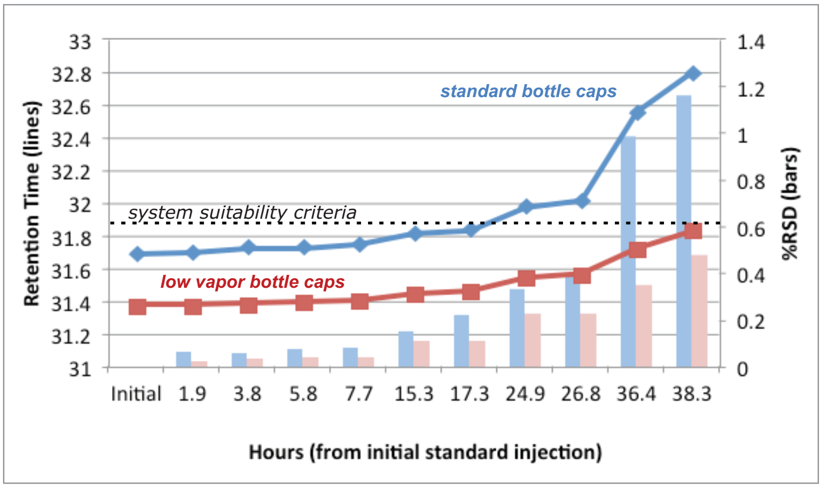


Figure 5. Retention time data and relative standard deviation over 38 hours, with standard and low vapor reservoir caps. Tests with low vapor reservoir caps produced stable retention times (red line) and lower %RSD (red bar) as compared to standard caps (blue line and blue columns). Using the standard bottle caps, the retention time RSD (Blue bar) was greater than the system suitability criteria (grey line) after 30+ hours.

USP ASSAY RESULTS

Through careful control of the mobile phase and the sample preparation, the USP assay for atorvastatin was performed successfully. Specifically, the USP criteria were met for this analysis (Table 3), including peak area and retention time %RSD.

	Criteria	Original results	New results*
Resolution	Not Less Than (NLT) 1.5 between peaks for atorvastatin related compound B and atorvastatin, system suitability solution	2.3	2.3
Tailing factor	Not More Than (NMT) 1.6%, standard solution	1.4	1.4
Relative standard deviation	Not More Than (NMT) 0.6%, standard solution	1.2% Retention time 0.6% area	0.5% Retention time 0.3% area

Table 3. System suitability criteria for USP assay of atorvastatin calcium with results. Without careful consideration of the variables that can affect long term sample and mobile phase stability, the assay did not meet the relative standard deviation requirements of the USP assay (original results). By aliquoting the sample into separate vials and using low vapor reservoir caps (new results) the system suitability criteria were all met.*

CONCLUSIONS

In the following study, a careful evaluation of the USP assay for atorvastatin was performed. The results demonstrate the impact of small changes in the composition of the sample and/or mobile phase over time. These changes, while slight, impact the method's ability to meet the system suitability criteria. Many of these changes are not due to the instrument or the column but instead are impacted by the lab environment. For example, both the room temperature and humidity can impact the volatility of the solvent and the sample diluent.

To minimize the impact of environmental conditions, both sample preparation and mobile phase composition were controlled. For example, initial analysis showed how aspiration of the standard multiple times out of a single vial could negatively impact peak area relative standard deviations. However, aliquoting the sample into separate vials increased the precision of the method over the 10 hours or five replicate injections due to minimal evaporation for the sample diluent. The long term stability of the mobile phase was also evaluated. Specifically, the impact of the reservoir cap on the evaporation and/or change in composition of the eluent was studied. In these studies low vapor reservoir caps provided more stable retention times over a longer period than standard reservoir caps. Both of these approaches are not prohibited within USP chapter 621.[2] These studies enabled a strategy to be devised, which was then used to minimize any variation in the method transfer of the USP method across multiple labs in different continents.

References

1. Official Monographs, USP 39-NF34. United States Pharmacopeia and National Formulary (USP 39-NF34) Baltimore, MD: United Book Press, Inc.; 2016. p. 2627.
2. <621> CHROMATOGRAPHY. United States Pharmacopeia and National Formulary (USP 39 NF34) Baltimore, MD: United Book Press, Inc.; 2016. p. 459–71.

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Stage 2

Procedure Performance Qualification

Once a method has been developed, the next stage of Method Lifecycle Management (MLCM) requires that the method is shown to be suitable for its intended use through performance qualification, including validation. Within this stage, the results from evaluation of the analyses are compared to the requirements previously identified in the analytical target profile (ATP).

Regulatory authorities expect that Stage 2 will be carried out using a protocol, experimental execution, and a formal report. The protocol should identify which analytical characteristics need to be evaluated and the acceptance criteria for the resulting data. The experimental procedure must be documented in compliant-ready software packages, taking into account the guidelines for data integrity. The report will document comparison of the outcomes from the experiments to the acceptance criteria in the protocol, leading to a conclusion about the suitability of the method. This is illustrated in "***Increasing Efficiency of Method Validation for Metoclopramide HCl and Related Substances with Empower 3 MVM Software***", page 59 and "***Streamline the Method Validation Process using Empower Method Validation Manager***", page 73.

An example of addressing specificity (both by way of retention time and using an independent detector) and injection repeatability is shown in "***Repeatability and Quantitation of a Method Using the Alliance HPLC System with the 2998 PDA Detector and the ACQUITY QDa***", page 67.

Tools, such as Empower Method Validation Manager (MVM), may simplify this process and improve laboratory productivity by simplifying the documentation process.

Increasing Efficiency of Method Validation for Metoclopramide HCl and Related Substances with Empower 3 MVM Software

Margaret Maziarz, Mark Wrona, and Sean M. McCarthy
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Automated method validation workflow
- Reduced time to complete the steps required to test and document a validated method
- Compliance with regulations on data security, different user privileges, audit trails, data traceability, and electronic signature sign-off requirements

WATERS SOLUTIONS

ACQUITY™ UPLC™ H-Class PLUS System

Empower™ 3 Chromatography Data Software

Empower 3 Method Validation Manager (MVM)

ACQUITY UPLC Columns

ACQUITY™ QDa™ Detector

KEYWORDS

UPLC, method validation, metoclopramide HCl, sub-2-µm particles, mass detection, ApexTrack™ peak integration

INTRODUCTION

Method validation, which demonstrates that a method is suitable for its intended purpose,[1,2] is an important regulatory requirement for pharmaceutical organizations and their supporting contract partners. A compliant laboratory must provide documented evidence and assurance that the analytical method used for testing a drug product's identity, quality, purity, and potency generates accurate and reliable results.

The validation process of an analytical method is a complex and demanding activity, consisting of many time-consuming steps. Some of these steps include creation of validation protocols, experimental work, reviewing and processing data, performing calculations, approving, and final reporting. Since some of these steps are prone to errors, a well-organized plan is essential for successful validation of an analytical method and to ensure that the appropriate regulations and guidelines are being followed.

Once validation is executed, adherence to the validation plan and specification is a critical compliance requirement. Any validation results not meeting the specifications must be clearly identified and addressed during the validation process. Raw and processed data must be appropriately stored and traceable by providing data security, audit trails, and automatic data documentation required for reviews and audits.

In this application note, we present validation of a UPLC method for metoclopramide HCl and related substances using Empower 3 Method Validation Manager (MVM), an option for Empower 3 Chromatography Data Software. We show how Empower 3 MVM tracked every step of the method validation process, identifying the steps and data that did not meet defined validation requirements. Overall, we demonstrate that Empower 3 MVM automates the method validation workflow within a single software environment, reducing time and ensuring conformance to the validation requirements and acceptance criteria defined in the protocol.

EXPERIMENTAL

Method conditions

LC system:	ACQUITY UPLC H-Class PLUS
Column:	ACQUITY UPLC CSH C_{18}, 2.1 × 50 mm, 1.7 µm (p/n 186005296)
Column temp.:	45 °C
Injection volume:	1.0 µL
Flow rate:	0.6 mL/min
Solvent A:	125 mM Formic acid in water
Solvent B:	Water
Solvent C:	Methanol
Separation:	Gradient

Step	Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
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	7.5	10	85.0	5.0

Purge/Sample wash:	50:50 water/methanol
Seal wash:	90:10 water/acetonitrile
UV detector:	ACQUITY UPLC PDA
UV setting:	210–400 nm (derived at 270 nm)
Mass detector:	ACQUITY QDa (Extended Performance)
Ionization mode:	ESI+, ESI
Acquisition range:	100–440 *m/z*
Sampling rate:	10 pts/sec
Data:	Centroid
System control, Data acquisition, and analysis:	Empower 3 FR4 CDS Software

RESULTS AND DISCUSSION

UPLC METHOD FOR METOCLOPRAMIDE HCL AND RELATED SUBSTANCES

The UPLC method validated in this study was developed using a systematic method development protocol.[3] An example of the UPLC chromatographic method for metoclopramide and related compounds is shown in Figure 1.

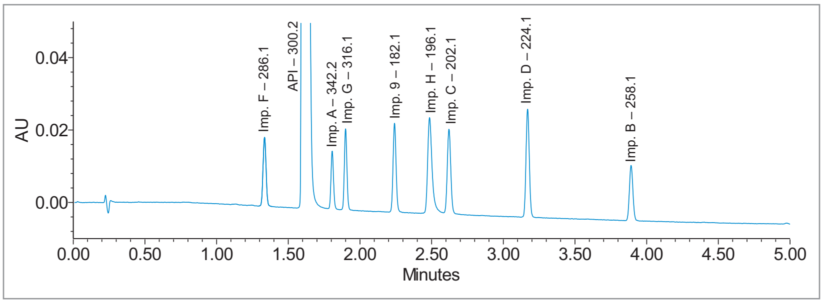


Figure 1. UPLC method for metoclopramide and related substances with UV at 270 nm.

ABOUT EMPOWER 3 MVM

Empower 3 Method Validation Manager (MVM) is software that automates the validation process and enables efficient validation of chromatographic methods to ensure compliance to the validation requirements defined by the analytical laboratory. As shown in Figure 2, a validation workflow consists of many steps. A validation protocol (Figure 3) is created and used to execute the study. Once executed, Empower 3 MVM checks data for adherence with the validation requirements and flags any results that do not meet specifications. Validation results can be displayed in a report using validation report templates specific for each test available in Empower MVM Software. The report templates can be customized as needed.

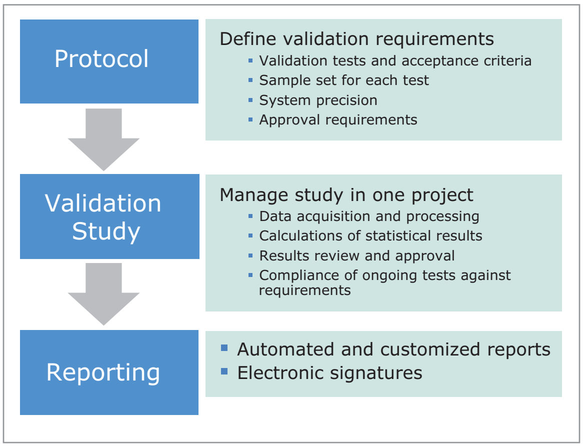
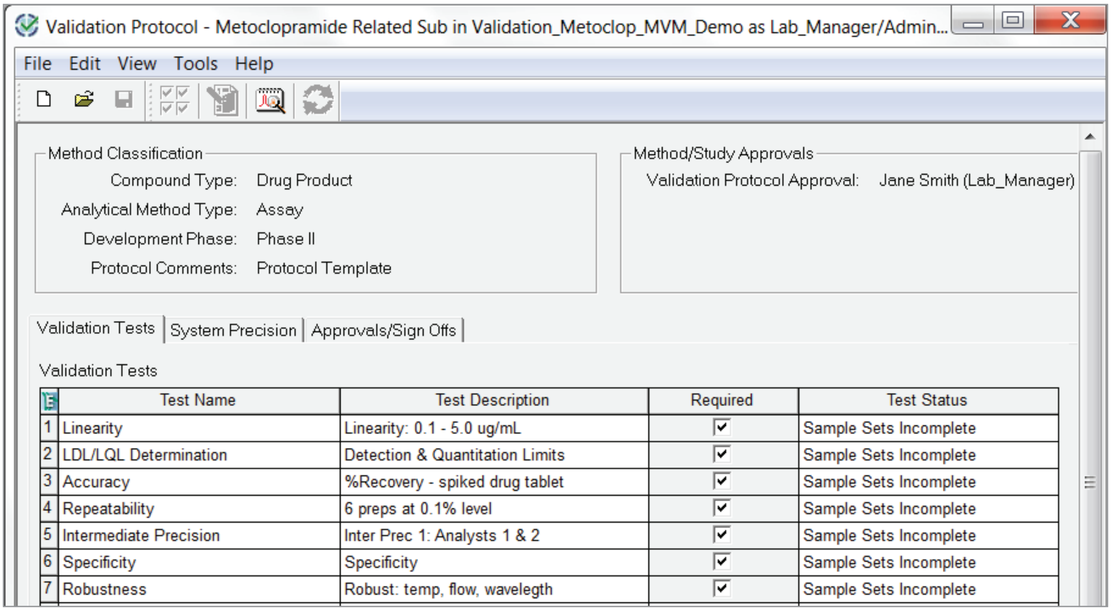


Figure 2. Workflow for validation of chromatographic methods with Empower 3 MVM.



Method Classification

Compound Type: Drug Product
Analytical Method Type: Assay
Development Phase: Phase II
Protocol Comments: Protocol Template

Method/Study Approvals

Validation Protocol Approval: Jane Smith (Lab_Manager)

Validation Tests

	Test Name	Test Description	Required	Test Status
1	Linearity	Linearity: 0.1 - 5.0 ug/mL	☑	Sample Sets Incomplete
2	LDL/LQL Determination	Detection & Quantitation Limits	☑	Sample Sets Incomplete
3	Accuracy	%Recovery - spiked drug tablet	☑	Sample Sets Incomplete
4	Repeatability	6 preps at 0.1% level	☑	Sample Sets Incomplete
5	Intermediate Precision	Inter Prec 1: Analysts 1 & 2	☑	Sample Sets Incomplete
6	Specificity	Specificity	☑	Sample Sets Incomplete
7	Robustness	Robust: temp, flow, wavelegth	☑	Sample Sets Incomplete

Figure 3. Validation protocol method created within Empower 3 MVM project. The validation tests, acceptance criteria for each validation test, and requirements for approval are defined in the validation protocol method. The validation protocol is approved by a lab manager via electronic signature.

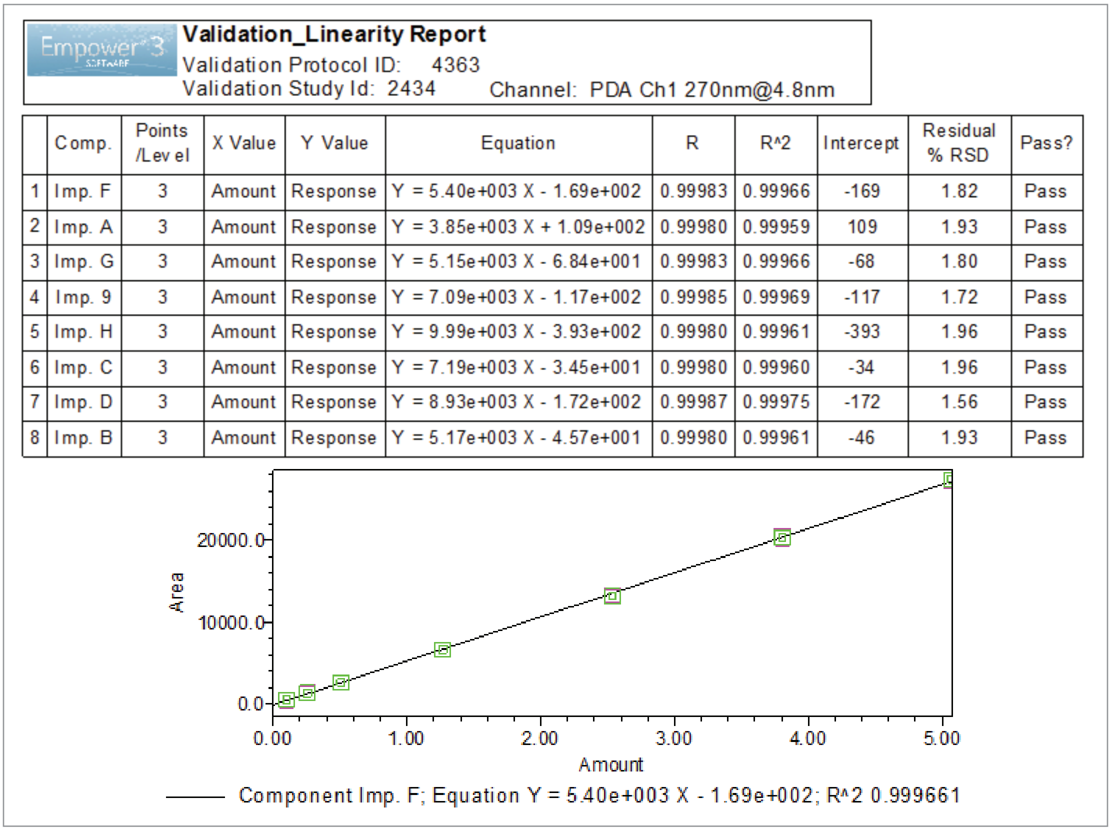
METHOD VALIDATION

We used an established validation protocol method approved by a lab manager (via electronic signatures) to execute the validation of our UPLC method for related substances of metoclopramide HCl in drug tablet formulation. The validation tests included linearity, detection and quantitation limits, accuracy, repeatability, intermediate precision, specificity, and robustness. System precision was evaluated for each validation test using five replicate injections of the sample, as recommended in the USP General Chapter <621> on Chromatography.[4] The system precision criteria include:

- %RSD of retention times: ≤1.0%
- %RSD of peak areas: ≤2.0%
- USP resolution: ≥1.5
- Peak tailing: ≤1.5

1. Linearity

Method linearity for related substances was evaluated by analyzing seven concentrations of standard solutions ranging from 0.1 to 5.0 µg/mL. These concentrations corresponded to 0.02, 0.05, 0.1, 0.25, 0.5, 0.75, and 1.0% of the metoclopramide HCl target concentration of 0.5 mg/mL. We used Empower 3 MVM to calculate regression equation and correlation coefficients for a plot of average peak areas against the concentrations. Method linearity results generated by the software are displayed in Figure 4. The method shows linear relationship between the peak areas and concentrations for all related compounds with the correlation coefficients (r^2) greater than 0.999.



Validation_Linearity Report
Validation Protocol ID: 4363
Validation Study Id: 2434 Channel: PDA Ch1 270nm@4.8nm

	Comp.	Points /Level	X Value	Y Value	Equation	R	R^2	Intercept	Residual % RSD	Pass?
1	Imp. F	3	Amount	Response	Y = 5.40e+003 X - 1.69e+002	0.99983	0.99966	-169	1.82	Pass
2	Imp. A	3	Amount	Response	Y = 3.85e+003 X + 1.09e+002	0.99980	0.99959	109	1.93	Pass
3	Imp. G	3	Amount	Response	Y = 5.15e+003 X - 6.84e+001	0.99983	0.99966	-68	1.80	Pass
4	Imp. 9	3	Amount	Response	Y = 7.09e+003 X - 1.17e+002	0.99985	0.99969	-117	1.72	Pass
5	Imp. H	3	Amount	Response	Y = 9.99e+003 X - 3.93e+002	0.99980	0.99961	-393	1.96	Pass
6	Imp. C	3	Amount	Response	Y = 7.19e+003 X - 3.45e+001	0.99980	0.99960	-34	1.96	Pass
7	Imp. D	3	Amount	Response	Y = 8.93e+003 X - 1.72e+002	0.99987	0.99975	-172	1.56	Pass
8	Imp. B	3	Amount	Response	Y = 5.17e+003 X - 4.57e+001	0.99980	0.99961	-46	1.93	Pass

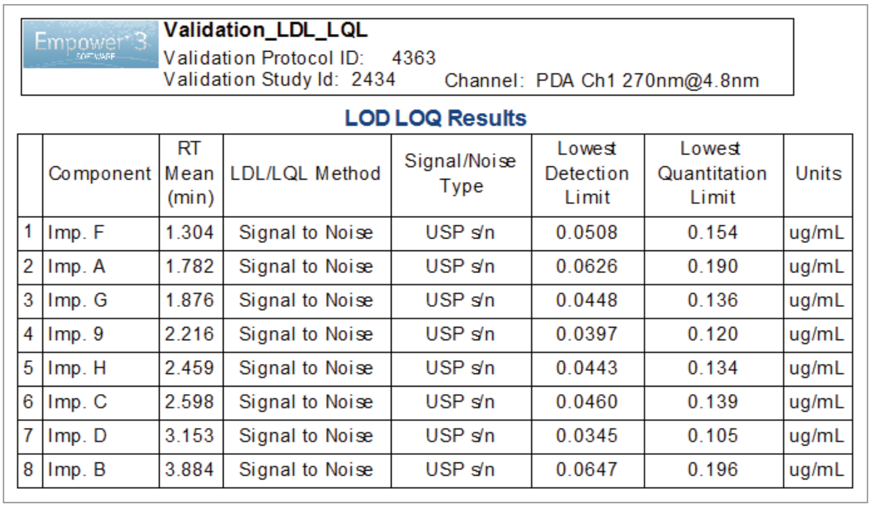
Component Imp. F; Equation Y = 5.40e+003 X - 1.69e+002; R^2 0.999661

Figure 4. Method linearity results for metoclopramide related substances generated by Empower 3 MVM software.

2. Detection and quantitation limits

We determined the lowest detection and quantitation limits (LDL and LQL) based on the USP signal-tonoise criteria of 3:1 and 10:1, respectively. In addition to signal-to-noise, other methods for LDL and LQL determination are available within Empower 3 MVM, such as based on linearity curve residual standard deviation and linearity curve intercept standard deviation.

As shown in Figure 5, the LDL and LQL for related substances ranged from 0.03 to 0.07 µg/mL and from 0.1 to 0.2 µg/mL, respectively.



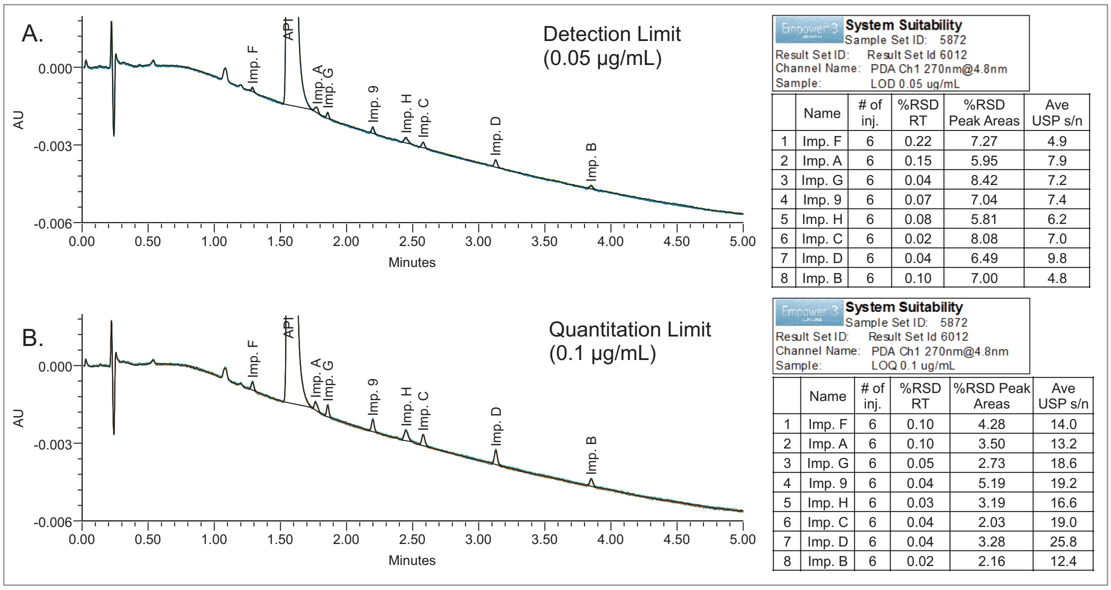
Validation_LDL_LQL
Validation Protocol ID: 4363
Validation Study Id: 2434 Channel: PDA Ch1 270nm@4.8nm

LOD LOQ Results

	Component	RT Mean (min)	LDL/LQL Method	Signal/Noise Type	Lowest Detection Limit	Lowest Quantitation Limit	Units
1	Imp. F	1.304	Signal to Noise	USP s/n	0.0508	0.154	ug/mL
2	Imp. A	1.782	Signal to Noise	USP s/n	0.0626	0.190	ug/mL
3	Imp. G	1.876	Signal to Noise	USP s/n	0.0448	0.136	ug/mL
4	Imp. 9	2.216	Signal to Noise	USP s/n	0.0397	0.120	ug/mL
5	Imp. H	2.459	Signal to Noise	USP s/n	0.0443	0.134	ug/mL
6	Imp. C	2.598	Signal to Noise	USP s/n	0.0460	0.139	ug/mL
7	Imp. D	3.153	Signal to Noise	USP s/n	0.0345	0.105	ug/mL
8	Imp. B	3.884	Signal to Noise	USP s/n	0.0647	0.196	ug/mL

Figure 5. Lowest detection and quantitation concentrations determined using USP signal-to-noise criteria.

We then validated the results by analyzing six replicate injections of the solutions prepared near the LDL (0.05 µg/mL) and LQL (0.1 µg/mL) to verify the performance, Figure 6. The LDL and LQL replicates tested in this study exceeded the USP signal-to-noise criteria.



System Suitability
Sample Set ID: 5872
Result Set ID: Result Set Id 6012
Channel Name: PDA Ch1 270nm@4.8nm
Sample: LOD 0.05 ug/mL

	Name	# of inj.	%RSD RT	%RSD Peak Areas	Ave USP s/n
1	Imp. F	6	0.22	7.27	4.9
2	Imp. A	6	0.15	5.95	7.9
3	Imp. G	6	0.04	8.42	7.2
4	Imp. 9	6	0.07	7.04	7.4
5	Imp. H	6	0.08	5.81	6.2
6	Imp. C	6	0.02	8.08	7.0
7	Imp. D	6	0.04	6.49	9.8
8	Imp. B	6	0.10	7.00	4.8

System Suitability
Sample Set ID: 5872
Result Set ID: Result Set Id 6012
Channel Name: PDA Ch1 270nm@4.8nm
Sample: LOQ 0.1 ug/mL

	Name	# of inj.	%RSD RT	%RSD Peak Areas	Ave USP s/n
1	Imp. F	6	0.10	4.28	14.0
2	Imp. A	6	0.10	3.50	13.2
3	Imp. G	6	0.05	2.73	18.6
4	Imp. 9	6	0.04	5.19	19.2
5	Imp. H	6	0.03	3.19	16.6
6	Imp. C	6	0.04	2.03	19.0
7	Imp. D	6	0.04	3.28	25.8
8	Imp. B	6	0.02	2.16	12.4

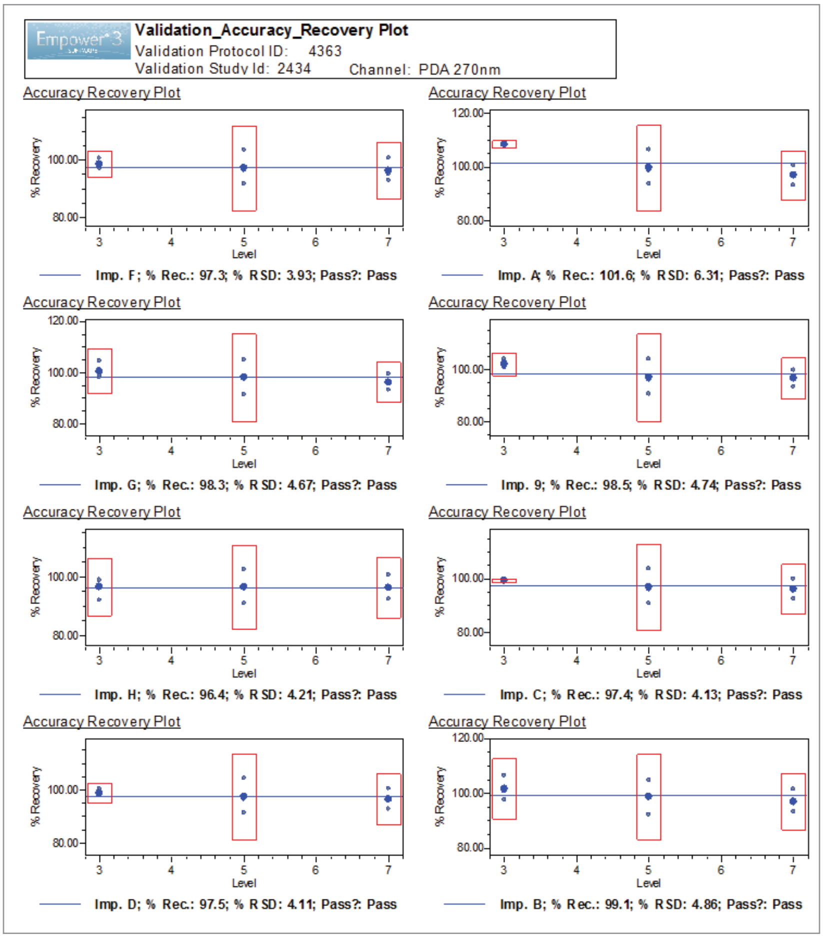
Figure 6. Overlay of six replicate injections of LDL and LQL solutions. The ApexTrack enabled consistent integration of all the peaks.

A. All components exceeded signal-to-noise criteria of 3:1 for detection.

B. All components exceeded signal-to-noise criteria of 10:1 for quantitation.

3. Accuracy

The accuracy of an analytical method includes quantitative determination of impurities in the presence of sample matrix components. Accuracy of our method was assessed by analyzing triplicate preparations of the drug tablet solutions spiked with related substances at 0.1, 0.5, and 1.0% levels in the presence of metoclopramide HCl concentration of 0.5 mg/mL. Accuracy results are summarized in Figure 7. The % recovery for all nine determinations ranged from 97 to 101% with %RSD ≤4.21%, which passes the acceptance criteria of 90–110% and %RSD ≤10%, respectively.



Validation_Accuracy_Recovery Plot
Validation Protocol ID: 4363
Validation Study Id: 2434 Channel: PDA 270nm

Imp. F; % Rec.: 97.3; % RSD: 3.93; Pass?: Pass
Imp. A; % Rec.: 101.6; % RSD: 6.31; Pass?: Pass
Imp. G; % Rec.: 98.3; % RSD: 4.67; Pass?: Pass
Imp. 9; % Rec.: 98.5; % RSD: 4.74; Pass?: Pass
Imp. H; % Rec.: 96.4; % RSD: 4.21; Pass?: Pass
Imp. C; % Rec.: 97.4; % RSD: 4.13; Pass?: Pass
Imp. D; % Rec.: 97.5; % RSD: 4.11; Pass?: Pass
Imp. B; % Rec.: 99.1; % RSD: 4.86; Pass?: Pass

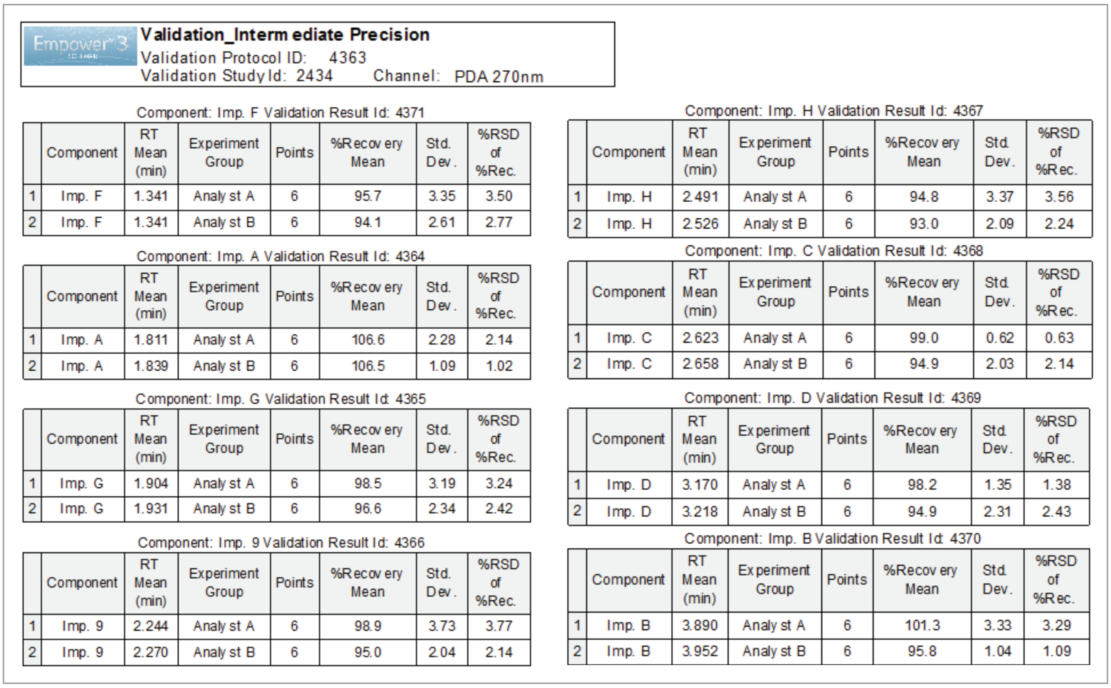
Figure 7. Accuracy results generated by Empower 3 MVM. Summary of 9 determination, 3 preparation at each levels: 0.1%, 0.5%, and 1.0%.

4. Repeatability

Method repeatability is a quantitative analysis of impurities from six independent preparations of the drug product by a single analyst. Repeatability of our method was demonstrated by spiking drug tablet sample solution with related substances at 0.1% level with respect to the metoclopramide HCl (API) concentration of 0.5 mg/mL. Repeatability results generated by analyst A (Figure 8) met the criteria for % recovery of 100 ±10% and %RSD ≤10%.

5. Intermediate precision

Intermediate precision was evaluated by a different analyst, on a different day, using different instrument and column. Summary of results from six individual spiked drug tablet sample preparations generated by analysts A and B are shown in Figure 8. Overall, the intermediate precision results met the criteria for % recovery of 100 ±10% and %RSD ≤10%.



Validation_Intermediate Precision
Validation Protocol ID: 4363
Validation Study Id: 2434 Channel: PDA 270nm

Component: Imp. F Validation Result Id: 4371

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. F	1.341	Analyst A	6	95.7	3.35	3.50
2	Imp. F	1.341	Analyst B	6	94.1	2.61	2.77

Component: Imp. A Validation Result Id: 4364

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. A	1.811	Analyst A	6	106.6	2.28	2.14
2	Imp. A	1.839	Analyst B	6	106.5	1.09	1.02

Component: Imp. G Validation Result Id: 4365

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. G	1.904	Analyst A	6	98.5	3.19	3.24
2	Imp. G	1.931	Analyst B	6	96.6	2.34	2.42

Component: Imp. 9 Validation Result Id: 4366

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. 9	2.244	Analyst A	6	98.9	3.73	3.77
2	Imp. 9	2.270	Analyst B	6	95.0	2.04	2.14

Component: Imp. H Validation Result Id: 4367

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. H	2.491	Analyst A	6	94.8	3.37	3.56
2	Imp. H	2.526	Analyst B	6	93.0	2.09	2.24

Component: Imp. C Validation Result Id: 4368

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. C	2.623	Analyst A	6	99.0	0.62	0.63
2	Imp. C	2.658	Analyst B	6	94.9	2.03	2.14

Component: Imp. D Validation Result Id: 4369

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. D	3.170	Analyst A	6	98.2	1.35	1.38
2	Imp. D	3.218	Analyst B	6	94.9	2.31	2.43

Component: Imp. B Validation Result Id: 4370

	Component	RT Mean (min)	Experiment Group	Points	%Recovery Mean	Std. Dev.	%RSD of %Rec.
1	Imp. B	3.890	Analyst A	6	101.3	3.33	3.29
2	Imp. B	3.952	Analyst B	6	95.8	1.04	1.09

Figure 8. Repeatability (analyst A) and intermediate precision (analysts A and B results generated by Empower 3 MVM.

6. Specificity

For the impurity test, specificity demonstrates that impurities can be separated and accurately measured in the presence of the sample matrix. This is typically done by spiking a drug substance or drug product with appropriate levels of impurities. In addition to demonstrating robust and reliable separation, it is important to identify that the desired components are not subject to interference with other species present in the sample. The UV peak purity determination is often used to show homogeneity of the chromatographic peak.

Specificity of our method was demonstrated by spiking drug tablet samples containing 0.5 mg/mL of metoclopramide HCl with related substances at 0.1% level. Accuracy and repeatability results show acceptable recoveries of each related substance. To demonstrate that the related substances are not coeluting with other components of the sample matrix, we used UV in conjunction with the MS spectral data as shown in Figure 9. Peak homogeneity was assessed using UV peak purity plot (Figure 9B). The peak purity angle is below the threshold angle, indicating the Impurity A peak is spectrally homogeneous. The mass spectral data provided additional information at the leading, apex, and tailing regions of the peak to confirm that only one mass is detected under the UV peak. The MS spectrum (Figure 9C) at the leading and tailing edge of the peak indicates the presence of an ion with mass of 342.0 *m/z*, which is specific to Impurity A. Overall, the UV peak purity plot and the MS spectrum shows that Impurity A is not coeluting with other peaks.

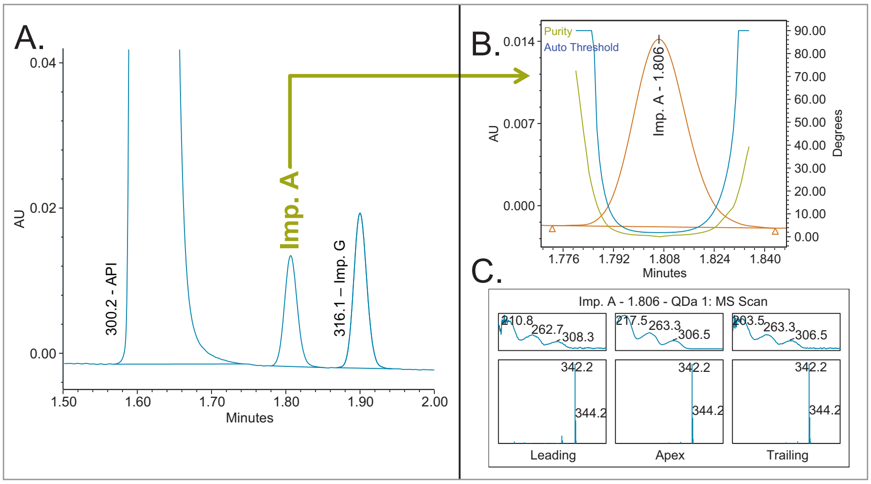


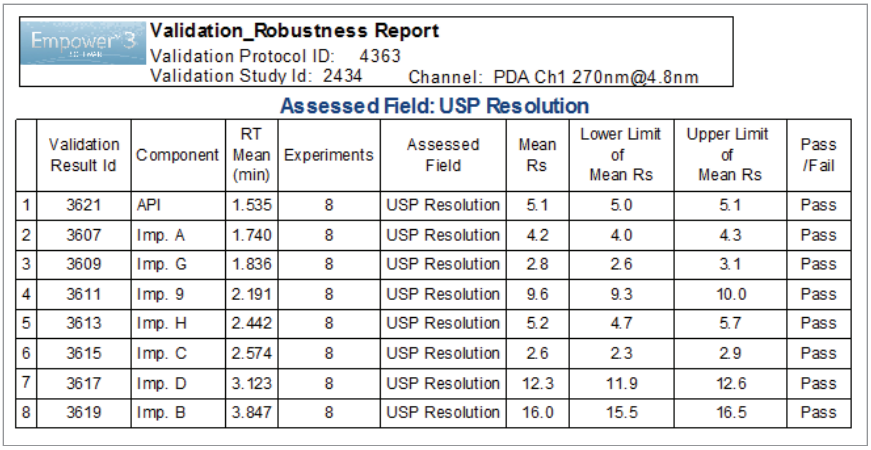
Figure 9. Peak homogeneity determination for specificity.
A. Accuracy sample with UV at 270 nm.
B. Peak purity plot of Impurity A.
C. UV and Mass profile of an Impurity A at the leading, apex, and tailing edge of the peak.

7. Robustness

Robustness is determined by the ability of the method to remain unaffected by the changes of chromatographic parameters. The parameters assessed in our study included:

- Column temperature: 45 ± 2.0 °C
- Flow rate: 0.6 ± 0.05 mL/min
- Wavelength: 270 ± 2 nm

Robustness was performed using a full factorial experimental design to investigate combination of different instrument conditions on the resolution between all the peaks, with a goal of achieving a minimum resolution of ≥2.0 for each peak. The robustness results in Figure 10 showed that the method met the criteria for resolution.



Validation_Robustness Report
Validation Protocol ID: 4363
Validation Study Id: 2434 Channel: PDA Ch1 270nm@4.8nm

Assessed Field: USP Resolution

	Validation Result Id	Component	RT Mean (min)	Experiments	Assessed Field	Mean Rs	Lower Limit of Mean Rs	Upper Limit of Mean Rs	Pass /Fail
1	3621	API	1.535	8	USP Resolution	5.1	5.0	5.1	Pass
2	3607	Imp. A	1.740	8	USP Resolution	4.2	4.0	4.3	Pass
3	3609	Imp. G	1.836	8	USP Resolution	2.8	2.6	3.1	Pass
4	3611	Imp. 9	2.191	8	USP Resolution	9.6	9.3	10.0	Pass
5	3613	Imp. H	2.442	8	USP Resolution	5.2	4.7	5.7	Pass
6	3615	Imp. C	2.574	8	USP Resolution	2.6	2.3	2.9	Pass
7	3617	Imp. D	3.123	8	USP Resolution	12.3	11.9	12.6	Pass
8	3619	Imp. B	3.847	8	USP Resolution	16.0	15.5	16.5	Pass

Figure 10. Robustness results. Resolution for each component was ≥2.0.

CONCLUSIONS

We successfully validated the UPLC method for related substances of metoclopramide HCl for linearity, detection and quantitation limits, accuracy, repeatability, intermediate precision, specificity, and robustness using Empower 3 MVM. Validation results showed that the method is linear, accurate, repeatable, precise, specific, and robust for all related substances tested in this study. The quantitation limit of all related compounds was below the reporting threshold of 0.1% or 0.5 µg/mL.

Empower 3 MVM software streamlined the entire validation process, from creating a validation protocol method to acquiring, reviewing, analyzing, approving, and reporting validation data. In addition, the software provided status of each validation test in a study and whether the results met the acceptance criteria, and flagged any out-of-specification results. Using ApexTrack for automated peak integration enabled consistent integration of all peaks during the validation process. Using the ACQUITY QDa Detector for mass detection in conjunction with UV detection enabled quick determination of peak purity using mass and UV spectral data. Finally, the validation results and validation study were reviewed and approved via electronic signatures.

Empower 3 MVM is compliant-ready software that can be easily adapted by any analytical laboratory to improve efficiency of the chromatographic method validation process and to ensure conformance to validation requirements.

References

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[APPLICATION NOTE]

Repeatability and Quantitation in a Method Using the Alliance HPLC System with the 2998 Photodiode Array Detector and the ACQUITY QDa Detector

Aparna Chavali, Thomas E. Wheat, Patricia McConville
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Orthogonal detection techniques provide confirmation of peak identity and homogeneity.
- The 2998 PDA and ACQUITY® QDa™ detectors can be configured with the Waters Isocratic Solvent Manager (ISM) with restrictor modules. This aligns the dynamic range and allows for the use of both detectors in the same analysis.
- Empower® Software provides a single user interface for both UV and mass data analysis.

WATERS SOLUTIONS

ACQUITY QDa Detector

Alliance® HPLC system
with 2998 PDA Detector

ACQUITY ISM with Alliance Splitter Kit

Waters® XSelect® HSS T3,
4.6 mm x 250 mm, 5 µm Column

Empower 3 FR2 CDS

KEY WORDS

Orthogonal detection, USP, dynamic range, quantitation, area repeatability

INTRODUCTION

High Performance Liquid Chromatography (HPLC) is one of the most widely applied analytical techniques, and can serve as a powerful separation technique for chemical mixtures consisting of a wide range of compounds including isomers. HPLC, with its choice of detection techniques, can separate analytes from a mixture, but provides little information about what a compound might be. Using photodiode array (PDA) detection can provide some information about peak homogeneity or presence of coeluting peaks, but can only provide identification by comparing standards in the same mobile phase. Adding a mass detector to an HPLC-PDA system can provide information for peak identification, for recognizing coeluting chromatographic peaks, and for confirming peak homogeneity. Mass spectrometry is, however, often perceived to be too complicated, time consuming, and expensive. Moreover, the two detectors have different sensitivity levels, and therefore combining the two detection techniques may require analyzing the sample on two different systems.

In the following application, orthogonal detection techniques – namely, PDA and mass detection – in combination with HPLC, were employed to identify and quantitate Irbesartan and related compound A. An automated solvent manager with flow splitting was included in the system configuration to align the dynamic ranges of the two detectors. The identity of the peaks was confirmed by mass detection with the ACQUITY QDa Detector. This chromatographic configuration displayed the required area and retention time reproducibility, in addition to reliable quantitation. The Alliance HPLC System, with orthogonal detection, and Waters CDS (Empower or MassLynx® Software), capable of combining data from orthogonal detectors, help increase confidence in compound identification of pharmaceutical products.

EXPERIMENTAL

Standard solutions

An accurately weighed quantity of Irbesartan standard was dissolved in methanol to obtain a 100 mL stock solution of 1 mg/mL. A series of different concentrations of Irbesartan-related compound A in the presence of a constant concentration of Irbesartan was analyzed. The calibration curve was run with different concentrations of Irbesartan-related compound A prepared at six levels (0.0005 mg/mL, 0.0010 mg/mL, 0.005 mg/mL, 0.010 mg/mL, 0.050 mg/mL, and 0.250 mg/mL), and a fixed amount of Irbesartan standard at 0.100 mg/mL in methanol.

HPLC conditions

LC system:	Alliance HPLC with ISM and Alliance Splitter Kit, 2998 PDA Detector, and ACQUITY QDa Detector
Run time:	25 min
Column:	XSelect HSS T3, 4.6 mm x 250 mm, 5 µm (L1) (p/n 186004793)
Column temp.:	40 °C
Mobile phase:	Water (Solvent line A) Acetonitrile (Solvent line B) 1% Formic acid in water (Solvent line C)
Separation Mode:	Gradient

Time (min)	%A	%B	%C
	70	10	20
5.0	30	50	20
8.0	30	50	20
12.0	0	80	20
18.0	0	80	20
18.1	70	10	20
25.0	70	10	20

Wash solvent:	70:30 water/acetonitrile
Flow rate:	1.0 mL/min
Injection volume:	10 µL

PDA conditions

Wavelength range:	210 nm to 400 nm at 1.2 nm bandwidth
Detection wavelength:	254 nm
Data rate:	5 points/s
Time constant:	0.4 s (normal)

ISM conditions

Solvent:	90:10 water/acetonitrile with 0.1% formic acid
Flow rate:	300 µL/min
Splitter kit:	Alliance Splitter Kit (p/n 205000917)

MS conditions

MS:	ACQUITY QDa
Detector Ionization mode:	ESI+
Capillary voltage:	0.8 V
Cone voltage:	15 V
Sampling rate:	2 points/s
Probe temp:	300 °C
Scan range:	100 – 600 Da
SIR masses [M + H]:	429.4 (Irbesartan API) 447.3 (Irbesartan-related compound A)
Data management:	Empower 3 FR2 CDS
USP System Suitability Criteria:	% Area RSD ≤1.5 for 5 replicate injections

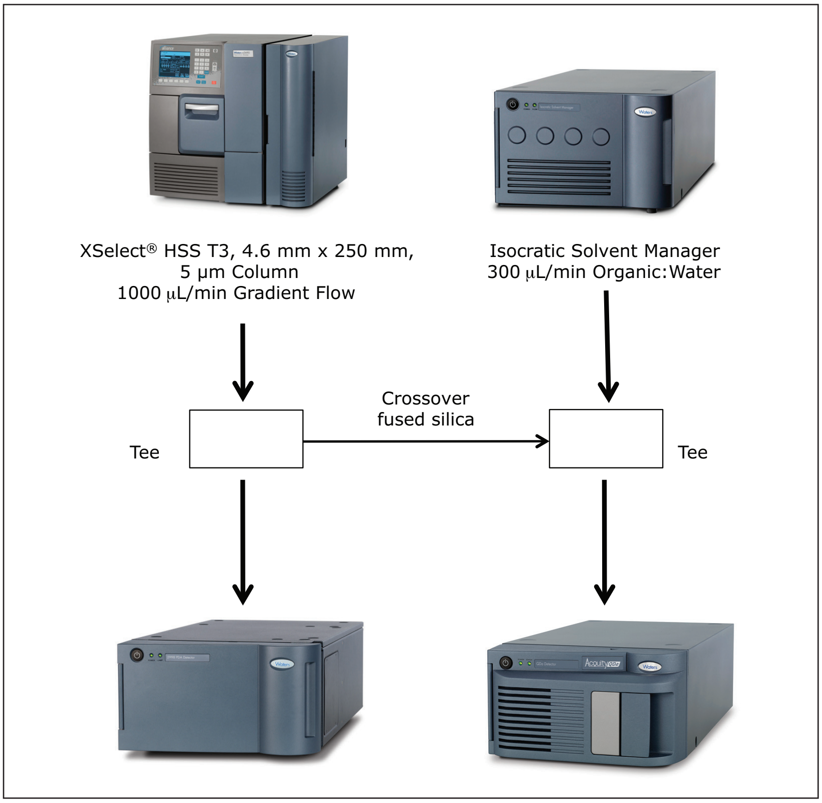
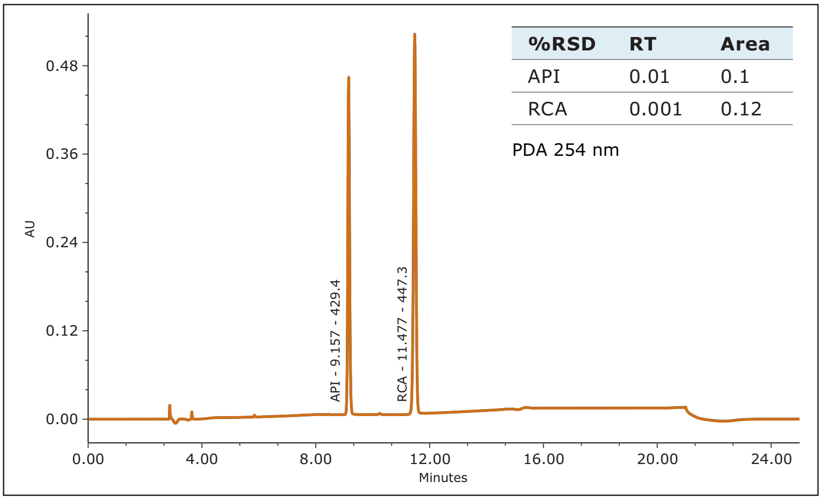


Figure 1. System configuration with Split-and-Dilute.

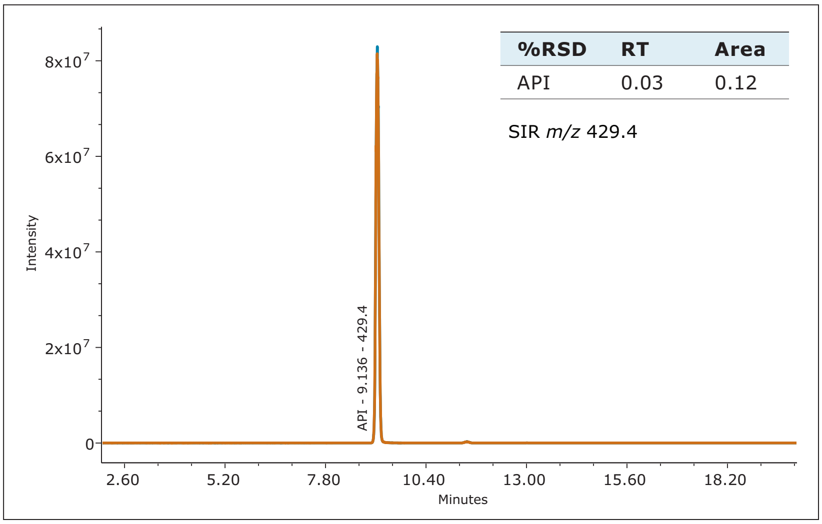
RESULTS AND DISCUSSION

The ACQUITY QDa Detector is a mass detector that detects a different physical property of an analyte, as compared to the Waters 2998 PDA Detector. In order to employ mass detection, the non-volatile USP-specified buffer was replaced with a volatile mobile phase containing formic acid, (see gradient table). The analytical method conditions separated irbesartan-related compound A from the standard. The identity of the peaks was confirmed using *m/z* of the analyte by the ACQUITY QDa Detector. The system's routine performance was evaluated using six injections prepared at 0.100 mg/ml concentration for the irbesartan API and related compound A. Retention time and area reproducibility are shown in figures 2, 3, and 4. All values meet the typical assay requirements. The area of the related compound A in the MS trace falls within acceptable limits (Figure 4). It is, however, slightly more variable than irbesartan API because the signal is smaller in intensity by a factor of 10 compared to the API.



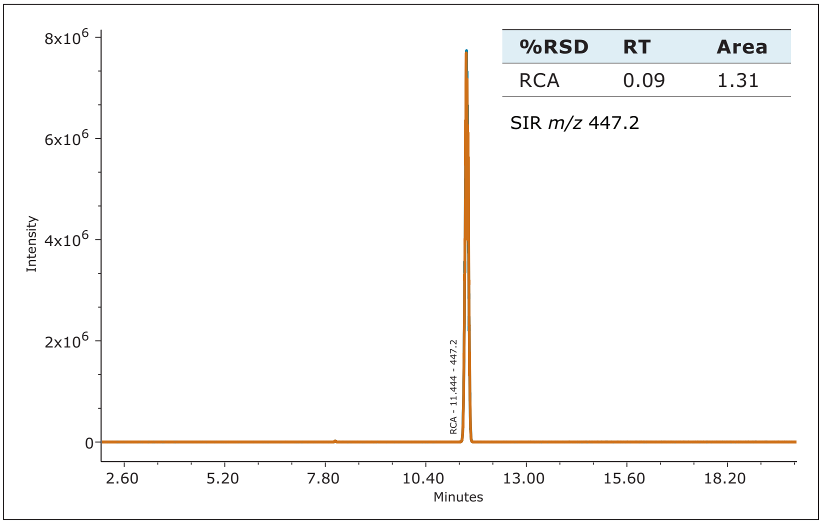
%RSD	RT	Area
API	0.01	0.1
RCA	0.001	0.12

Figure 2. Retention time and Area % RSD for irbesartan API and related compound A (2998 PDA Detector).



%RSD	RT	Area
API	0.03	0.12

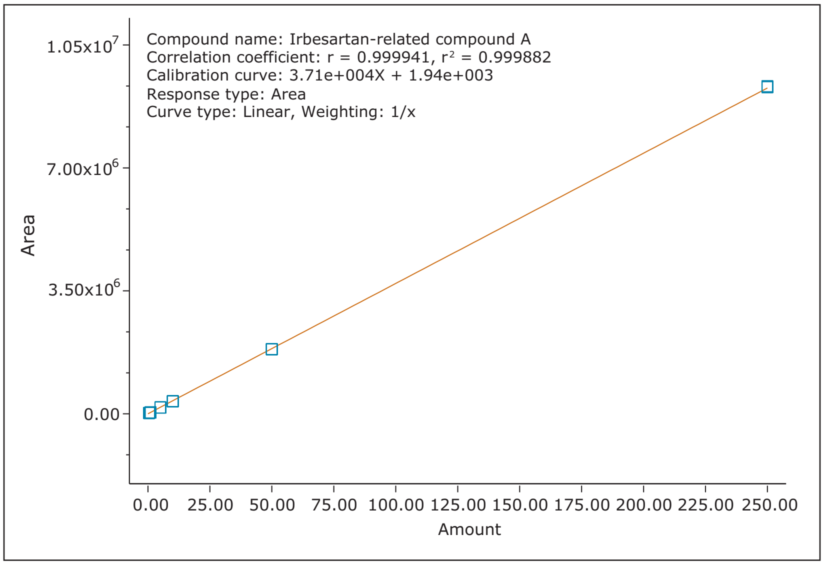
Figure 3. Retention time and Area % RSD for irbesartan API (ACQUITY QDa Detector).



%RSD	RT	Area
RCA	0.09	1.31

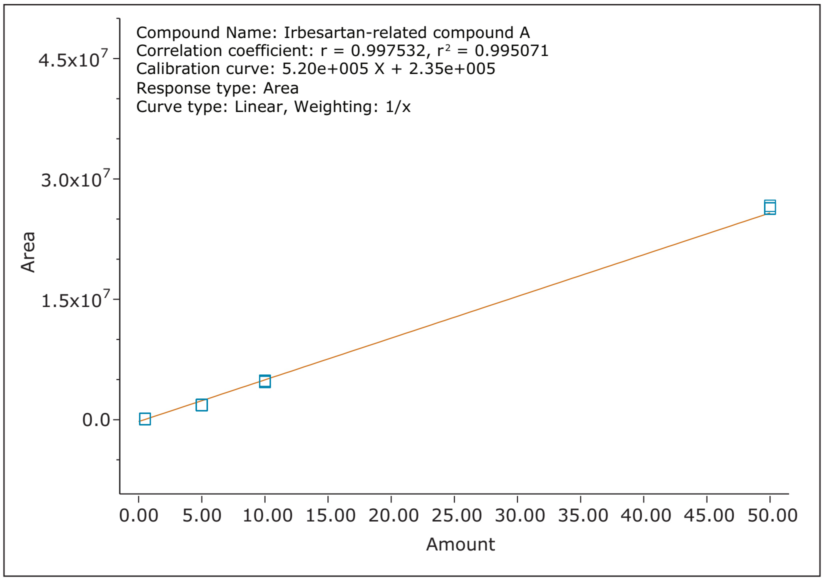
Figure 4. Retention time and Area % RSD for irbesartan-related compound A (ACQUITY QDa Detector).

The ISM with restrictor module was used to divide the flow from the column between the 2998 PDA Detector and the ACQUITY QDa Detector. With this split and dilute configuration (Figure 1), the dynamic linear ranges of the two detectors were aligned. Linearity was evaluated using triplicate injections prepared at six levels on the PDA detector and five levels on the mass detector. The resulting calibration was linear across the range of concentrations, 0.0005 mg/mL to 0.250 mg/mL with a correlation coefficient (r^2) of 0.9998 on the PDA detector and 0.0005 mg/mL to 0.050 mg/mL with a correlation coefficient (r^2) of 0.995 on the mass detector, (Figure 5 and 6). The use of split and dilute resulted in achieving equivalent sensitivity for irbesartan related compound A on the PDA and the mass detectors.



Compound name: Irbesartan-related compound A
Correlation coefficient: r = 0.999941, r^2 = 0.999882
Calibration curve: 3.71e+004X + 1.94e+003
Response type: Area
Curve type: Linear, Weighting: 1/x

Figure 5. Irbesartan-related compound A calibration curve range from 0.0005 mg/mL to 0.250 mg/mL, with the 2998 PDA Detector.



Compound Name: Irbesartan-related compound A
Correlation coefficient: r = 0.997532, r^2 = 0.995071
Calibration curve: 5.20e+005 X + 2.35e+005
Response type: Area
Curve type: Linear, Weighting: 1/x

Figure 6. Irbesartan-related compound A calibration curve range from 0.0005 mg/mL to 0.050 mg/mL, with the ACQUITY QDa Detector.

CONCLUSIONS

- The addition of a mass detector to an existing LC system equipped with a PDA detector increases confidence in compound identification.
- The dynamic linear range of the 2998 PDA Detector can be aligned with the ACQUITY QDa Detector with the use of an isocratic solvent manager.
- Linear calibration was observed on both the 2998 PDA Detector as well as the ACQUITY QDa Detector.
- Retention time reproducibility was less than 0.1% for both the compounds, and area count repeatability for the related compound A was less than 0.15% and 1.5% on the 2998 PDA Detector and ACQUITY QDa Detector, respectively.
- Empower Software enables simultaneous acquisition and analysis of data acquired from PDA and mass detectors in a single user interface.

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Streamline the Chromatographic Method Validation Process using Empower Method Validation Manager

INTRODUCTION

Chromatographic method validation is a critical step in the workflow of pharmaceutical, food safety, chemical, and environmental laboratories that can adversely impact regulatory compliance, product development, and ultimately product release and availability. The current process of validating chromatographic methods is time-consuming and prone to errors, which can decrease laboratory productivity and increase the time and costs associated with bringing new and existing products to market.

Waters™ Empower™ Method Validation Manager (MVM) Software not only automates the chromatographic method validation workflow but also addresses the limitations and bottlenecks faced with the typical chromatographic method validation process. By streamlining the method validation workflow, Method Validation Manager software offers up to an 80% reduction in the time and cost associated with the method validation process.

The typical validation process in use today requires the use of a variety of disparate software packages and is riddled with inefficiencies. MVM allows the entire chromatographic method validation process – from protocol planning through data acquisition, data processing, data review and approval, as well as final reporting – to be efficiently performed within Empower. So many of the manual and error prone steps present in the typical process – exporting data to additional software applications, checking for transcription errors, verifying data placement and calculation syntax, data review and approval, and final data reporting – are either streamlined or eliminated altogether when using MVM. Ultimately, this results in many significant business benefits, including a lower total cost of ownership.

- Fewer software applications need be deployed, validated, and maintained.
- The number of software applications on which users need to be trained is minimized.
- Ongoing software support is minimized.
- Software can be deployed more quickly and efficiently.
- Allows organization to reduce costs and focus the efforts of scientists on analyses.

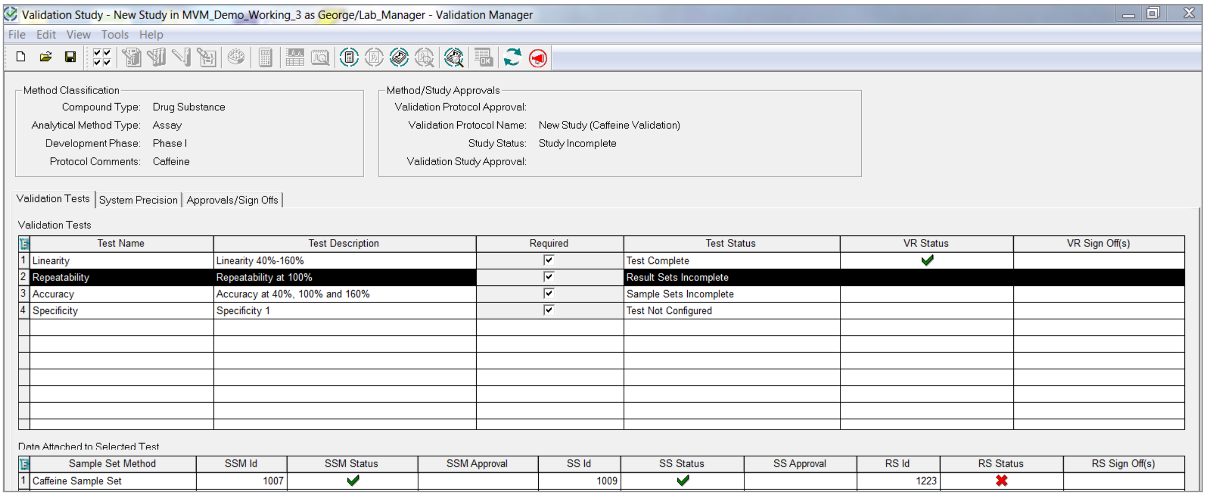
Because Method Validation Manager is built on Empower, it is compliant-ready software and provides the tools that allow you to easily satisfy compliance requirements and efficiently locate information requested in an audit.

- MVM automatically tracks and manages your validation data for complete traceability and data mining.
- Validation calculations and statistics are structurally validated and performed within Empower.
- All data is securely stored in the Empower database; no flat files are used.

This paper explores how the challenges encountered in the typical chromatographic method validation process are addressed by Empower Method Validation Manager and how its advanced functionality translates to distinct operational advantages for the laboratories that employ the software.

USING EMPOWER METHOD VALIDATION MANAGER TO OPTIMIZE YOUR CHROMATOGRAPHIC METHOD VALIDATION WORKFLOW

MVM allows you to streamline your chromatographic method validation process. When using Empower, the entire method validation process can be performed within the Validation Manager window.



Validation Study - New Study in MVM_Demo_Working_3 as George/Lab_Manager - Validation Manager

Method Classification
Compound Type: Drug Substance
Analytical Method Type: Assay
Development Phase: Phase I
Protocol Comments: Caffeine

Method/Study Approvals
Validation Protocol Approval:
Validation Protocol Name: New Study (Caffeine Validation)
Study Status: Study Incomplete
Validation Study Approval:

Validation Tests

	Test Name	Test Description	Required	Test Status	VR Status	VR Sign Off(s)
1	Linearity	Linearity 40%-160%	☑	Test Complete	✓	
2	Repeatability	Repeatability at 100%	☑	Result Sets Incomplete		
3	Accuracy	Accuracy at 40%, 100% and 160%	☑	Sample Sets Incomplete		
4	Specificity	Specificity 1	☑	Test Not Configured		

Data Attached to Selected Test

	Sample Set Method	SSM Id	SSM Status	SSM Approval	SS Id	SS Status	SS Approval	RS Id	RS Status	RS Sign Off(s)
1	Caffeine Sample Set	1007	✓		1009	✓		1223	✗	

Figure 1. Empower Validation Manager window allows you to perform chromatographic method validation from start to finish.

STREAMLINE THE CHROMATOGRAPHIC METHOD VALIDATION PROCESS

MVM provides all the tools that are required to perform and manage your method validation workflow completely from start to finish – from validation planning through data acquisition, processing and reporting in one comprehensive, automated application.

MVM clearly displays the status of each validation test in a validation study. Whether you need to acquire data, process data, or approve data, you can see at a glance what you need to do next. Hence, you don't need to manually document and continually refer back to your laboratory notebook. Simply look at the Test Status field to check what you need to do next.

Throughout the entire method validation process, MVM continually monitors the data that you are using for each validation test and tells you whether your data adheres to your validation requirements (Figure 1). An ✓ or ✗ indicates that your data does or does not adhere to your validation requirement, respectively. A ⚠ indicates that your results are out of specification. With MVM, you have assurance that the data used for each validation test adheres to the requirements you have specified in your corporate validation SOP.

MVM allows you to quickly process your validation results– there is no complicated configuration at this step. Structurally validated results are calculated with the simple click of a button. This is an astronomical time savings when compared to the use of spreadsheets to perform these calculations.

Sign-offs and approvals can be required and provide you with checks in the method validation workflow. These show that someone has reviewed the data and then entered their username and password, as an indication of their approval or e-signature. This enforces the workflow because if a sign-off or approval is required, MVM will not allow the next step in the process to be performed until the sign-off or approval has taken place.

MVM provides the data management, documentation, and traceability so the analyst can work on other value added tasks.

Throughout the entire method validation process, Empower Method Validation Manager monitors and tracks all of your method validation activity. MVM *tells you* what activity you need to do next. MVM *tells you* if your data adheres to your validation requirements. MVM *tells you* if your results are out of specification. All of this while *providing you* documentation and traceability in a compliant-ready environment.

EFFICIENTLY ACQUIRE METHOD VALIDATION DATA USING VALIDATION TEST COMPOSITES

When performing chromatographic method validation, the potential exists to minimize the number of chromatographic injections required and still maintain result quality and integrity while also providing automatic traceability of your validation data.

This can be readily accomplished with Method Validation Manager by using chromatographic injections in a sample set for multiple validation tests. For example, it is commonly desired to use some of the same injection data for linearity, accuracy and repeatability testing. The benefits of acquiring sample sets as validation test composites are obvious; efficient acquisition yields savings of the following:

- Amount of sample consumed
- Sample preparation time
- Instrument usage time
- Volume of solvent waste disposal

However, the method validation workflow in use today does not typically promote this efficiency because it results in difficulty of data documentation and traceability. Consider that the method validation workflow typically includes the use of third-party spreadsheets or statistical software. When this approach is used, the chromatographic result data must be transferred from the chromatographic data system (CDS) to a spreadsheet. Because each validation test; accuracy, linearity, robustness, specificity, and so on, requires a different set of validation calculations, the chromatographic data pertaining to each validation test is typically transferred individually to the proper spreadsheet independent from the other data for the other validation tests.

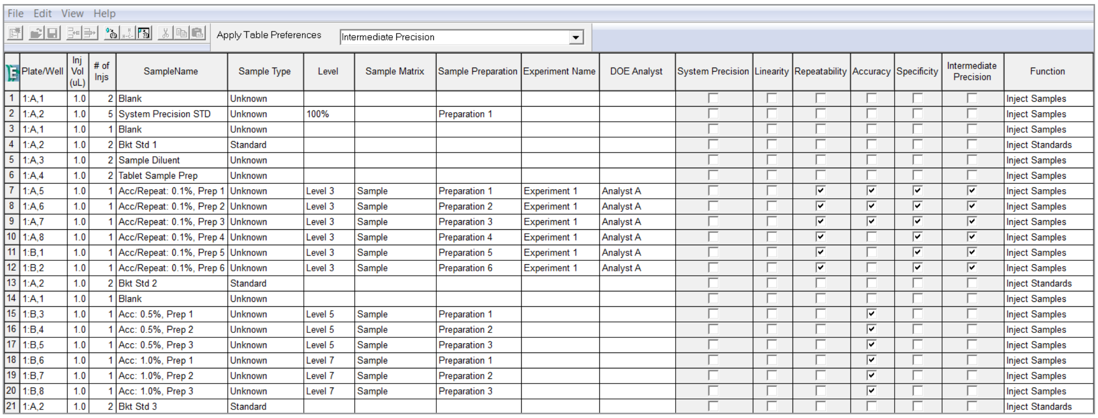
STEPS IN METHOD VALIDATION WORKFLOW

1. Within the sample sequence(s), locate the correct chromatographic results pertaining to a particular validation test.
2. Accurately transfer the result data to the region of the spreadsheet that is appropriate for that validation test.
3. Confirm the data transfer and placement on the spreadsheet.
4. Document the source and location of raw data, result data, and metadata pertaining to the validation test.
5. Repeat steps 1–4 for each validation test.

THERE IS NO LONGER A CHOICE BETWEEN EFFICIENT ACQUISITION AND EFFICIENT DOCUMENTATION AND TRACEABILITY

It is much easier for the analyst to maintain discrete sample sequences for each validation test as opposed to the validation test composite approach because he can transfer each set of data in its entirety, rather than transfer a subset of the data for each validation test. Ultimately, acquisition efficiency is replaced in lieu of the efficiency of data transfer, data processing, and data documentation.

When using Empower MVM, there is no need to make a choice between these trade-offs. During the creation of a sample set, the user has the ability to assign injections to each validation test. In this manner, the software is aware of what injections pertain to which validation tests and not only automatically uses the appropriate data when performing the calculations specific to each validation test, but also maintains full traceability of this data. Figure 2 displays an example of a validation test composite where injections from within one sample set will be used for a variety of validation tests.



Apply Table Preferences: Intermediate Precision

E	Plate/Well	Inj Vol (uL)	# of Injs	SampleName	Sample Type	Level	Sample Matrix	Sample Preparation	Experiment Name	DOE Analyst	System Precision	Linearity	Repeatability	Accuracy	Specificity	Intermediate Precision	Function
1	1:A,1	1.0	2	Blank	Unknown						☐	☐	☐	☐	☐	☐	Inject Samples
2	1:A,2	1.0	5	System Precision STD	Unknown	100%		Preparation 1			☐	☐	☐	☐	☐	☐	Inject Samples
3	1:A,1	1.0	1	Blank	Unknown						☐	☐	☐	☐	☐	☐	Inject Samples
4	1:A,2	1.0	2	Bkt Std 1	Standard						☐	☐	☐	☐	☐	☐	Inject Standards
5	1:A,3	1.0	2	Sample Diluent	Unknown						☐	☐	☐	☐	☐	☐	Inject Samples
6	1:A,4	1.0	2	Tablet Sample Prep	Unknown						☐	☐	☐	☐	☐	☐	Inject Samples
7	1:A,5	1.0	1	Acc/Repeat: 0.1%, Prep 1	Unknown	Level 3	Sample	Preparation 1	Experiment 1	Analyst A	☐	☐	☑	☑	☑	☑	Inject Samples
8	1:A,6	1.0	1	Acc/Repeat: 0.1%, Prep 2	Unknown	Level 3	Sample	Preparation 2	Experiment 1	Analyst A	☐	☐	☑	☑	☑	☑	Inject Samples
9	1:A,7	1.0	1	Acc/Repeat: 0.1%, Prep 3	Unknown	Level 3	Sample	Preparation 3	Experiment 1	Analyst A	☐	☐	☑	☑	☑	☑	Inject Samples
10	1:A,8	1.0	1	Acc/Repeat: 0.1%, Prep 4	Unknown	Level 3	Sample	Preparation 4	Experiment 1	Analyst A	☐	☐	☑	☐	☑	☑	Inject Samples
11	1:B,1	1.0	1	Acc/Repeat: 0.1%, Prep 5	Unknown	Level 3	Sample	Preparation 5	Experiment 1	Analyst A	☐	☐	☑	☐	☑	☑	Inject Samples
12	1:B,2	1.0	1	Acc/Repeat: 0.1%, Prep 6	Unknown	Level 3	Sample	Preparation 6	Experiment 1	Analyst A	☐	☐	☑	☐	☑	☑	Inject Samples
13	1:A,2	1.0	2	Bkt Std 2	Standard						☐	☐	☐	☐	☐	☐	Inject Standards
14	1:A,1	1.0	1	Blank	Unknown						☐	☐	☐	☐	☐	☐	Inject Samples
15	1:B,3	1.0	1	Acc: 0.5%, Prep 1	Unknown	Level 5	Sample	Preparation 1			☐	☐	☐	☑	☐	☐	Inject Samples
16	1:B,4	1.0	1	Acc: 0.5%, Prep 2	Unknown	Level 5	Sample	Preparation 2			☐	☐	☐	☑	☐	☐	Inject Samples
17	1:B,5	1.0	1	Acc: 0.5%, Prep 3	Unknown	Level 5	Sample	Preparation 3			☐	☐	☐	☑	☐	☐	Inject Samples
18	1:B,6	1.0	1	Acc: 1.0%, Prep 1	Unknown	Level 7	Sample	Preparation 1			☐	☐	☐	☑	☐	☐	Inject Samples
19	1:B,7	1.0	1	Acc: 1.0%, Prep 2	Unknown	Level 7	Sample	Preparation 2			☐	☐	☐	☑	☐	☐	Inject Samples
20	1:B,8	1.0	1	Acc: 1.0%, Prep 3	Unknown	Level 7	Sample	Preparation 3			☐	☐	☐	☑	☐	☐	Inject Samples
21	1:A,2	1.0	2	Bkt Std 3	Standard						☐	☐	☐	☐	☐	☐	Inject Standards

Figure 2. Validation test composite showing injections to be used for system precision, linearity, accuracy, repeatability, intermediate precision, reproducibility, and stability testing.

AUTOMATIC TRACEABILITY

The user is responsible for specifying which injections pertain to each validation test. Beyond that, MVM takes full control. MVM performs the required validation calculations while completely and automatically maintaining traceability of all related data so the user can easily trace a validation calculation back to its raw data (Could there be an outlier?), to its acquisition parameters (Was the column temperature set correctly?), to its system information (What system was used? Had it just been serviced? Was it due for service?), to its user information (Was the user that acquired the data properly trained on this procedure?), to custom information (What Batch Number was used?) and so on.

ELIMINATE THE USE OF SPREADSHEETS DURING METHOD VALIDATION USING EMPOWER METHOD VALIDATION MANAGER

Analytical method validation is the process of establishing through experimentation that a method is suitable for its intended use. It is an important regulatory requirement for pharmaceutical and other industries as it provides documented evidence and assurance that the methods in use are suitable for the determination of identity, quality, strength, purity, and potency of their products.

The validation process can be a time-consuming and repetitive task, consisting of several sequential steps. These steps include, planning (protocol generation), sample preparation and experiment setup, data acquisition, calculation of results (both chromatographic and validation results), and report generation. Of these, one of the most time-consuming, tedious, and error-prone steps is that of calculating validation results. Currently, this step is typically accomplished by importing the chromatographic results generated by the CDS to a third-party software package such as Microsoft Excel or SAS's JMP Statistical software.

SHORTCOMINGS OF TYPICAL APPROACH

The approach described above is problematic for the following reasons:

- Method validation requires the calculation of many different validation results; thus the development of the many formulas on multiple spreadsheets is a daunting task.
- Formulas must be validated to ensure they are correctly created and produce correct results.
- Spreadsheets pose data integrity and security concerns in a compliant environment.
- The burden of spreadsheet lifecycle maintenance is upon the user.
- Every time a method is validated, it is required to verify that the data transfer from the CDS to the spreadsheets is free of transcription errors. This is typically done in duplicate by the user and an additional peer review.
- In producing the final report, data must be tediously configured and compiled using various software applications including MS Word, MS Excel, and the CDS.
- Data mining becomes unmanageable because there is no traceability between the different software applications used.

The routine use of spreadsheets for validation becomes a management headache. With Method Validation Manager software, all of these concerns and manual step are completely eliminated. MVM allows you to perform the chromatographic method validation process completely from protocol planning and data acquisition through reporting, all within Empower. All validation calculations are performed within the Empower CDS and thus are structurally validated, secure, audit trailed, and traceable.

DATA MANAGEMENT

There are a total of eleven possible validation tests that may need to be assessed during method validation:

- Linearity and range
- Accuracy
- Specificity
- LOD and LOQ
- Repeatability
- Intermediate precision
- Reproducibility
- System precision
- Robustness
- Stability
- Filter validation

Each of these tests requires a different number of sample preparations and injections. Many validation tests include the variation of factors such as analyst, column, day, laboratory, instrument, pH, temperature, flow rate, and so on. Some validation tests may require multiple sample sets to be acquired. Additionally, a subset of the injections used for one test may be also used for another test, either alone, or in conjunction with other injections. All of this adds up to a total of tens or even hundreds of injections that need to be managed and ultimately processed to determine the validation-specific calculations. Moreover, the calculations necessary for each validation test are unique. For example, the calculations for a linearity test are different than those necessary for a robustness or accuracy test.

A BETTER WAY TO SEARCH, CONFIRM, AND DOCUMENT YOUR DATA

The analyst must exercise great care when transferring data from the chromatography data software to the spreadsheet. Assuming the spreadsheet calculations have been previously validated, the analyst needs an efficient way to:

- Find the correct chromatographic data for each validation test independent from the other validation test data.
- Accurately transfer each validation test's data to the correct region of the appropriate spreadsheet.
- Confirm the data transfer and placement on the spreadsheet.
- Confirm whether results are within specification.
- Transfer data from spreadsheet to software application to be used for final report.
- Document the source and location of raw data, result data, and metadata pertaining to the validation test.

ASSURED REGULATORY COMPLIANCE WITH EMPOWER METHOD VALIDATION MANAGER

REGULATORY COMPLIANCE CHALLENGES

The current chromatographic method validation process in use today is riddled with inefficiencies and regulatory compliance concerns. Using MVM provides confidence that your method validation process is streamlined and meets all compliance regulations.

Managing regulatory compliance and data security are some of your lab's biggest challenges. Empower Software was engineered to maintain security, data integrity and compliance. Empower is a compliant-ready software solution that allows you to configure the system to comply with GxP and 21 CFR Part 11 regulations. Multi-level security is provided for every job – from assigning user access privileges, automated log-outs due to inactivity, expired passwords, as well as and the ability to limit the number of password entry attempts to establishing audit trails and regulating project access. Empower Software ensures the utmost integrity for all of your data.

METHOD VALIDATION MANAGER ASSURANCE

Since Method Validation Manager is powered by Empower, MVM contains the same regulatory and compliant-ready benefits:

- Complete traceability of all validation data – result, raw data, methods, and all metadata.
- All data is stored in the embedded Oracle relational database which provides security and data integrity.
- Audit trails capture all chromatographic and method validation activity.
- A full set of user privileges control both chromatographic and method validation activities, as well as data access.
- Corporate method validation requirements can be approved and locked.
- All calculations are performed within Empower and are structurally validated by Waters.
- No flat files are used.
- No third-party software is required to manage, maintain, or validate.
- Data traceability is automatically maintained, so the user isn't burdened with this error-prone task.

ROBUSTNESS TESTING

Robustness is the capacity of a method to remain unaffected by small, *deliberate* variations in method parameters; it is a measurement of the reliability of a method. In robustness, you challenge your method by varying certain method parameters, with the intention of determining which parameters, or factors, must be tightly controlled when running your method on a routine basis. Investing in a thorough and properly designed robustness study can help ensure successful method implementation and transfer down the road. Investing a little time up-front can save a lot of time, energy, and expense later.

In liquid chromatography, examples of typical robustness factors are:

- Mobile phase composition
- Number, type, and proportion of organic solvents
- Buffer composition and concentration
- pH of the mobile phase
- Temperature
- Flow rate
- Wavelength
- Gradient variations
- Hold times
- Slope
- Length

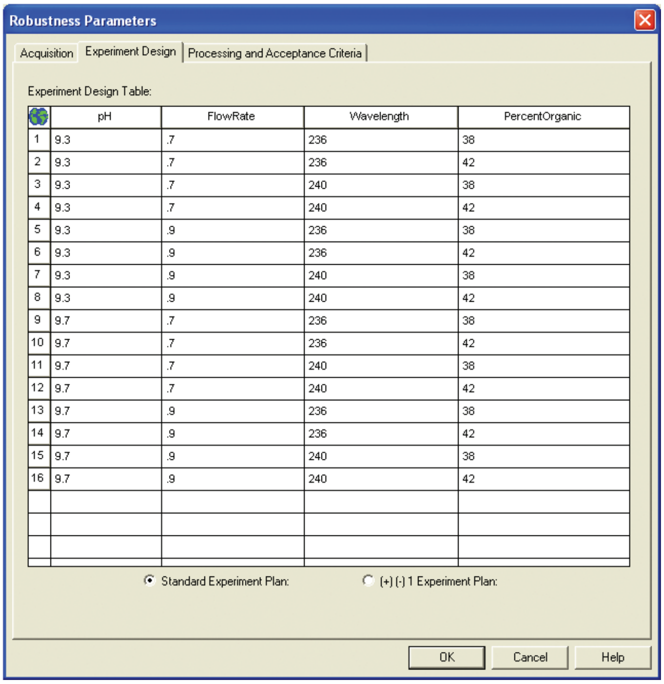
Factors that are varied as part of a robustness study are typically parameters that are specified in the method itself. If any of these factors cause variability in the resulting data, this information is typically documented in the method procedure so that the analyst knows to take measures to tightly control these factors, thereby ensuring that consistent results are obtained every time the analysis is run.

DESIGN OF EXPERIMENT (DOE)

DOE is the use of factorial experiments where multiple factor variations can be combined together in a single chromatographic run instead of the vary one-factor-at-a-time approach which has historically been popular. Performing experiments in the latter manner most likely resulted from being trained as scientists (one variable at a time) as opposed to a statistician, which also allows for simplistic data reduction. However, this approach is time consuming due to the necessity of large numbers of chromatographic runs. Additionally, possible interactions between factors such as pH changes, temperature, or ionic strength, remain undetected. The DOE approach allows for the acquisition of a minimal amount of chromatographic runs, thus saving on the amount of sample, analyst time, instrument time, and solvent waste disposal. Furthermore, this approach allows for a full statistical data analysis, providing much more comprehensive information including the determination of factor interactions.

Method Validation Manager allows the use of the DOE approach to robustness without the necessity for a resident statistician or the use of a third-party statistical software which would need to be learned, validated, and maintained. Unlike third-party software, the Empower CDS has the advantage that the data is traceable, secure, and audit trailed.

There are different types of experimental designs available. In a full factorial experiment, all possible combinations of factors are measured. A common full factorial design is one with all factors set at two levels each, a high and low value. If there are k factors, each at two levels, a full factorial design then has 2^k runs. In other words, using four factors, there would be 2^4 or 16 design points or runs. To further illustrate the point, Figure 3 shows a full factorial design robustness study for four factors; pH, flow, wavelength, and percent organic in the mobile phase.



Experiment Design Table:

	pH	FlowRate	Wavelength	PercentOrganic
1	9.3	.7	236	38
2	9.3	.7	236	42
3	9.3	.7	240	38
4	9.3	.7	240	42
5	9.3	.9	236	38
6	9.3	.9	236	42
7	9.3	.9	240	38
8	9.3	.9	240	42
9	9.7	.7	236	38
10	9.7	.7	236	42
11	9.7	.7	240	38
12	9.7	.7	240	42
13	9.7	.9	236	38
14	9.7	.9	236	42
15	9.7	.9	240	38
16	9.7	.9	240	42

● Standard Experiment Plan: ○ (+) (-) 1 Experiment Plan:

Figure 3. Full factorial design of experiment using four factors.

Full factorial design runs can really start to add up when investigating large number of factors; for nine factors, 512 runs would be needed, without even taking into account replicate injections. In addition, the design presented in Figure 1 assumes linear responses between factors. In many cases, curvature is possible, necessitating center point runs (runs at the nominal conditions) further increasing the number of injections. For this reason, fractional factorial designs are commonly used for robustness studies incorporating more than five factors.

A fractional factorial DOE is a statistically chosen fraction or subset of the total factor combinations. In the example above, with nine factors resulting in 512 runs for a full factorial design, fractional factorial designs can accomplish the same evaluation in as little as 32 runs.

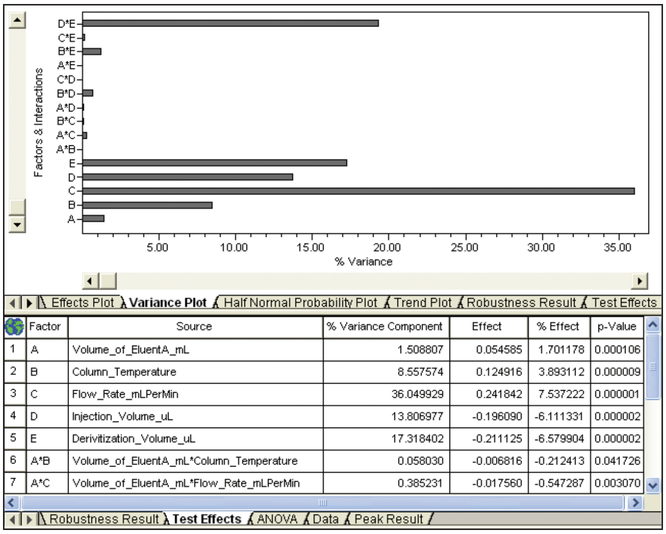
The available experimental design types in MVM are as follows: Full Factorial, 1/2 Factorial, 1/4 Factorial, 1/8 Factorial, 1/16 Factorial, 1/32 Factorial, and Plackett Burman. These designs provide main effect information as well as some 2-factor interaction effects. These designs allow for up to nine different factors to be varied in a robustness test with a maximum of only 32 experiments required.

ROBUSTNESS RESULTS

Creating robustness results in MVM is as easy as clicking a button. The resulting data is presented in a logical and interactive manner in both tabular and plot format. Effects plots and percent variance plots are particularly useful in robustness data assessment.

PERCENT VARIANCE PLOT

Similar to a bar chart, or histogram, the Variance plot shows the factors and factor interactions on the Y axis plotted against percent variance on the X axis. The percent variance looks at the variability in your data due to each factor and factor combinations and compares this to the total variability in the data set, the sum of which is always 100%. An example percent variance plot is shown in Figure 4.

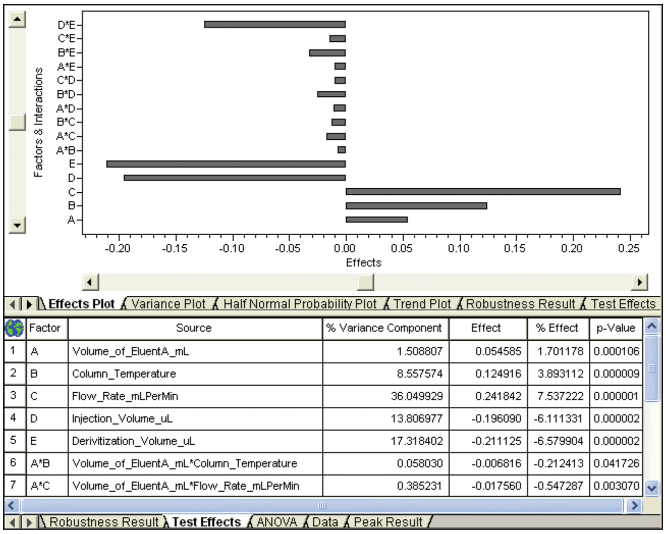


	Factor	Source	% Variance Component	Effect	% Effect	p-Value
1	A	Volume_of_EluentA_mL	1.508807	0.054585	1.701178	0.000106
2	B	Column_Temperature	8.557574	0.124916	3.893112	0.000009
3	C	Flow_Rate_mLPerMin	36.049929	0.241842	7.537222	0.000001
4	D	Injection_Volume_uL	13.806977	-0.196090	-6.111331	0.000002
5	E	Derivitization_Volume_uL	17.318402	-0.211125	-6.579904	0.000002
6	A*B	Volume_of_EluentA_mL*Column_Temperature	0.058030	-0.006816	-0.212413	0.041726
7	A*C	Volume_of_EluentA_mL*Flow_Rate_mLPerMin	0.385231	-0.017560	-0.547287	0.003070

Figure 4. Percent variance plot.

EFFECTS PLOT

The effect is the change in a measured response due to the change of a factor. The effect value is one-half of the average response at the high level minus the average response at the low factor level. Like the percent variance plot, you can use this information to see which factors cause the greatest change or variation in your data. But rather than being based on a scale of 100, like the percent variance, the units of the effect value are the same as the value that you are measuring so it is easy to see the magnitude of the change in your data due to the change of a factor. Figure 5 displays an example effects plot.



	Factor	Source	% Variance Component	Effect	% Effect	p-Value
1	A	Volume_of_EluentA_mL	1.508807	0.054585	1.701178	0.000106
2	B	Column_Temperature	8.557574	0.124916	3.893112	0.000009
3	C	Flow_Rate_mLPerMin	36.049929	0.241842	7.537222	0.000001
4	D	Injection_Volume_uL	13.806977	-0.196090	-6.111331	0.000002
5	E	Derivitization_Volume_uL	17.318402	-0.211125	-6.579904	0.000002
6	A*B	Volume_of_EluentA_mL*Column_Temperature	0.058030	-0.006816	-0.212413	0.041726
7	A*C	Volume_of_EluentA_mL*Flow_Rate_mLPerMin	0.385231	-0.017560	-0.547287	0.003070

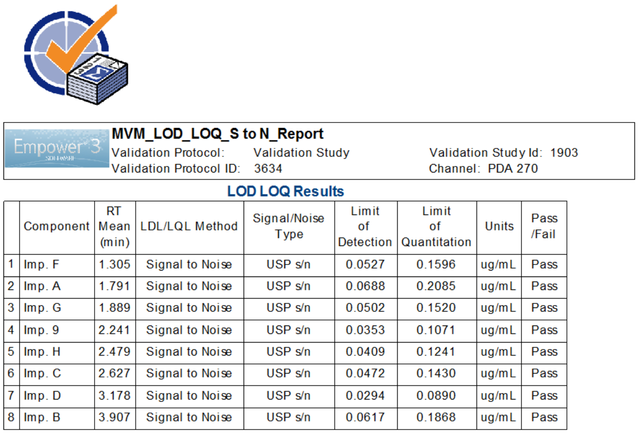
Figure 5. Robustness effects plot.

Effect data, provides an overview of not only what factors cause the greatest change in the response of your data, but also the magnitude of this change. If you are assessing retention time, the X axis for this plot would be in the units of minutes and you could easily determine how much your retention time changed relative to your factor changes. If you are assessing area, the X axis would be in area units and you could directly determine the area change in response to each factor and factor interaction change. The actual effect value is shown in the test effects table.

Empower Method Validation Manager software provides an efficient, statistically sound approach to robustness testing. This capability is provided directly within the Empower Software, eliminating the need for third-party statistical software and the associated concerns while providing all of the security and compliance benefits of the Empower CDS.

REPORTING VALIDATION RESULTS

The Method Validation Manager default project has 32 report methods which can be copied into your own working project and tailored to your SOP (Figure 6).



MVM_LOD_LOQ_S to N_Report

Validation Protocol: Validation Study

Validation Protocol ID: 3634

Validation Study Id: 1903

Channel: PDA 270

LOD LOQ Results

	Component	RT Mean (min)	LDL/LQL Method	Signal/Noise Type	Limit of Detection	Limit of Quantitation	Units	Pass /Fail
1	Imp. F	1.305	Signal to Noise	USP s/n	0.0527	0.1596	ug/mL	Pass
2	Imp. A	1.791	Signal to Noise	USP s/n	0.0688	0.2085	ug/mL	Pass
3	Imp. G	1.889	Signal to Noise	USP s/n	0.0502	0.1520	ug/mL	Pass
4	Imp. 9	2.241	Signal to Noise	USP s/n	0.0353	0.1071	ug/mL	Pass
5	Imp. H	2.479	Signal to Noise	USP s/n	0.0409	0.1241	ug/mL	Pass
6	Imp. C	2.627	Signal to Noise	USP s/n	0.0472	0.1430	ug/mL	Pass
7	Imp. D	3.178	Signal to Noise	USP s/n	0.0294	0.0890	ug/mL	Pass
8	Imp. B	3.907	Signal to Noise	USP s/n	0.0617	0.1868	ug/mL	Pass

Figure 6. Empower method validation report method supplied with the default project.

CONCLUSION

The typical process used for validating chromatographic methods is time-consuming, error-prone, and riddled with compliance concerns; it can decrease laboratory productivity so much that the time and costs associated with bringing products to market are significantly increased. Method Validation Manager is a business-critical software that reduces the time and costs required to perform chromatographic method validation by as much as 80%.

Because Method Validation Manager allows the entire chromatographic method validation process to be efficiently performed within Empower, fewer software applications need be deployed, validated, and maintained. Software training and support is also minimized. When less software is required, the software that is business-essential can be deployed more quickly and efficiently. In addition, Method Validation Manager allows you to be fully compliant with governmental regulations by providing data security, a full set of user privileges, audit trails, and automatic data documentation; providing you with the necessary information and complete data traceability required for final reports and to pass audits and data reviews.

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Stage 3

Continued Procedure Performance Verification

Routine/QC use of an analytical method, Stage 3 of Method Lifecycle Management (MLCM), often extends over many years. Although validation experiments have demonstrated that the method is suitable, it is prudent to verify that the method continues to perform well during routine use. Trending of performance parameters has proven valuable in accomplishing this task. "***Continued Performance Verification of Analytical Procedures Using Control Charts of the Empower Chromatography Data Software***", page 83, shows how control charts can be used to monitor system suitability test results or instrument pressure.

Transferring methods between different chromatographic systems, maybe derived from different instrument vendors, or moving methods from one laboratory to another, may not always be straight forward, but the MLCM approach encompasses a well-defined change control pathway to address challenges that may arise. "***Method Transfer from Agilent 1100 Series LC System to the ACQUITY UPLC H-Class System: The Effect of Temperature***", page 89, demonstrates that moving from one instrument to another may involve returning to Stage 1 to gain better understanding of the method parameters, then moving to Stage 2 for re-validation before putting the method into routine use. Additional differences across systems are discussed in both "***Method Transfer from an Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System with Gradient SmartStart Technology*** ", page 93 and "***Dwell Volume and Extra-Column Volume: What Are They and How Do They Impact Method Transfer***", page 97.

During this stage, in keeping with the concept of continuous improvement, modifications to the method may be appropriate. One way to improve laboratory productivity is to reduce the particle size and column dimensions and change category of LC systems, as shown in "***USP Method Transfer (Modification) of Levonorgestrel and Ethinyl Estradiol Tablets from HPLC to UPLC***", page 115. This application note illustrates that, with the assistance of the ACQUITY UPLC Column Calculator, revised method conditions are easily selected and the changed conditions are within the USP Chapter <621> allowable adjustments. System suitability requirements of injection repeatability and resolution were met. Changes to older USP methods, such as introducing more modern column technologies or separations platforms can help to reduce variability of results as well as reducing analytical run times and overall costs. In this case, monitoring system pressure resulted in the addition of a gradient wash step, which further improved the method. In another example, "***USP Method Transfer (Modification) and Routine Use Analysis of Budesonide Nasal Spray from HPLC to UPLC***", page 107, modifications to the sample preparation are discussed.

Risk assessment may include modifying or scaling LC methods along with different LC instruments as shown in "***Scaling of a USP Assay for Quetiapine Fumarate Across Different Liquid Chromatographic Systems***", page 121. This example shows the impact of instrument characteristics, such as dwell volume and extra-column volume, when performing method modification based on USP guidelines and chromatographic principles.

Continued Performance Verification of Analytical Procedures Using Control Charts of Empower Chromatography Data Software

Margaret Maziarz, Paul Rainville, Sherri Naughton
Waters Corporation, Milford, MA, USA

INTRODUCTION

Analytical procedures (methods) are a critical element of the overall quality control to ensure the identity, quality, purity, and efficacy of drug substances and drug products. A method that is used for quality testing of the manufactured product must be validated to demonstrate it is suitable for its intended purpose by meeting the proper standard of accuracy, sensitivity, specificity, and reproducibility. After implementation in a QC laboratory, a validated method should be monitored during its lifecycle to continually assure that it remains fit for its intended purpose.[1]

The United States Pharmacopeia (USP) proposed a new general chapter: *The Analytical Procedure Lifecycle* <1220>. It is to introduce a holistic approach to controlling an analytical procedure throughout its lifecycle.[2] The approach starts with establishing predefined performance requirements for the analytical procedure or the analytical target profile (ATP). Depending on the intended use of the method, the ATP requirements may include the performance criteria such as accuracy, precision, specificity, and linearity. In addition, the quality of the reportable value produced by an analytical procedure, based on the product acceptance criteria, may also be a requirement specified in the ATP.[1]

The overall analytical procedure lifecycle approach is based on three stages that include design and development, performance qualification, and continued verification. The first stage, design and development, is built around a series of steps that include procedure development, risk assessment, experimental robustness studies, and knowledge gathering. The design of experiments (DoE) approach is often applied to investigate variables that may affect the performance characteristics of the procedure. The output of the DoE study may be a design space of operational conditions, within which the procedure performance is ensured. In the second stage, performance qualification, method validation is performed to show that the procedure is suitable for its intended use. This is done by conducting a validation study to confirm the procedure generates results that consistently meet the performance criteria defined in the ATP. In the final stage, procedure performance continues to be verified during routine use in the quality control (QC) laboratory to assure that the method remains fit-for-purpose and meets the ATP criteria throughout its lifecycle. Overall, the ATP is a reference point for assessing the fitness of a method throughout all stages of its lifecycle, starting from the development to continued verification.

This white paper describes the use of Empower™ 3 Control Charts to facilitate the continued procedure performance verification stage of the analytical procedure lifecycle approach. Trend analysis of the system suitability results of replicate injections, as well as of the assay results for the related substances of metoclopramide drug substance, are demonstrated using Empower 3 Control Charts. Data from separate chromatographic runs are compared against the predefined performance goals and assessed for any adverse trends. Utilizing system suitability calculation and control charts of the Empower Software streamlines trending analysis of the method performance characteristics to assure that the method continuously meets the predefined goals, or to predict performance issues before they happen.

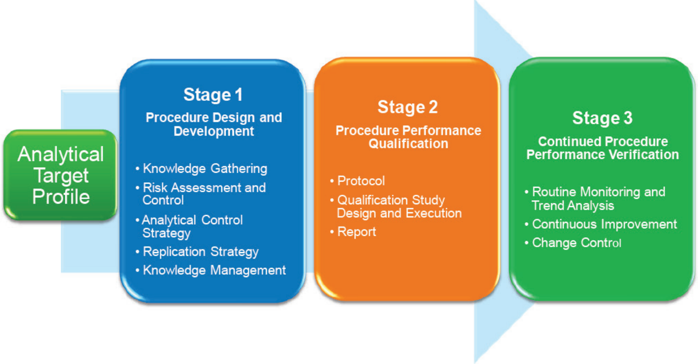


Figure 1. The analytical procedure lifecycle approach as illustrated in the USP proposed chapter <1220>.[2]

CONTINUED PROCEDURE PERFORMANCE VERIFICATION

Continued performance verification of the lifecycle approach evaluates how the method operates during routine use in a QC laboratory and confirms that the generated data continues to meet the requirements defined in the ATP.

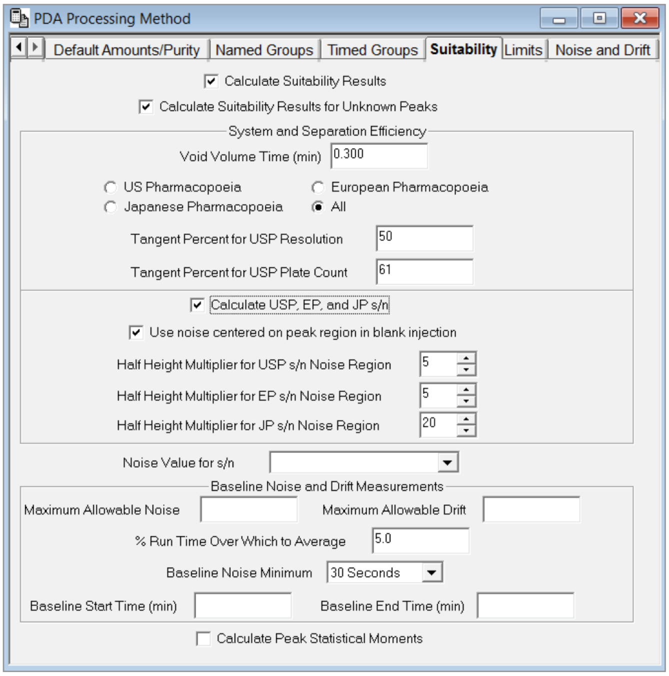
Routine monitoring may include trending of the system suitability data, tracking analytical results of real samples or standards, out-of-specification or out-of-trend investigations, stability trends, or other parameters as appropriate.[2,3] The trend analysis of the method performance should be conducted on a regular basis to assure conformance with performance requirements in the ATP. When a method is demonstrated to generate data that meets the performance requirements, it is considered acceptable. However, if frequent modifications of the operating conditions are required to meet the performance goals, or adverse trends are identified, it may be an indication that the procedure is not in control. In this case, an investigation should be performed with a goal of identifying the root cause and taking corrective action. Changes to the method or the control strategy may be required to improve operational performance. Therefore, the method should be reevaluated, revalidated, or amended, as appropriate.[1]

SYSTEM SUITABILITY TREND ANALYSIS

System suitability is a regulatory requirement to verify that the chromatographic system is performing as intended at the time of use. As stated in the USP General Chapter, <621> Chromatography, system suitability tests are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.[4]

Acceptable criteria for the system suitability test are defined as a quality check to ensure that the system functions as expected on a given day and typically include repeatability of the retention times and peak areas, USP resolution between peaks (if there are multiple peaks), and peak symmetry. The peaks of interest are often identified using retention time, therefore it is important that the retention time for a peak is constant throughout the chromatographic run. Setting up a retention time window ensures correct peak identification, while allowing for some variations in mobile phase, batch-to-batch compositions or change in column characteristics. Precision measures the closeness of repeated measurements; therefore, it is an important parameter of the system suitability test to assure confidence in the generated data. Resolution confirms acceptable separation between closely eluting peaks under method operating conditions. For some applications, tailing peaks can have a negative impact on the separation, and decrease sensitivity. Therefore, including tailing factor will ensure that the method performs acceptably over routine use.

Measuring system suitability parameters provides assurance that the method is running as expected at the time of use. The system suitability option of the Empower 3 processing method calculates system suitability parameters according to the guidelines defined by the United States, European, and Japanese Pharmacopeias (Figure 2).



☑ Calculate Suitability Results
☑ Calculate Suitability Results for Unknown Peaks

System and Separation Efficiency
Void Volume Time (min) 0.300
○ US Pharmacopoeia ○ European Pharmacopoeia
○ Japanese Pharmacopoeia ● All
Tangent Percent for USP Resolution 50
Tangent Percent for USP Plate Count 61

☑ Calculate USP, EP, and JP s/n
☑ Use noise centered on peak region in blank injection
Half Height Multiplier for USP s/n Noise Region 5
Half Height Multiplier for EP s/n Noise Region 5
Half Height Multiplier for JP s/n Noise Region 20

Noise Value for s/n

Baseline Noise and Drift Measurements
Maximum Allowable Noise
Maximum Allowable Drift
% Run Time Over Which to Average 5.0
Baseline Noise Minimum 30 Seconds
Baseline Start Time (min)
Baseline End Time (min)

☐ Calculate Peak Statistical Moments

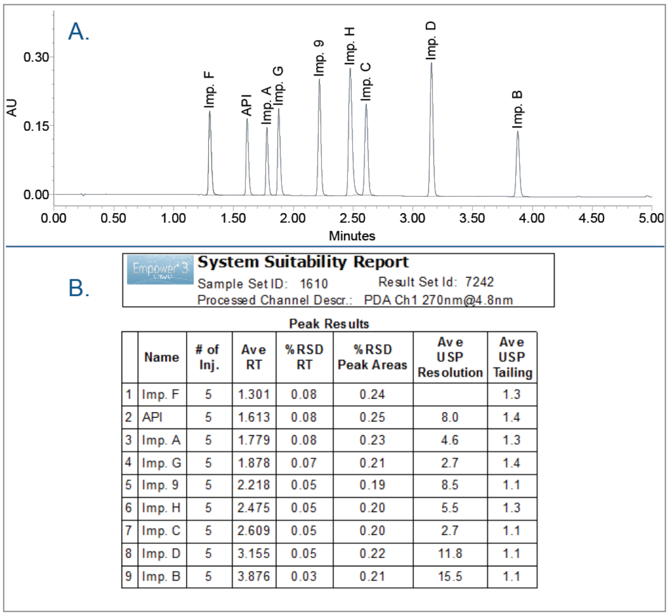
Figure 2. System suitability option in the processing method of the Empower 3 Software.

System suitability test for each chromatographic run should be conducted as per specifications defined in the individual analytical procedure. When such specifications are not available, performance can be verified according to the system suitability recommendations listed in the USP General Chapter, <621> Chromatography.[4]

In case of a UPLC™ method for related substances analysis of metoclopramide,[5] the system suitability parameters with the predefined performance goals or acceptance criteria include:

- %RSD of peak areas ≤2.0
- %RSD of retention times ≤0.5
- USP resolution of ≥2.0 between the peaks
- USP peak tailing of ≤1.5

Example of the system suitability determination for five replicate injections of a standard solution for a single chromatographic run is shown in Figure 3. The repeatability of retention times and peak areas are excellent. The USP resolution between all components and tailing for each peak pass the system suitability acceptance criteria.



System Suitability Report

Sample Set ID: 1610 Result Set Id: 7242

Processed Channel Descr.: PDA Ch1 270nm@4.8nm

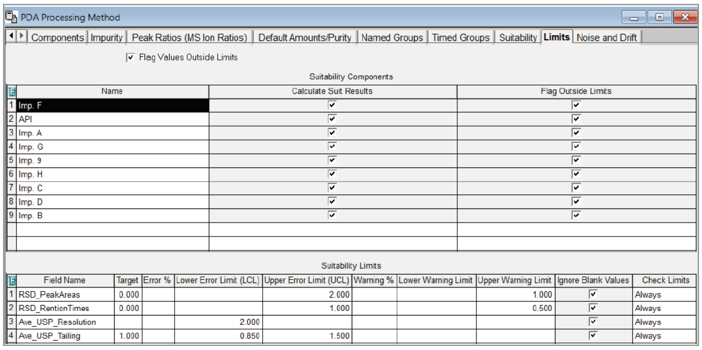
Peak Results

	Name	# of Inj.	Ave RT	%RSD RT	%RSD Peak Areas	Ave USP Resolution	Ave USP Tailing
1	Imp. F	5	1.301	0.08	0.24		1.3
2	API	5	1.613	0.08	0.25	8.0	1.4
3	Imp. A	5	1.779	0.08	0.23	4.6	1.3
4	Imp. G	5	1.878	0.07	0.21	2.7	1.4
5	Imp. 9	5	2.218	0.05	0.19	8.5	1.1
6	Imp. H	5	2.475	0.05	0.20	5.5	1.3
7	Imp. C	5	2.609	0.05	0.20	2.7	1.1
8	Imp. D	5	3.155	0.05	0.22	11.8	1.1
9	Imp. B	5	3.876	0.03	0.21	15.5	1.1

Figure 3. UPLC method for analysis of related substances of metoclopramide drug.. Five replicate injections standard solution at 0.06 mg/mL (A) and system suitability results (B).

When an analytical procedure is implemented in a QC laboratory for quality testing of the manufactured product, it should be monitored during its lifecycle to assure that it continuously meets the predefined performance goals, in this instance, system suitability requirements. The control charts of the Empower Software enable quick and easy data trending over time against the performance characteristics for any analytical procedure. The Empower Software compares the processed data against the system suitability acceptance criteria defined by the user, flagging any out-of-trend results.

For related substances of the metoclopramide method, control charts of the Empower 3 Software are used to monitor system suitability trending over 20 separate chromatographic runs. Performance target goals for each parameter, lower and upper limits, as well as the warning limits are entered in the "Limit" tab of the Empower 3 Software processing method (Figure 4). These limits include %RSD of peak areas, %RSD of retention times, average USP resolution, and average USP peak tailing for five replicate injections of standard solution. The control charts are then used to evaluate system suitability trending against the predefined limits. Example of trend analysis of %RSD for impurity A peak areas from five replicate injections over 20 separate chromatographic runs is displayed in Figure 5. The performance target, lower and upper limits, as well as warning limit lines are displayed in a plot. The trend plot shows that the repeatability of impurity A peak areas is below the target performance goal, but two of the chromatographic runs reached the upper warning limit. Approaching a warning limit may be an indication that some preventative and corrective action might be needed to prevent getting out-of-specification results. A complete system suitability trend analysis of %RSD of peak areas, %RSD of retention times, average USP resolution, and average USP peak tailing for impurity A (Figure 6) show that all results meet the target performance goals. Using control charts, system suitability trending displayed in a plot enables quick data examination for any out-of-trend results over the lifetime of the analytical procedure.



☑ Flag Values Outside Limits

Suitability Components

	Name	Calculate Suit Results	Flag Outside Limits
1	Imp. F	☑	☑
2	API	☑	☑
3	Imp. A	☑	☑
4	Imp. G	☑	☑
5	Imp. 9	☑	☑
6	Imp. H	☑	☑
7	Imp. C	☑	☑
8	Imp. D	☑	☑
9	Imp. B	☑	☑

Suitability Limits

	Field Name	Target	Error %	Lower Error Limit (LCL)	Upper Error Limit (UCL)	Warning %	Lower Warning Limit	Upper Warning Limit	Ignore Blank Values	Check Limits
1	RSD_PeakAreas	0.000			2.000			1.000	☑	Always
2	RSD_RentionTimes	0.000			1.000			0.500	☑	Always
3	Ave_USP_Resolution			2.000					☑	Always
4	Ave_USP_Tailing	1.000		0.850	1.500				☑	Always

Figure 4. Limits for the system suitability parameters defined in the processing method of the Empower Software.

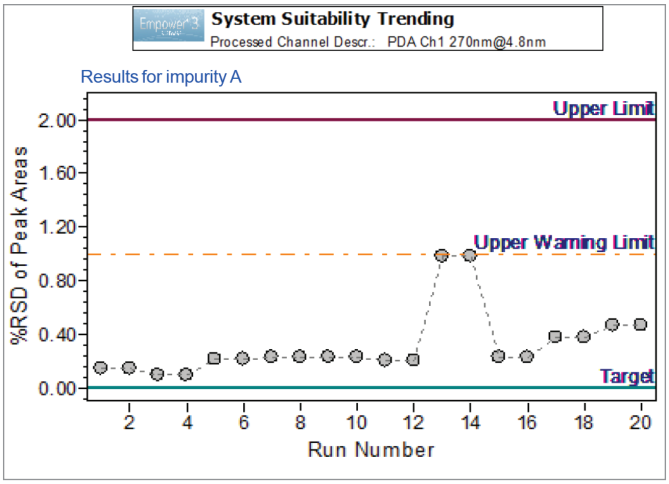


Figure 5. Trend analysis using control chart of the Empower 3 Software showing %RSD for impurity A peak areas from five replicate injections over 20 separate chromatographic runs.

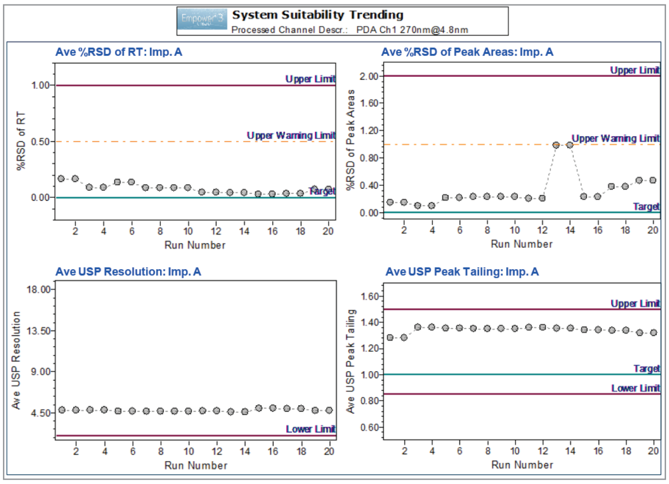


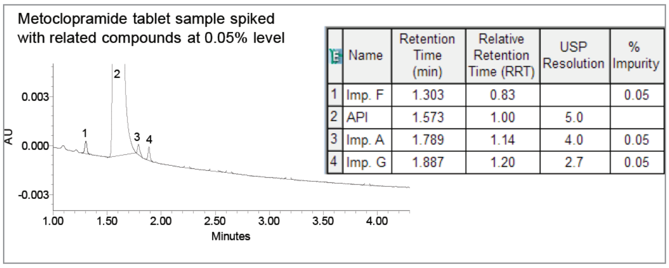
Figure 6. Control charts of the Empower 3 Software for system suitability trend analysis for impurity A from five replicate injections of standard over 20 separate chromatographic runs.

ASSAY RESULTS TREND ANALYSIS

Reportable values generated using qualified analytical procedures via laboratory testing provide the basis for key decisions regarding compliance of the test article with the regulatory, compendia, and manufacturing limits.[1] The laboratory testing is necessary to ensure that the components, containers and closures, in-process materials, and finished products conform to the specifications. Hence, reportable assay results are used to confirm whether the manufactured product meets the prescribed standards for identity, quality, safety, purity, and potency.

Trend analysis of reportable values over the lifetime of the drug product enables a quick identification of out-of-trend results or deviations from the acceptance criteria. Charts visually display the data, which helps to examine patterns, predict likelihood of future out-of-specification results, and suggest corrective measures.

Control charts of the Empower 3 Software facilitate trend analysis of the reportable assay results over separate chromatographic runs against the product specification or acceptance criteria. Trend analysis of the reportable results is demonstrated using assay results for related substances analysis in a metoclopramide tablet sample.[5] An example of a related substances analysis (% impurity) in a spiked metoclopramide tablet sample is shown in Figure 7. Using the control charts of the Empower 3 Software, the trending is performed over 20 chromatographic runs to examine how the assay results compared to the predefined acceptable limit or specification (Figure 8). For this analysis, the specification includes related substance content (or % impurity) of less than 0.1% (the upper limit) with a warning limit of 0.075% in sample injection. The trend plot clearly indicates results that meet and exceed the allowable limits, respectively. Using control charts, trending reportable results helps to identify results that deviate or exceed specifications over the lifetime of the drug product.



Metoclopramide tablet sample spiked with related compounds at 0.05% level

	Name	Retention Time (min)	Relative Retention Time (RRT)	USP Resolution	% Impurity
1	Imp. F	1.303	0.83		0.05
2	API	1.573	1.00	5.0	
3	Imp. A	1.789	1.14	4.0	0.05
4	Imp. G	1.887	1.20	2.7	0.05

Figure 7. Results for related substances analysis in metoclopramide tablet sample.

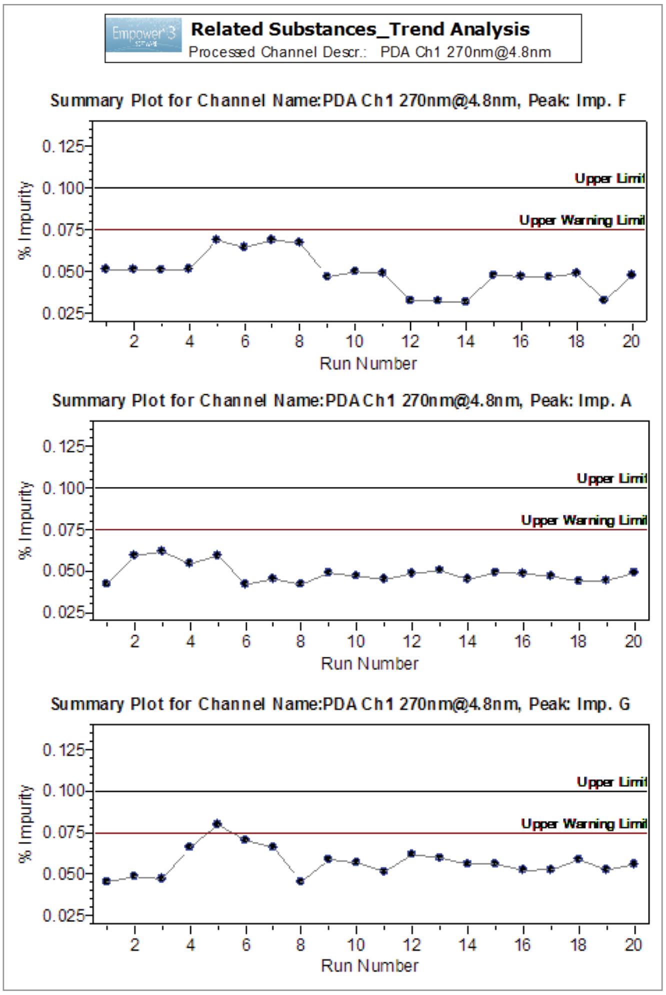


Figure 8. Trend analysis of assay results for related substances content (% impurity) in metoclopramide tablet sample over 20 chromatographic runs.

COLUMN ROUTINE USE

The performance of the LC column may deteriorate over long-term use due to the stress applied by the separation conditions or samples analyzed. When the LC column is used for a specific assay, tracking the injection count and column pressure will help to decide if a corrective action is needed to prevent issues before they happen or when the column is approaching end of life so that a new column should be installed. This may subsequently prevent failures of the sample assay runs or a need to start a new chromatographic run.

As demonstrated in a routine use study of a UPLC assay method for Telmisartan Tablets, a control chart in the Empower Software is used to display system pressure trending over 3000 injections of standard and tablet preparation solutions on a single column lot.[6] The system pressure monitored for a routine use study shows slight increase in overall system pressure at approximately 2100 injections (Figure 9). This increase indicated a need to wash the column, which regenerated and returned it to the original operating conditions.

Monitoring other parameters such as column theoretical plates, USP resolution, and peak tailing in control charts may also help to predict whether a corrective action is needed to prevent problems or for how many injections is a column expected to last. Overall tracking column performance characteristics over routine use will help to reduce instrument downtime caused by column failures, hence improve routine QC laboratory efficiency.

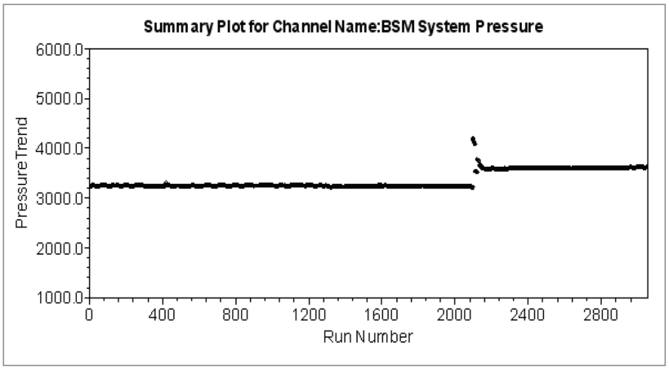


Figure 9. System pressure trend analysis for routine use over 3000 injections on the ACQUITY™ UPLC HSS T3 Column.[6]

CONCLUSION

Continued performance verification of the analytical procedure lifecycle approach routinely examines performance data of a method over its lifecycle. Therefore, large amounts of data must be reviewed to confirm that the method continuously meets the performance goals. Trending using control charts visually displays the data and the target performance goals, which helps to quickly identify patterns and out-of-trends results that may go unnoticed when using summary reports.

Control charts from the Empower 3 Software provide graphical representation of the performance characteristics data trends over time. They provide an easy way to perform trend analysis of data generated by a method, which allows quick identification of any out-of-trend results or deviations from the performance goals. This helps to predict any potential performance problems or take preventive and corrective actions before a major problem occurs. Whether examining system suitability trending, column performance characteristics, or reportable results for a specific product, Empower control charts will help to assure that a method continuously generates fit-for-purpose results.

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[TECHNOLOGY BRIEF]

Method Transfer from Agilent 1100 Series LC System to the ACQUITY UPLC H-Class System: The Effect of Temperature

Paula Hong and Patricia R. McConville

GOAL

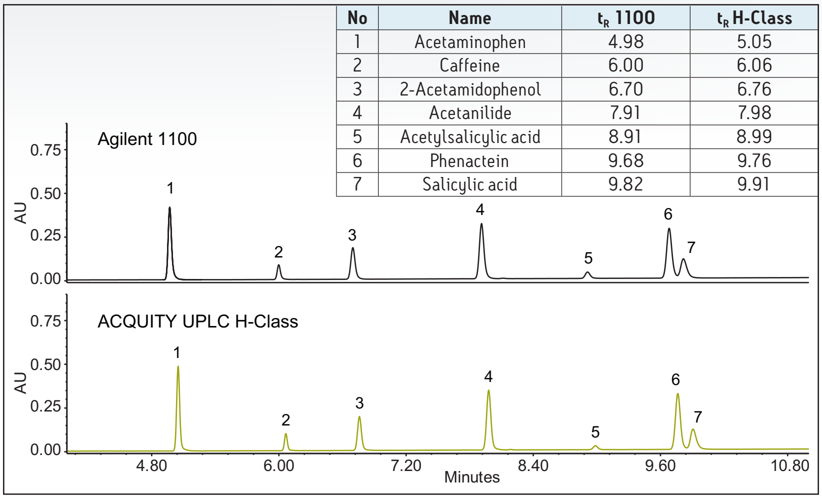
To demonstrate the comparability of temperature selectivity effects on HPLC method transfer from an Agilent 1100 Series LC System to an ACQUITY UPLC® H-Class System.

BACKGROUND

Given the high investment in instrumentation, analytical laboratories often have the need or desire to transfer methods across available systems, regardless of whether those systems come from a single or from multiple instrument manufacturers. For methods that are temperature-sensitive, transferring legacy HPLC methods across systems from different manufacturers can be challenging. Instrument modes for heat transfer can vary: instrument attributes can include active or passive pre-heating of the mobile phase and/or static or convection heating and cooling of the column. The differences in heat transfer approaches can affect the fidelity of the separation.

If the separation is not preserved, the simplest approach to resolving poor method transfer is to adjust the set column temperature.[1] If this option is not permitted, the method may need to be redeveloped, or deemed untransferable, both resulting in added costs from the loss of instrument time and the loss of the analytical chemist's time.

Methods transfer from an Agilent 1100 Series LC System to a Waters ACQUITY UPLC H-Class System with a CH-A can be successful over a range of temperatures.



No	Name	t_R 1100	t_R H-Class
1	Acetaminophen	4.98	5.05
2	Caffeine	6.00	6.06
3	2-Acetamidophenol	6.70	6.76
4	Acetanilide	7.91	7.98
5	Acetylsalicylic acid	8.91	8.99
6	Phenacetein	9.68	9.76
7	Salicylic acid	9.82	9.91

Figure 1. Separation of an analgesic mix on an Agilent 1100 Series LC System and an ACQUITY UPLC H-Class System at 36 °C. Comparable retention times were observed on both instruments.

THE SOLUTION

Method transfer from the Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System with a single column compartment (CH-A) was performed. To account for differences across the systems, each instrument's dwell volume was measured[2] and, for the ACQUITY UPLC H-Class System, the appropriate gradient delay was entered using gradient SmartStart Technology.[3] To evaluate the effect of temperature control, each system was tested both with and without mobile phase pre-heating. The Agilent 1100 Series LC System included a passive pre-heater (3 µL) and the ACQUITY UPLC H-Class System contained an active

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pre-heater. For analyses without pre-heating, the Agilent 1100 Series LC System was plumbed to bypass the passive pre-heater (directly from the injector valve to the column inlet), and the ACQUITY UPLC H-Class System's active pre-heater was set to active, disabled mode in the instrument console (i.e., no active pre-heating).

On an Agilent 1100 Series LC System, an HPLC separation of analgesics was found to produce selectivity differences over a temperature range of 36 °C to 60 °C. Given the impact of temperature on the separation, method transfer to the ACQUITY UPLC H-Class System was also evaluated over the same temperature range as shown by a representative chromatogram at 36 °C (Figure 1). The results showed comparable retention times and similar retention time trends on both systems over the temperature range (Figure 2, red and blue lines). Further confirmation of the equivalency was provided by the retention time behavior of phenacetin and salicylic acid (Figure 3). The same selectivity changes were observed at temperature intervals of 2 °C, confirming the high precision across the two systems.

While mobile phase pre-heating at higher temperatures can improve column performance by reducing axial and radial temperature gradients,[4] not every legacy HPLC method may use this type of temperature control. Therefore, the same experiments were repeated on both systems without mobile phase pre-heating. Under these conditions, comparable retention times were observed on both the Agilent 1100 Series LC System and the ACQUITY UPLC H-Class System (Figure 4). However, over the entire temperature range (36 °C to 60 °C), both sets of analyses produced minimal changes in retention time as a function of temperature (Figure 2, green and purple lines). In addition, without mobile phase pre-heating, no selectivity changes were observed above 42 °C as in the first set of experiments (i.e., those which used mobile phase pre-heating) (Figure 4). It is important to note: for this example, at temperatures above 42 °C, methods transfer – even on the same system

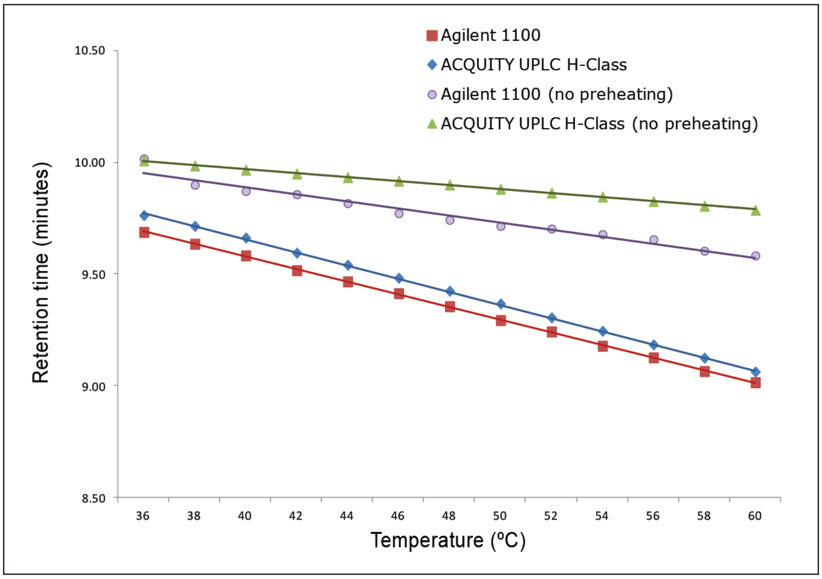

Figure 2. The effect of column temperature control on retention time for phenacetin. Both the ACQUITY UPLC H-Class System and the Agilent 1100 Series LC System exhibited a reduction in retention time with increasing temperature. For each system, mobile phase pre-heating resulted in greater change in retention time as a function of temperature.

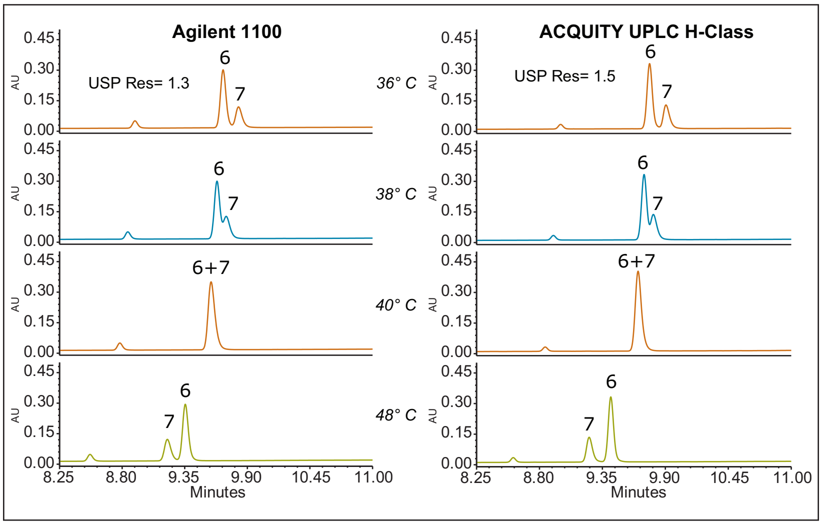

Figure 3. The effect of temperature on the separation of an analgesic mix. Both the Agilent 1100 Series LC System and the ACQUITY UPLC H-Class System showed similar selectivity changes with temperature for phenacetin (6) and salicylic acid (7).

– would be unsuccessful if the changes are made to mobile phase pre-heating. Thus, when transferring a method the use - or lack - of mobile-phase pre-heating should be considered.

SUMMARY

Temperature control in an HPLC method can have a significant impact on the success of methods transfer. In this discussion, an HPLC method was successfully transferred from an Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System with a single column compartment. The effect of temperature upon the separations was found to be comparable. However, each system produced different results depending on whether or not mobile phase pre-heating was used. For this set of experiments, temperature selectivity effects were only observed using mobile phase pre-heating. Thus, the impact of temperature control should be considered in methods transfer. However, with or without mobile phase pre-heating, we have demonstrated successful method transfer from an Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System with a single column compartment (CH-A).

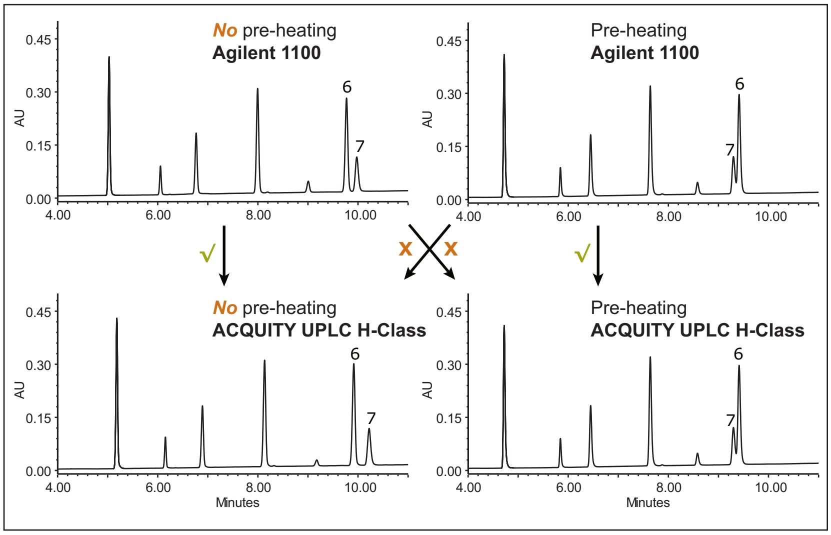

Figure 4. *Effect of mobile phase pre-heating on an analgesic separation at 46 °C. With the same pre-heating configuration, the Agilent 1100 Series LC System and the ACQUITY UPLC H-Class System produced comparable retentivity (√). However, selectivity differences for phenacetin and salicylic acid were observed between those separations run with mobile phase-preheating and those run without (X).*

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[NOTES]

Method Transfer from an Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System with Gradient SmartStart Technology: Analysis of an Active Pharmaceutical Ingredient and Related Substances

Paula Hong and Patricia R. McConville

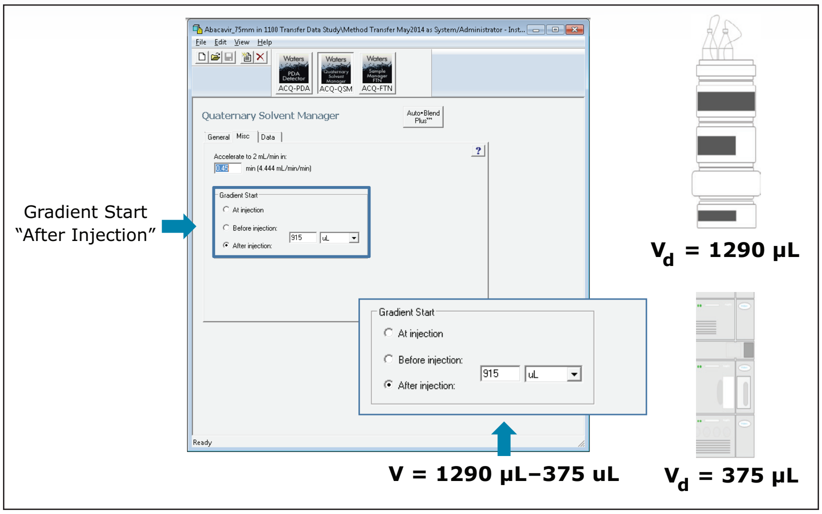
GOAL

To transfer a reversed-phase LC gradient method for an API and its related substances from an Agilent 1100 Series LC system to an ACQUITY UPLC® H-Class System.

BACKGROUND

The analysis of an active pharmaceutical ingredient (API) and its related substances by HPLC is often conducted throughout the life cycle of a drug to ensure safety and efficacy. These assays are typically performed in regulated laboratories, in which changes to the method are either limited[1] or not permitted and may result in the need for a complete revalidation. While many legacy methods were originally developed on traditional HPLC systems, there may be a desire to transfer the method to newer UHPLC instrumentation. This need may be driven by the available resources or by an overall drive to modernize. When transferring the method to a different HPLC/UHPLC instrument, the new instrumentation must typically produce the same separation and meet the system suitability requirements of the original method/instrument.[1] However, there are a number of instrument attributes that can affect the success of the method transfer. For gradient separations, the impact of dwell volume can be dramatic. The dwell volume, which is affected by the mixer, valves, and injector, varies from instrument to instrument whether the instrument is from the same or different manufacturers. For gradient method transfer, these instrument characteristics should be considered and compensated for in a gradient table.[1]

Facilitate methods transfer to an ACQUITY UPLC H-Class System using gradient SmartStart Technology.



Abacavir_75mm in 1100 Transfer Data Study\Method Transfer May2014 as System/Administrator - Inst...

Quaternary Solvent Manager

Accelerate to 2 mL/min in: 0.45 min (4.444 mL/min/min)

Gradient Start

○ At injection

○ Before injection:

● After injection: 915 uL

Ready

Figure 1. Instrument method editor for ACQUITY UPLC H-Class Quaternary Solvent Manager. To assist in methods transfer the instrument method allows the analyst to adjust the gradient start either before or after the injection using gradient SmartStart Technology. In this example, the system volumes for both instruments were measured and the difference was entered into ACQUITY UPLC H-Class System method.

Agilent 1100 Series LC System		ACQUITY UPLC H-Class System	
Module	Part Number	Module	Part Number
Degasser	G1322A		
Quaternary Pump	G1311A	Quaternary Solvent Manager	186015018
Autosampler	G1313A	Sample Manager FTN	186015017
Column Compartment	G1316A	Column Heater (CH-A)	186015042
DAD Detector	G1315B	PDA Detector	186015032

Table 1. System modules and part numbers
Chromatography Data System (both): Empower3 FR2.

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THE SOLUTION

A previously published method for abacavir and related substances[2] was transferred from an Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System (Table 1). The Agilent 1100 Series LC System was configured with a passive mobile phase pre-heater (3 µL), while the ACQUITY UPLC H-Class System was configured with an active mobile phase pre-heater. To account for gradient delay differences, each instrument's dwell volume was measured.[3] The measured dwell volume was greater on the Agilent 1100 Series LC System. Therefore, to compensate for the differences in dwell volume between the Agilent 1100 Series LC System and the target ACQUITY UPLC H-Class System, a 915-µL "after injection" delay was used for the analysis on the ACQUITY UPLC H-Class System. This delay was entered directly in units of volume or µLs using gradient SmartStart Technology (Figure 1) in the instrument method.[4] This feature compensated for the differences in dwell volume in methods transfer, eliminating the need to make manual adjustments to the gradient table, an action which could trigger a full revalidation of the method. The volume entry into the gradient SmartStart Technology was the only adjustment to the method.

Both the Agilent 1100 Series LC System and the ACQUITY UPLC H-Class System produced comparable separations (Figure 2). Specifically, the retention times for abacavir and related compounds were all within 0.2 min or less than 3% deviation across the two instruments (Table 2). The relative retention times, which were calculated relative to the API, were all within 0.01% deviation for the related substances. The USP resolution for the critical pair (API and compound 3) was ≥2.5 on both systems, indicating no substantial loss of resolution in method transfer. The % area of the related substances and the API were within 0.2% on both instruments.

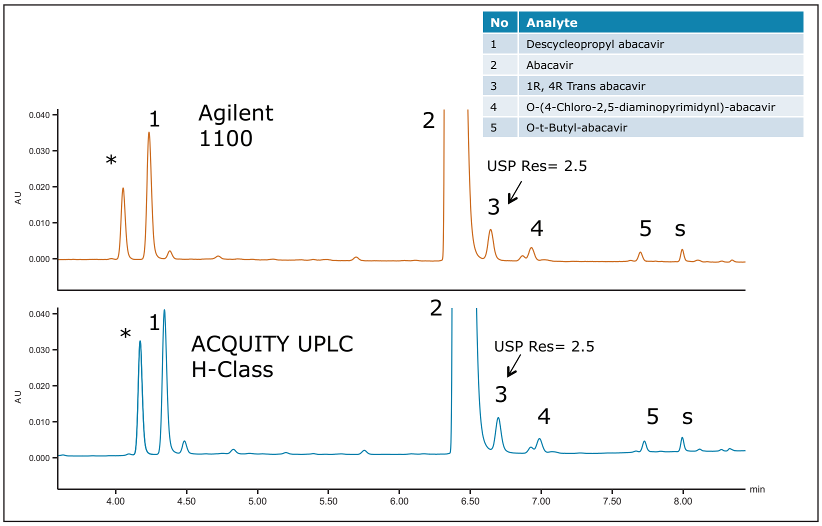

*Figure 2. Method transfer of the analysis of abacavir and related substances. The separation was performed on an Agilent 1100 Series LC System using a CORTECS® C_{18}, 2.7 µm, 4.6 x 75 mm column. Method transfer to an AQUITY UPLC H-Class resulted in a comparable separation and similar resolution for the API (2) and related compound (3). (s=solvent peak, *=unknown analyte)*

Compound	Retention time (min)			Relative retention time			% Area		
	Agilent 1100	ACQUITY UPLC H-Class	Percent Deviation	Agilent 1100	ACQUITY UPLC H-Class	Absolute Deviation	Agilent 1100	ACQUITY UPLC H-Class	Absolute Deviation
unknown	4.05	4.17	2.96	0.64	0.65	0.01	1.84	1.75	0.09
1	4.23	4.35	2.84	0.67	0.68	0.01	2.4	2.42	0.02
2-Abacavir	6.34	6.4	0.95	1.00	1.00	n/a	94.23	94.38	0.15
3	6.64	6.7	0.90	1.05	1.05	0.00	0.68	0.66	0.02
4	6.93	6.99	0.87	1.09	1.09	0.00	0.34	0.31	0.03
5	7.70	7.73	0.39	1.21	1.21	0.00	0.16	0.16	0.00

Table 2. Comparison of average retention times and peak areas for abacavir and related substances on an Agilent 1100 Series LC System and an ACQUITY UPLC H-Class System. Five replicate injections were performed. Retention times on both systems were within 3% deviation. Relative retention times and peak area percent (%) were within 0.2 absolute deviation.

Compound	Retention time				Peak area			
	Agilent 1100		ACQUITY UPLC H-Class		Agilent 1100		ACQUITY UPLC H-Class	
	Standard deviation	%RSD	Standard deviation	%RSD	Standard deviation	%RSD	Standard deviation	%RSD
1	4.64E-03	0.10	1.10E-03	0.03	2.22E+03	2.57	8.27E+02	0.88
2-Abacavir	6.12E-03	0.10	2.12E-03	0.03	5.25E+04	1.55	1.61E+03	0.04
3	5.81E-03	0.08	1.52E-03	0.02	4.17E+02	1.70	3.77E+01	0.15
4	5.54E-03	0.08	1.92E-03	0.03	2.49E+02	2.05	4.88E+01	0.40
5	4.34E-03	0.06	1.10E-03	0.01	1.06E+02	1.85	4.58E+01	0.72

Table 3. Comparison of retention time and peak area repeatability for analysis of abacavir and related substances on Agilent 1100 Series LC System and ACQUITY UPLC H-Class System. For five replicate injections, both systems produced relative standard deviations of less than 0.2% for retention time and less than 3% for peak area.

Many methods use retention time and relative retention time for identification purposes only.[5,6] When transferring a method from one manufacturer's system to another, a generally accepted criterion for retention time variance is within 3–5%.[7] The results obtained for the method transfer described meet this criterion.

To evaluate repeatability of the method, five replicate injections were performed on both instruments. The standard deviations and percent relative standard deviations (%RSD) were calculated (Table 3). On both systems, the retention time RSD's were less than 0.2% and the peak area RSD's were less than 3% for all the known analytes. In addition, the analysis on the ACQUITY UPLC H-Class System produced lower peak area RSD's for all the analytes, as compared to that run on the Agilent 1100 Series LC System. While the injection repeatability on both systems was acceptable (<3% RSD), the ACQUITY UPLC H-Class System had slightly lower injection-to-injection variability as measured by the peak area %RSD.

SUMMARY

An assay for the analysis of abacavir and related substances was successfully transferred from an Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System. The fidelity of the separation was preserved: the retention times were within 2%, and the relative retention times were within 2%. For this assay, the %RSD's for injection repeatability were lower on the ACQUITY UPLC H-Class System as compared to the Agilent 1100 LC Series System. In addition, by using gradient SmartStart Technology in the instrument method, the differences in system volume were factored into the method – without the need to make any manual adjustments to the gradient table. These results demonstrate how gradient method transfer from Agilent 1100 Series LC System to an ACQUITY UPLC H-Class System can be performed with minimal adjustments to the method and/or the system.

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[NOTES]

Dwell Volume and Extra-Column Volume: What Are They and How Do They Impact Method Transfer?

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Waters Corporation, Milford, MA, USA

A chromatographic separation is impacted by numerous factors, including the LC system and its characteristics. The impact of the chromatographic system on the separation may not be obvious until the method is transferred to another LC system or scaled to a different column dimension. By characterizing the system and understanding these differences, strategies can be undertaken to increase the success of methods transfer. These approaches can use software or hardware tools – including the ACQUITY™ instrument control software – or follow the USP <621> Chromatography guidelines for scaling to translating methods.[1]

INTRODUCTION

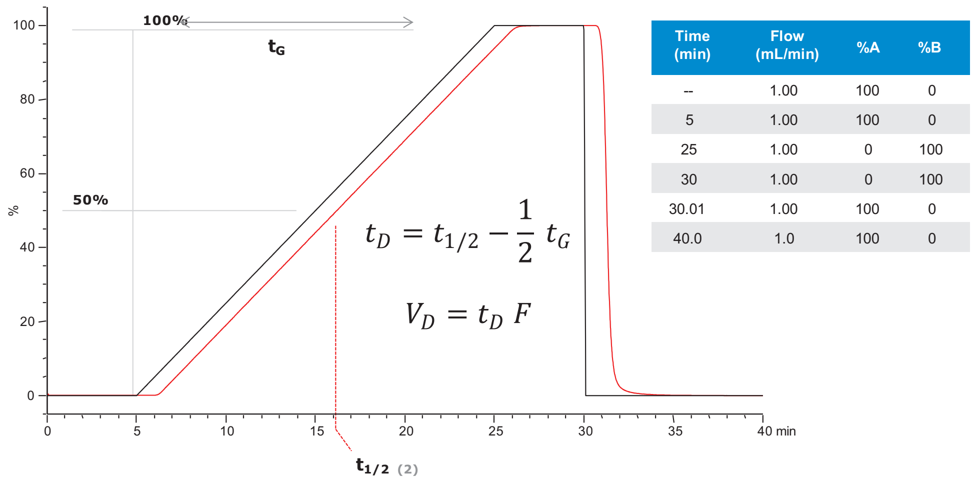
Method transfer or method scaling across different instrument platforms can be affected by both dwell volume and extra-column dispersion. Each characteristic will have a very different effect on the chromatographic separation. Dwell volume is the volume required for the change in a gradient to reach the column, or the volume difference between the point of mixing and the head of the column. Dwell volume impacts the retention times of a gradient separation, but can also affect selectivity, particularly for early eluting compounds. Extra-column dispersion is a measurement of the broadening of a peak that occurs between the injector and the detector and excludes the column. Extra-column dispersion or volume impacts peak width, resolution, and the overall efficiency of a separation. In this review, the differences of these attributes across a variety of instruments will be examined, specifically for their impact on methods transfer. We will also review strategies to minimize the impact on the method transfer. These recommendations will follow the USP <621> guidelines.

DWELL VOLUME

MEASURING DWELL VOLUME

For every chromatographic system, the dwell volume is a physical characteristic that is primarily a function of the pump. It represents the volume difference between the device that controls the delivery of the gradient and the head of the column. It is also commonly referred to as gradient delay volume. Dwell volume is impacted not only by the tubing (length and internal diameter (I.D.)), but also by any valves or mixers in the fluidic path up to the head of the column. To understand the variations among systems, we measured the volume difference of a programmed gradient and the delivery of said gradient to the detector (Figure 1). This was accomplished by using a gradient from 0–100% B where B contains a UV absorbent compound (in this case caffeine). Using a UV detector, we can then record the response of the UV absorber over time, which reflects the delay in the gradient delivery. For a more accurate assessment, the delay was calculated at 50% of the gradient. The analyses were conducted in triplicate with the average values recorded. This procedure has been extensively described in literature.[2,3]

The measured values (Figure 2), cover a range from <100 µL to over 1 mL for UPLC,™ UHPLC, and HPLC quaternary and binary systems. For each type of system, the pump characteristics determine the dwell volume. For quaternary pumps, changes in the gradient occur at the gradient proportioning valve (GPV). This valve, which is typically before the mixer, results in a greater dwell volume than in a binary system with the same mixer, tubing and valves, because the GPV is located *before* the solvent is pressurized by the pump. The total dwell volume not only includes the valve and the mixer, but also the internal volume of the pump heads themselves. In binary systems, the gradient change occurs at the mixer after the solvent has been pressurized by the pump; there is no gradient proportioning valve that affects the dwell volume. Rather, the gradient is formed by varying the flow rate of the A and B pumps to form the desired flow rate and composition.



Time (min)	Flow (mL/min)	%A	%B
--	1.00	100	0
5	1.00	100	0
25	1.00	0	100
30	1.00	0	100
30.01	1.00	100	0
40.0	1.0	100	0

Figure 1. Method for determining a system's dwell volume. A UV absorbent compound was placed in mobile phase B using a programmed gradient from 0–100% B. The chromatogram above shows the overlay of the programmed gradient (black) and the UV signal (red). The calculations were performed by determining the time at which the UV trace reached 50% of total absorbance ($t_{½}$) and then subtracting ½ of the programmed gradient (in time) to determine the dwell time (t_D). This value was converted to volume by multiplication with the flow rate (F).

Conditions: Mobile phase A: water; Mobile phase B: 10 mg/L caffeine in water; Wavelength: 273 nm. For all systems, a restrictor was used to ensure that the measurements were within a systems recommended operating pressures.

System	Dwell Volume (V_D) (mL)
Alliance HPLC (Quaternary) System	1.15
ACQUITY Arc™ (Quaternary) System	1.19 (Path 1) 0.770 (Path 2)
ACQUITY UPLC H-Class PLUS (Quaternary) System	0.375
ACQUITY UPLC I-Class PLUS (Binary) System	0.073

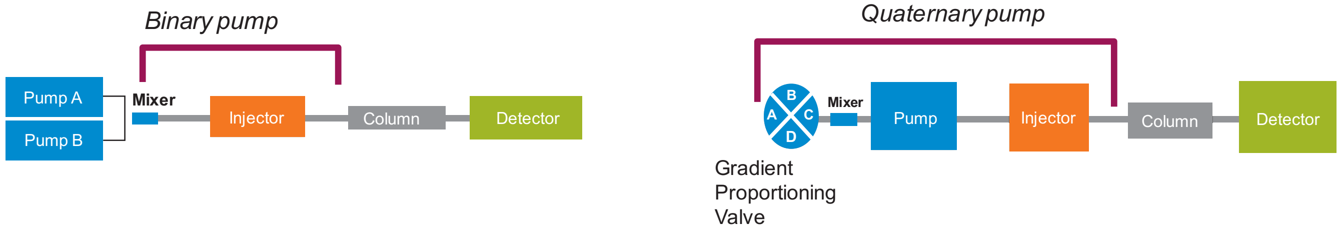


Figure 2. Measured dwell volumes for specific systems. All measurements were performed with the system in its default configuration. Values may vary from system to system based on column heater, tubing, and flow cell configuration. Red brackets indicate the parts of the system contributing to dwell volume.

COMPENSATING FOR DWELL VOLUME WHEN TRANSFERRING A METHOD

For each pump, differences in dwell volume and mixing behavior can impact retention times in gradient methods transfer. To illustrate these effects, the separation of flavonoids in orange extract was transferred from an ACQUITY UPLC H-Class PLUS (Quaternary) System to an Alliance™ HPLC (quaternary) System. When the method is transferred, the difference in retention time is apparent (Figure 3). In this separation, the retention time difference from UPLC (3A) to the HPLC (3B) is approximately 1 minute. The delay is throughout the gradient and also affects the time at which the gradient ends and the re-equilibration begins. Therefore, improper re-equilibration may occur, if the method is transferred to a system with larger dwell volume and no adjustments are made to the overall run time.

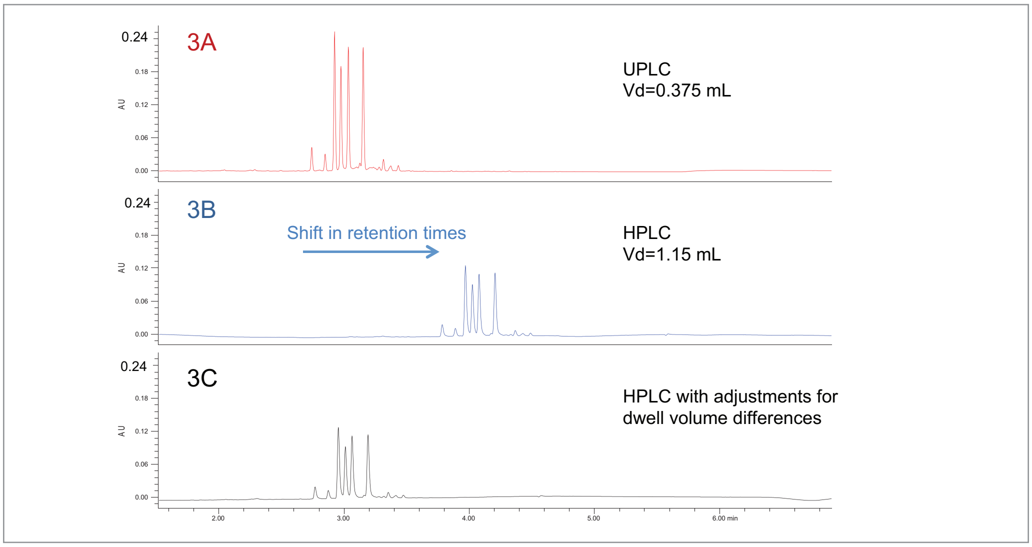
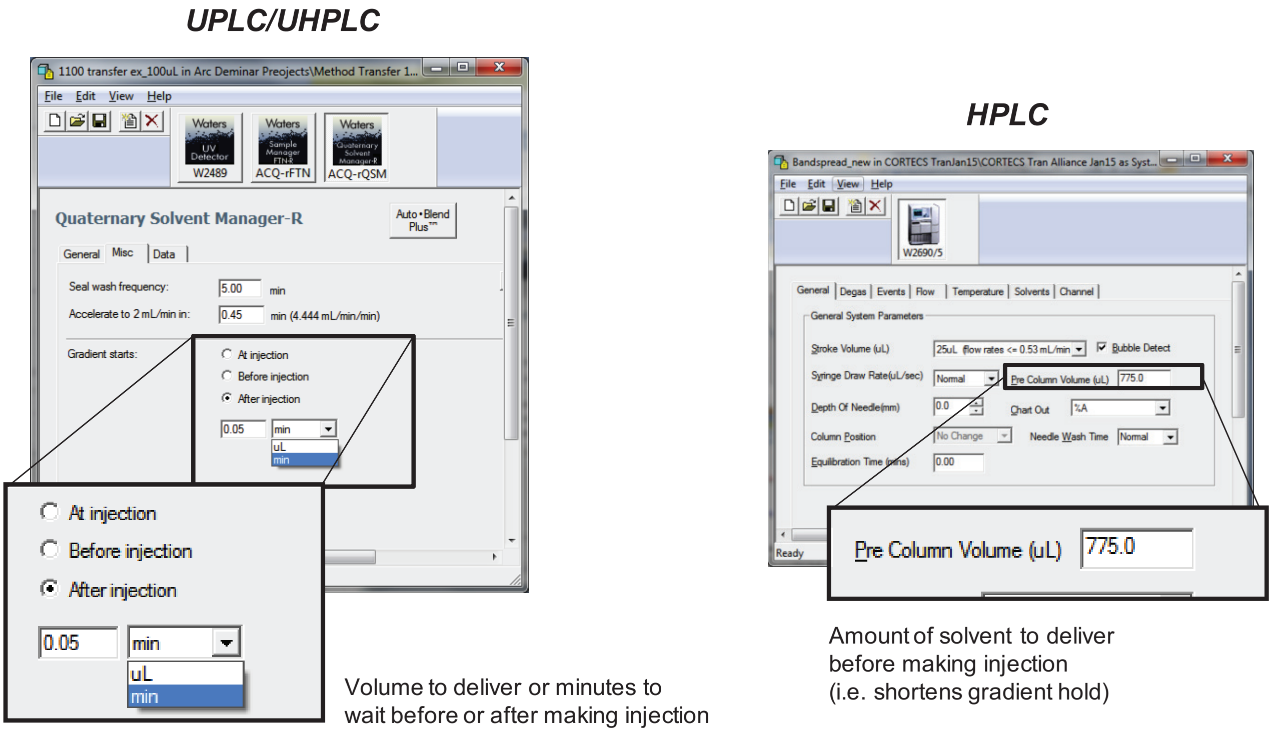


Figure 3. Effect of dwell volume on methods transfer. The separation of flavonoids in orange extract was performed on a UPLC and an HPLC system. The retention time offset is approximately 1.5 min between the two systems. This shift is expected given the dwell volume differences (0.775 mL) across the two systems. By adjusting the HPLC injection to occur after the gradient start, the dwell volume (and retention time) differences can be minimized.

Conditions: Column: CORTECS™ C_{18}+, 2.7 µm, 2.1 x 75 mm (p/n 186007396); Mobile phase A: 0.1% (v/v) HCOOH in H_2O; Mobile phase B: 0.1% (v/v) HCOOH in ACN; Flow rate: 0.6 mL/min; Gradient: 5–90% B in 4 min, 90% B for 1 min, 90–5% B in 1 min, 5% B for 5 min.

Note: The extra-column volume of the HPLC system (3B and 3C) negatively impacts the peak widths and resolution of the separation.

Adjustments for dwell volume differences are allowed per the USP <621>.[4] The typical approach to address dwell volume differences is to alter the gradient table, such as increasing the initial isocratic hold or shortening the gradient duration to emulate another system with different dwell volume. However, this approach requires making changes to the gradient table, which may result in the need to revalidate the method if operating in a regulated environment. As illustrated in the ACQUITY UPLC H-Class PLUS System and Alliance HPLC System method editor software (Figure 4), another approach towards addressing these differences is to adjust the gradient start time relative to the injection, through software. Using this feature, the previously described separation (Figure 3A) on an ACQUITY UPLC H-Class PLUS System was emulated on the Alliance HPLC System (3C). A pre-column volume of 775 µL (1.15–0.375 mL) was entered into the Alliance instrument method. The resulting chromatogram (3C) produces a shift in retention time on the Alliance HPLC System, with all the peaks eluting earlier (3B versus 3C). The new retention times are comparable to those observed on the UPLC PLUS System, illustrating an approach to minimize retention time difference across the two systems without changing the gradient.


UPLC/UHPLC

1100 transfer ex_100uL in Arc Deminar Preojects\Method Transfer 1...

Quaternary Solvent Manager-R

Seal wash frequency: 5.00 min

Accelerate to 2 mL/min in: 0.45 min (4.444 mL/min/min)

Gradient starts:

○ At injection

○ Before injection

● After injection

0.05 min

Volume to deliver or minutes to wait before or after making injection

HPLC

Bandspread_new in CORTECS TranJan15\CORTECS Tran Alliance Jan15 as Syst...

General System Parameters

Stroke Volume (uL): 25uL (flow rates <= 0.53 mL/min

☑ Bubble Detect

Syringe Draw Rate(uL/sec): Normal

Pre Column Volume (uL): 775.0

Depth Of Needle(mm): 0.0

Chart Out: %A

Column Position: No Change

Needle Wash Time: Normal

Equilibration Time (mins): 0.00

Amount of solvent to deliver before making injection (i.e. shortens gradient hold)

Figure 4. Tools for gradient adjustments between HPLC and UPLC. Instrument method editor options are available to adjust gradient start relative to the injection to compensate for dwell volume adjustments across systems.

EXTRA-COLUMN VOLUME

MEASURING EXTRA-COLUMN EFFECTS

Band broadening in HPLC is a function of both intra-column and extra-column effects. Extra-column effects are related to the volume from the injector to the detector. These effects are influenced by physical components of the system, including the needle seat, tubing connectors to the column, detector flow cells, preheaters, etc., as well as method conditions, such as injection volume and detector filtering.[5,6,7] Lastly, detector settings can also impact band variance.[8] The sum of all these factors can be represented by the band variance (σ) in the following equation.[5,6]

$$\sigma^2_{obs} = \sigma^2_{col} + \sigma^2_{extra\ -col}$$

where

$$\sigma^2_{extra\ -col} = \sigma^2_{needle\ \ seat} + \sigma^2_{injector\ \ volume} + \sigma^2_{injector\ \ valve} + \sigma^2_{tubing} \\ + \sigma^2_{pre\,heater} + \sigma^2_{detector\ \ volume} + \sigma^2_{detector\ \ filter} + ..$$

To understand and characterize each LC's system, extra-column band broadening or dispersion was measured for HPLC, UHPLC, and UPLC instruments. Measurements were performed with each system in the standard configurations (tubing, flow cells, etc.) at a sampling rate of 40 Hz (enough to ensure adequate points across the peak). A zero dead volume union was used in place of a column (Figure 5). Measurements were calculated at 4σ (13.4% peak height) and 5σ (4.4% peak height) (Figure 6).

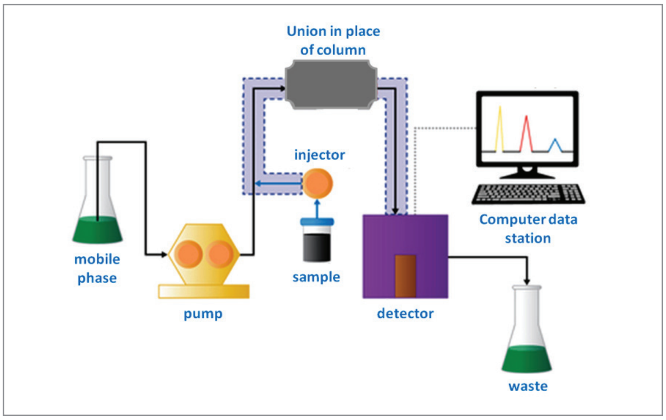


Figure 5. Typical system set up for measuring extra-column band broadening. Dotted blue lines represent potential areas of dispersion within the sample flow path. Each system was configured with standard tubing and flow cells, unless otherwise specified.

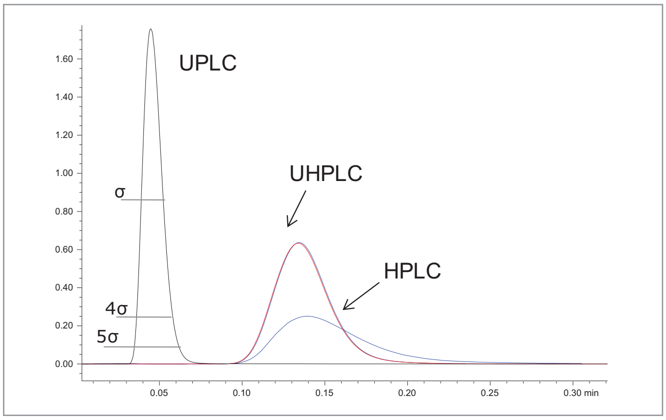


Figure 6. Extra-column effects. Using the Empower™ 3 Chromatography Data System, peak widths at 13.4% peak height (4σ) and 4.4% peak height (5σ) were recorded. The extra-column dispersion was determined by multiplying the peak width and the flow rate. The extra-column dispersion of the UHPLC (ACQUITY Arc System) is represented by the overlaid chromatograms of both Path 1 and Path 2.

Conditions: Flow rate: 0.3 mL/min; Mobile phase: 30:70 water:acetonitrile; Sample: 0.16 mg/mL caffeine in 1:9 water:acetonitrile (p/n 700002642), solution 7; Wavelength: 273 nm; Sampling rate: 40 pt/s (Hz); Injection volume: 1 µL; Low volume V-detail union (p/n 700002636).

The measured values vary significantly across HPLC to UPLC systems (Table 1). As the measurements show, column heaters and detectors can significantly impact extra-column effects of an LC system. Many LC systems have multiple column heater configurations with different I.D. and length of inlet and outlet tubing – all of which impact band broadening. System components, such as switching valves, preheaters, or flow cells, can also significantly affect these values. However, it should be noted that any volume before the injector, such as the Multi-flow path™ in the ACQUITY Arc System, do not impact extra-column dispersion. Given the influence of specific components, the injector, column heater, and detector configuration should be specified when reporting these values.

System	Extra-column band broadening @ 5 σ	Extra-column band broadening @ 4 σ
Alliance HPLC with 30 cm CH and HPLC PDA Detector	43–45	30–34
ACQUITY Arc System (Path 1 and 2) with 30 cm CHC and HPLC PDA Detector	25	19
ACQUITY UPLC H-Class PLUS with CH-A and ACQUITY PDA Detector	8	7
ACQUITY UPLC I-Class PLUS SM-FTN with CH-A and ACQUITY PDA Detector	7.5	5

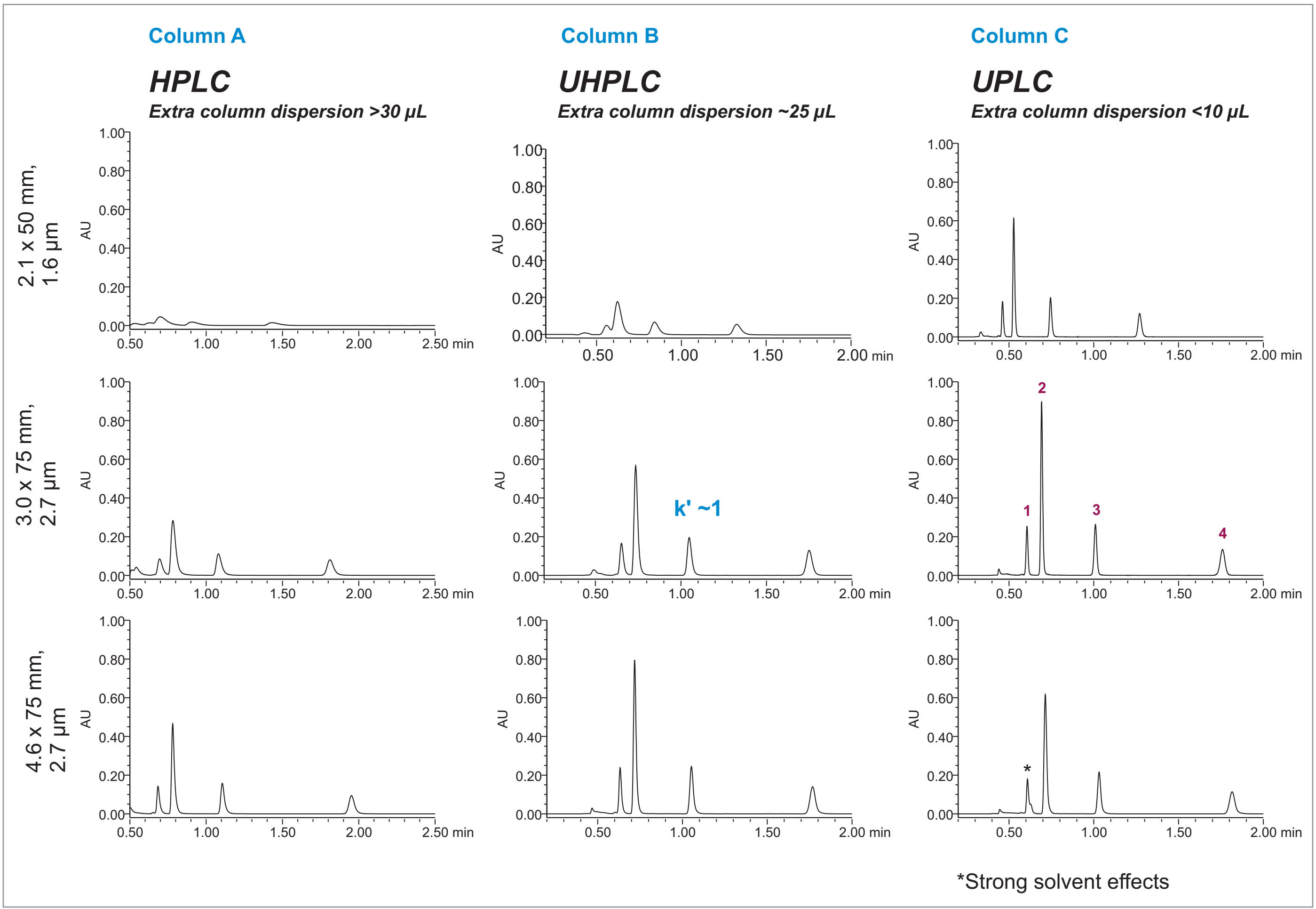
Table 1. Extra-column band broadening of HPLC, UHPLC, and UPLC systems. Measurements were performed following the conditions previously specified. All systems were in the standard configuration unless otherwise specified. All detectors had standard flow cells.

**Ranges reflect differences in preheater and column compartment configurations.*

IMPACT OF EXTRA-COLUMN EFFECTS ON AN ISOCRATIC SEPARATION

The loss of efficiency for a separation due to extra-column effects varies with column dimensions and particle size.[5,9] Lower column volumes will be impacted more by extra-column band broadening then HPLC (4.6 mm I.D.) columns. This is because the ratio of on-column to extra-column band broadening is low, meaning extra-column effects are a significant portion of the overall band broadening.

To illustrate these effects, an isocratic separation (Figure 7) was performed on UPLC, UHPLC, and HPLC systems. Three different column configurations – scaled to keep L/d_P (length/particle size) constant – were analyzed on each system. On the UPLC System (Column C), extra-column band broadening was not significant (<10 µL), therefore, the efficiency and resolution of the separation was minimally impacted, regardless of the column dimensions.



*Strong solvent effects

Figure 7. Isocratic separation and extra-column effects. An isocratic separation was evaluated on HPLC, UHPLC, and UPLC systems using 2.1, 3.0, and 4.6 mm I.D. columns with the appropriate method scaling.[1] In general, the HPLC system produced the broadest peaks and lowest sensitivity. However the differences varied with column dimensions. The greatest loss in efficiency was observed for the 2.1 mm column, while differences on the 4.6 mm were not as evident. In addition, some strong solvent effects were observed for the 4.6 x 75 mm column on the UPLC system due to sample diluent mismatch. These effects can be eliminated by matching the diluent to the mobile phase, adding pre-column volume or reducing injection volume.

Conditions: Sample: imipramine, chlorimipramine, 11-α-hydroxyprogesterone, and 17-α-hydroxyprogesterone at 0.1 mg/mL in 1:1 Water:methanol; Isocratic: 1:1 0.1% (v/v) TFA in water/0.1% (v/v) TFA in ACN; Wavelength: 254 nm; Column temperature: 35 °C; Method scaling: Column: CORTECS UPLC C_{18}, 1.6 µm, 2.1 x 50 mm (p/n 186007093), Flow rate: 0.3 mL/min, Injection volume: 1 µL; Column: CORTECS C_{18}, 2.7 µm, 3.0 x 75 mm (p/n 186007371), Flow rate: 0.612 mL/min, Injection volume: 3.1 µL; Column: CORTECS C_{18}, 2.7 µm, 4.6 x 75 mm (p/n 186007376), Flow rate: 1.44 mL/min, Injection volume: 7.2 µL.

On the HPLC and UHPLC system (Column A and B), the separation on the 2.1 x 50 mm column (top row) was noticeably impacted by the extra-column band broadening of each system. The resulting chromatography produced much lower efficiencies (USP plate count), and resolution than that observed on the UPLC system, as illustrated for peak 4 in Figure 8. On both the HPLC and UHPLC systems, a significant loss of efficiency (>50%) was observed as compared to the UPLC System.

Scaling the method to HPLC columns (middle and bottom rows) resulted in improved efficiencies on both the HPLC and UHPLC systems. In fact, the loss of efficiency for peak 4 on a 3.0 mm I.D. column was significantly less than observed with the 2.1 mm I.D. column (Figure 8). Scaling the separation to a 4.6 x 75 mm column resulted in comparable efficiencies on all systems. For the 4.6 mm I.D. column, the ratio of column volume to system dispersion is much more favorable, reducing the negative impact of the system dispersion on column efficiency.

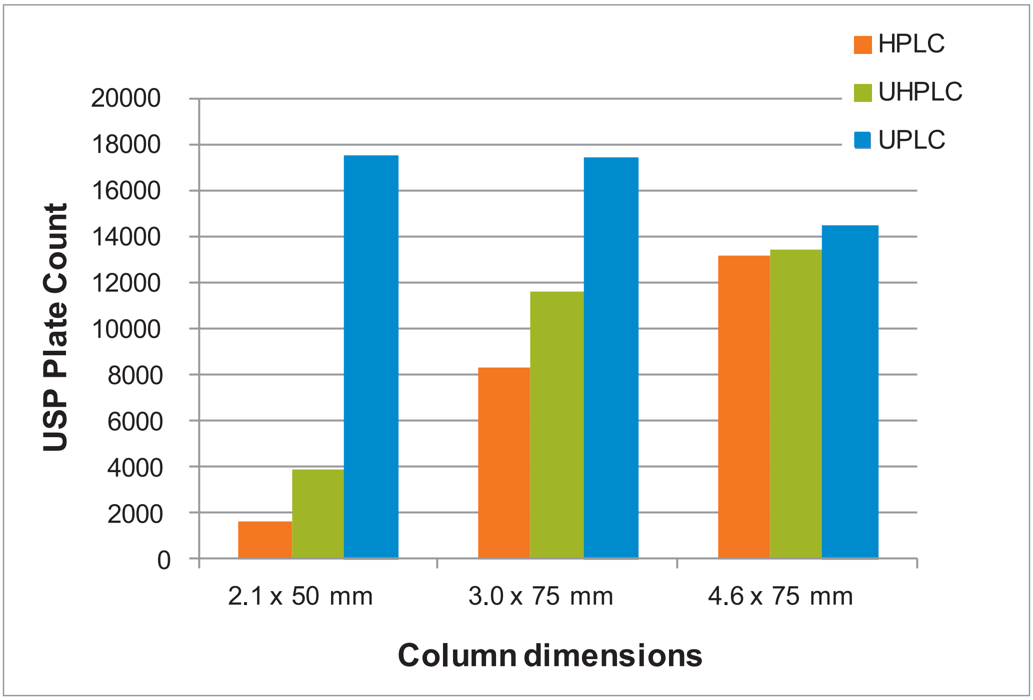


Figure 8. USP plate count for Peak 4. The loss of efficiency on the CORTECS UPLC C_{18}, 1.6 µm Column, 2.1 x 50 mm (p/n 186007093), is over 50% when comparing the UPLC to either the UHPLC or HPLC system. However, with the CORTECS C_{18}, 2.7 µm Column, 4.6 x 75 mm (p/n 186007376) the difference in USP plate count is much lower. The extra-column effects of the different systems have a much lower impact on performance as compared to the sub-2-µm 2.1 mm I.D. column.

IMPACT OF EXTRA-COLUMN DISPERSION ON A GRADIENT SEPARATION

While the effects of extra-column volume are particularly noticeable for isocratic separations, gradient separations are more forgiving of extra-column dispersion due to peak focusing at the head of the column. However, extra-column volume can affect resolution – particularly for critical pairs – since resolution is based on peak widths.

In this example, the separation on flavonoids in orange extract was performed on a 2.1 x 75 mm column on an HPLC system using a standard flow cell (8.4 µL, 10 mm path length) for the PDA detector (Figure 9A). The USP resolution for the flavonoids was between 1.1–1.8. To minimize extra-column band broadening and increase resolution, the same system was configured with a microbore flow cell (2.7 µL, 8 mm path length) (9B). Keeping all the inlet and outlet tubing the same and changing only the flow cell, resolution increased by almost 50%. The lower dispersion flow cell not only produced narrower peak widths, but similar sensitivity despite the shorter path length.

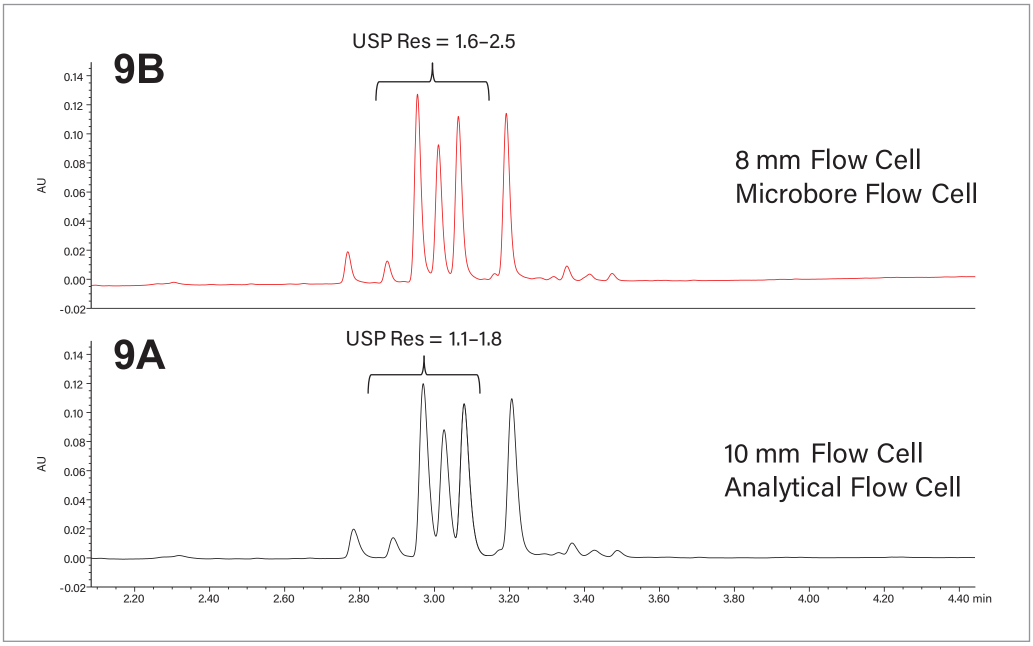


Figure 9. Effect of post column dispersion on a separation of orange extract. The same separation was performed on an HPLC system with two different flow cells. The lower dispersion (microbore) flow cell produced increased resolution of approximately 50%. Sensitivity was comparable despite the shorter path length due to the narrower peak widths observed in the lower dispersion flow cell.

Conditions: Alliance HPLC with 2998 Detector; Gradient: 5–90% B in 4 min; Column: CORTECS C_{18}*+, 2.7 µm Column, 2.1 x 75 mm (p/n 186007396); Wavelength: 315 nm, Sampling rate: 20 pts/s (Hz), Filter time constant: Normal.*

CONCLUSIONS

The success or failure of method transfer can be impacted by a wide range of system characteristics. Two of the more prominent attributes include dwell volume and extra-column volume. Each of these characteristics has a very different impact on the separation. While dwell volume can impact the time and slope of the delivery of the gradient, extra-column effects impact peak width, efficiency, and resolution of the separation. The impact of both of these characteristics can be minimized with proper adjustments. For dwell volume, adjusting the gradient start relative to the injection, whether through software or changes to the gradient table, helps minimize retention time differences. For dispersion effects, understanding the extra-column volume of each system and taking advantage of the instrument options, can improve the results of methods transfer. However, for smaller column dimensions and smaller particle size columns, the impact of extra-column volume may still affect the separation due to the unfavorable ratio of the column volume to extra-column effects of the system.

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[NOTES]

USP Method Modification and Routine Use Analysis of Budesonide Nasal Spray from HPLC to UPLC

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APPLICATION BENEFITS

- 87% reduction in run time while preserving peak resolution and efficiency
- More than 90% reduction in solvent and sample consumption
- Availability of wide selection of sub-2 µm column chemistries
- Proven column performance of over 2800 injections on the ACQUITY UPLC® column

WATERS SOLUTIONS

Alliance® HPLC system

ACQUITY UPLC system

ACQUITY UPLC BEH C_{18} and XBridge™ C_{18} columns

Method Transfer kits

Waters Pre-column filter

Empower™ 2 CDS software

KEY WORDS

ACQUITY UPLC Columns Calculator, Waters Reversed-Phase Column Selectivity Chart, method transfer, USP, budesonide, nasal spray, UPLC, BEH, routine use

INTRODUCTION

Compendial methods originally written, several years ago, do not take advantage of the advancements in chromatographic chemistries and instrumentation. With the advent of UPLC® technology, it is desirable to modify USP methods following chromatographic principles and yielding same analytical results. These migrated methods however are only valuable when they are robust enough for routine use.

In this study we use the USP monograph for budesonide as an example to modify the long HPLC run to a short UPLC run. Budesonide formulation is available as a nasal spray, analytically separated under low-pH conditions with phosphate buffer. By effectively managing chromatographic variables such as, proper handling of the nasal spray formulation and operating conditions delivered by the instrument, a routine use study of over 2800 injections is shown. Following the process described here will help with the successful adoption of modern UPLC technology and its benefits, to test products described in the Pharmacopeial monographs.

EXPERIMENTAL

HPLC Conditions

LC System:	Alliance HPLC with 2489 UV/Visible detector
Run Time:	32 minutes
Column:	XBridge C_{18} 4.6 x 150 mm, 5 µm (USP designation: L1), part number 186003116
Mobile Phase:	Acetonitrile and Solution A (32:68); Solution A: 3.17 mg/mL of monobasic sodium phosphate and 0.23 mg/mL of phosphoric acid; pH 3.2 +/- 0.1
Separation Mode:	Isocratic
Flow Rate:	1.5 mL/min
Injection Volume:	20 µL
Detection:	UV at 254 nm

UPLC Conditions

LC System:	ACQUITY UPLC with TUV detector
Run Time:	4 minutes
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm (USP designation: L1), part number 186002350
Mobile Phase:	Acetonitrile and Solution A (32:68); Solution A: 3.17 mg/mL of monobasic sodium phosphate and 0.23 mg/mL of phosphoric acid; pH 3.2 +/- 0.1

Standard Preparation

A 12.8 µg/mL working standard of budesonide was prepared in a solution of 70:30 Solution A/acetonitrile. (Please refer to Mobile-Phase description in HPLC Conditions for details on Solution A.)

Sample Preparation

The concentration of the working standard and sample specified in the USP monograph, is 0.5 mg/mL.[1] This monograph, however, is for drug substance analysis. For drug product analysis, the sample preparation protocol adopted use of a lower concentration to ensure that the budesonide formulation dissolves in its diluent. Rhinocort AQUA® (budesonide) nasal spray formulation contains a micronized suspension of budesonide in an aqueous medium consisting of microcrystalline cellulose, carboxy-methyl cellulose sodium, dextrose anyhydrous, polysorbate 80, disodium edentate, potassium sorbate, HCl and water.

An amount equivalent to 1.0 g of Rhinocort AQUA® (budesonide) nasal spray was accurately weighed and transferred to a 50 mL volumetric flask. 16 mL of acetonitrile was added to this flask. This mixture was mechanically shaken in the Burrell Wrist-Action® shaker, Model 75 for 15 minutes. The mixture was diluted with Solution A to volume and mechanically shaken for an additional 10 minutes. This mixture shown in 1(b) was then subjected to centrifugation at 3,220 rcf (4,000 rpm) for 15 minutes. The supernatant shown in Figure 1(c) was aliquoted into a 2 mL Waters Certified Glass Screw Cap Vial with bonded pre-slit PTFE/silicone Septum (Part number 186000307C). Final concentration of the working sample was 12.8 µg/mL.

(a) (b) (c)

Figure 1. RHINOCORT AQUA® (budesonide) nasal spray sample preparation. (a) RHINOCORT AQUA® (budesonide) nasal spray formulation; (b) budesonide formulation in diluent; (c) budesonide sample after centrifugation.

Wash Solvents:	70:30 water/ acetonitrile (weak wash) 100% acetonitrile (strong wash)
Separation Mode:	Isocratic
Flow Rate:	0.92 mL/min
Injection Volume:	1.4 µL
Detection:	UV at 254 nm
Data Management:	Empower 2 CDS

USP System Suitability Criteria

USP Resolution:	NLT 1.5
USP Plate Count:	NLT 5500

$RT_{\text{epimer A}} = 1.1 \times RT_{\text{epimer B}}$

RESULTS AND DISCUSSION

The USP monograph for budesonide requires the use of a 4.6 x 150 mm, 5 µm packing (L1) column.[1] As per the USP monograph, the original methodology was developed and submitted using a Supelcosil™ LC C_{18} column. The Waters XBridge C_{18} column was selected in regards to similar selectivity with the guidance of the Waters Reversed-Phase Column Selectivity chart. The separation of the budesonide standard run using Waters XBridge C_{18} 4.6 x 150 mm, 5 µm (L1) column on an Alliance HPLC system is as shown in Figure 2.

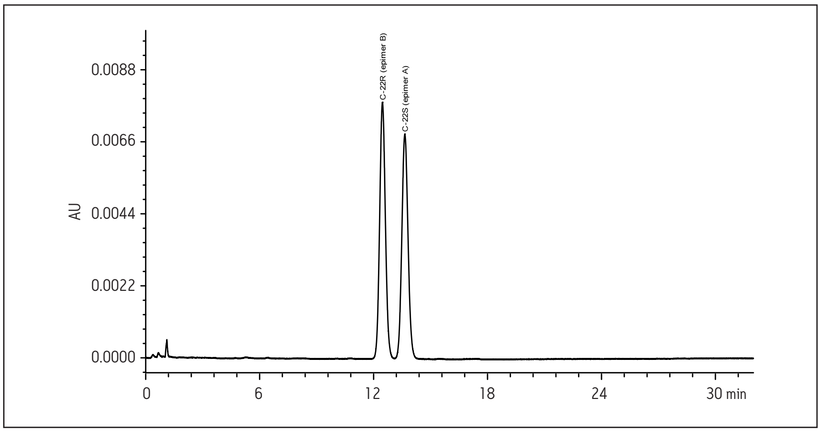

Figure 2. USP budesonide standard method run on XBridge 4.6 x 150 mm, 5 µm column on the Alliance HPLC system.

The USP method was then modified from HPLC to UPLC using the Waters ACQUITY UPLC Columns Calculator as described in the application note "*Implementation of Methods Translation between Liquid Chromatography Instrumentation*", part number 720003721en.[3] The retention factor (k') of budesonide, calculated from the separation on Alliance, was input into the columns calculator to optimally scale the method and generate UPLC method conditions while preserving the resolving power of the separation. The run time generated by the ACQUITY UPLC Columns Calculator is based on the retention factor (k') and may be adjusted to account for total run time (Figures 3 and 4).

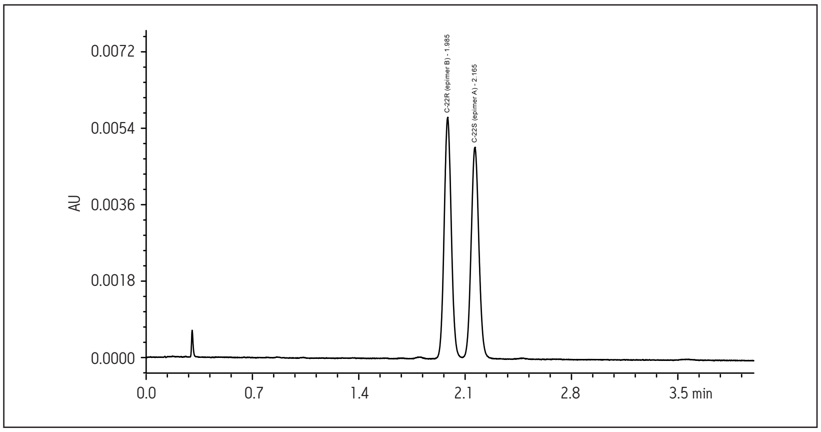

Figure 3. USP budesonide standard run on an ACQUITY UPLC BEH C_{18} column on the ACQUITY UPLC system.

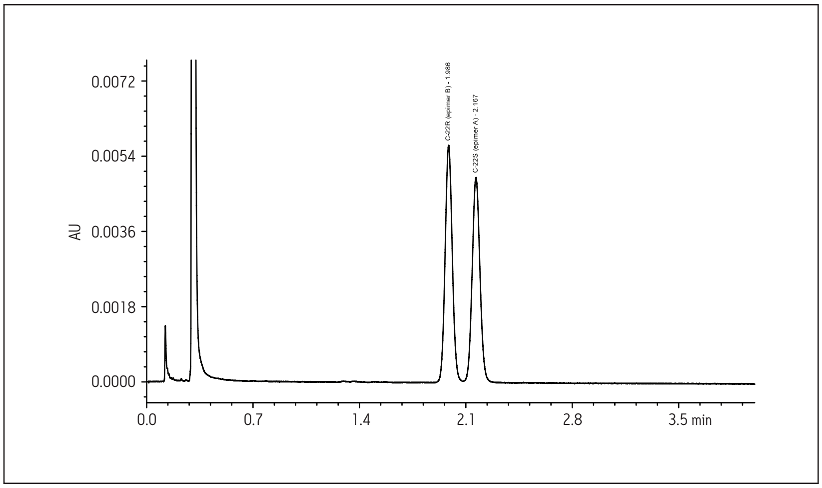

Figure 4. USP budesonide sample run on an ACQUITY UPLC BEH C_{18} column on the ACQUITY UPLC system.

System suitability requirements for the budesonide separation on the Alliance HPLC system and the ACQUITY UPLC system were met (Table 1).

USP Requirements	Budesonide Standard (HPLC)	Budesonide Standard (UPLC)	Budesonide Sample (UPLC)
USP Resolution (≥ 1.5)	2.03	1.96	1.97
USP Plate Count from Epimer B peak (≥ 5500)	7962	8539	8540
Relative Retention Time (Epimer A = 1.1 X Epimer B)	1.1	1.1	1.1

Table 1. System suitability results for six replicate injections of budesonide standard and sample on Alliance HPLC and ACQUITY UPLC systems.

ROUTINE USE STUDY

Following the protocol that a typical QC lab would use to perform the USP monograph testing, both standards and samples were injected to evaluate routine use. The sample set was constructed with two replicate injections of standard bracketing 6 replicate injections of sample.

Two system suitability studies were conducted. In the first study, it was observed that the working standard gave higher plate count values than the sample (figure 5). It should be noted that the plate count and resolution values at the start of the study for this column were only 6477 and 1.78, respectively. System suitability results for the first study are reported in table 2. After about 830 injections, USP plate count values for Epimer B began to fail the USP specification. In an effort to revive the column, the column was washed overnight with 100% acetonitrile at 0.1mL/min for about 8 hours followed by a mobile-phase composition of 50:50 water:acetonitrile, respectively at a flow rate of 0.1mL/min for another 8 hours. USP plate count for Epimer B continued to fail the USP specification. The column inlet frit was replaced to determine if it experienced blockage leading to the reduced column lifetime. This approach alone did not retain USP specifications for budesonide Epimer B (table 2).

A second study was then conducted after making the following three minor modifications. First, within the sample preparation protocol, the time required to centrifuge the budesonide sample was increased from 15 minutes to 25 minutes. This change was made to ensure appropriate filtration of the sample. Next, a pre-column filter (part number 700002775) was connected to the head of the column for preventative maintenance and to prolong column lifetime. Finally, a constant column temperature of 30 °C was used in the method to improve retention time reproducibility. With the above mentioned improvements, plate count and resolution values for a new ACQUITY UPLC BEH 2.1 x 50 mm, 1.7 µm column began at 8540 and 1.97 respectively. Both plate count and resolution values were maintained throughout the course of the study, for over 2846 injections. System pressure remained stable at around 11,800 psi throughout the course of the study (figure 6). System suitability results are shown in table 3.

Injection #/ System Suitability Requirements	**52 - 57**	**506 - 511**	824 - 829	1199 - 1204	1228 – 1233 (New Column Inlet Frit)
USP Resolution (≥ 1.5)	1.78	1.72	1.62	1.58	1.50
USP Plate Count from Epimer B Peak (≥ 5500)	6477	6137	5508	4992	4897
Relative Retention Time (Epimer A = 1.1 X Epimer B)	1.1	1.1	1.1	1.1	1.1

Table 2. System suitability results from routine use study 1 of only about 1000 injections on ACQUITY UPLC BEH C_{18} column and ACQUITY UPLC system.

Injection #/ System Suitability Requirements	**10 - 15**	**506 - 511**	1002 - 1007	1502 - 1507	2006 - 2011	2504 - 2509	2840 - 2846
USP Resolution (≥ 1.5)	1.97	1.96	1.95	1.99	1.95	1.97	2.00
USP Plate Count from Epimer B Peak (≥ 5500)	8540	8515	8419	8750	8591	8737	8857
Relative Retention Time (Epimer A = 1.1 X Epimer B)	1.1	1.1	1.1	1.1	1.1	1.1	1.1

Table 3. System suitability results from routine-use study 2 of over 2800 injections on ACQUITY UPLC BEH C_{18} column and ACQUITY UPLC system.

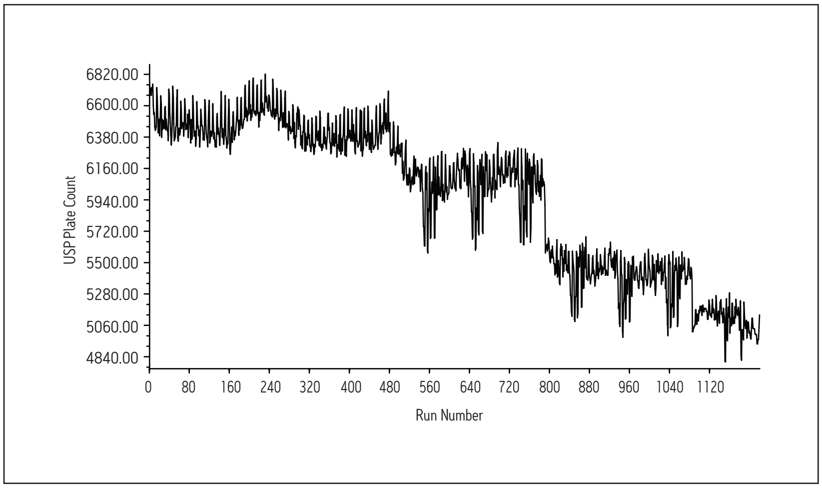

Figure 5. USP plate count trend plot from routine use study 1 on an ACQUITY UPLC BEH C_{18}, 1.7 µm column.

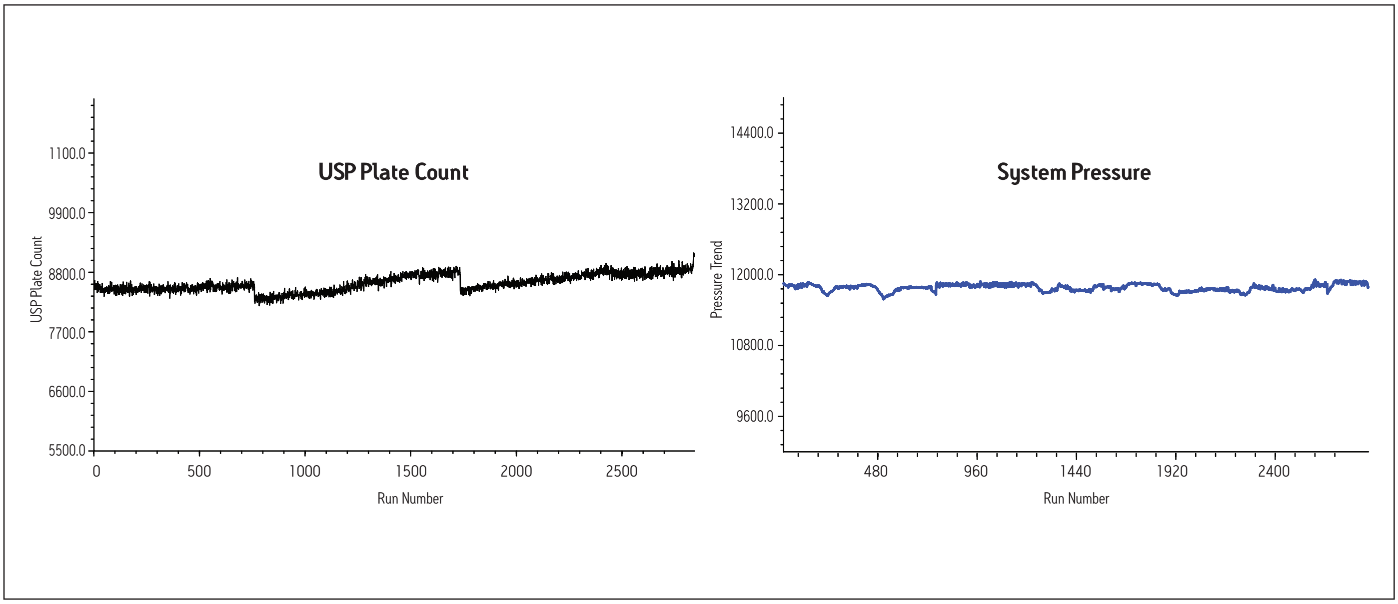

Figure 6. USP plate count and system pressure trend plot from routine use study 2 of over 2800 injections on ACQUITY UPLC BEH C_{18} column.

CONCLUSIONS

USP monograph for Budesonide was used to successfully modify the HPLC method to a UPLC platform using appropriate software tools and modern sub-2 µm column chemistry. System suitability requirements for the USP method transferred from the Alliance HPLC system to the ACQUITY UPLC system were met. Optimizing the sample preparation protocol (i.e. increasing centrifugation time), to ensure maximum sample clean-up, adding a pre-column filter to protect the column from particulates and maintaining a constant column temperature, resulted in excellent column performance for over 2800 injections. The ACQUITY UPLC BEH C_{18} 1.7µm column demonstrated robustness under low-pH (3.2) conditions with phosphate buffer. With proper sample preparation, one can successfully analyze USP methods for generic drugs on UPLC systems. Other benefits realized through this modification were an 87% reduction in run time and more than 90% reduction in solvent and sample consumption. The ease of method modification using the simplistic workflow design[3] helps in the seamless adoption of UPLC technology with conspicuous gains in overall laboratory efficiency and productivity for running USP monographs.

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[NOTES]

USP Method Modification of Levonorgestrel and Ethinyl Estradiol Tablets from HPLC to UPLC

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APPLICATION BENEFITS

- Updating USP methods from HPLC to UPLC® using sub-2 µm columns
- 85% decrease in analysis time, faster throughput for routine sample analysis
- 92% reduction in solvent usage and sample injected

WATERS SOLUTIONS

- ACQUITY UPLC® H-Class and Alliance® systems
- ACQUITY UPLC BEH C_8 and XBridge™ C_8 columns
- Empower™ 2 CDS
- Method Transfer kit
- Waters Levonorgestrel-Ethinyl Estradiol USP standard

KEY WORDS

Method transfer, USP methods, HPLC, UPLC, levonorgestrel, ethinyl estradiol, birth control, ACQUITY UPLC Columns Calculator, quality control

INTRODUCTION

Chromatographic assays for the analysis of generic drugs are frequently based on USP compendial methods. The HPLC methods described in the USP are not routinely updated and do not take advantage of sub-2 µm particle technology, which provides faster run times and increased productivity of analysis. Additionally, routine analysis of drug formulations can result in more frequent and costly replacement of columns due to the chemical diversity of excipients and other formulation agents. USP methods developed on HPLC are often isocratic to eliminate the need for re-equilibration, thereby increasing sample throughput. However, if the sample is not properly eluted off of the column, it can build up on the column bed, resulting in increased backpressure and potentially premature failure of the column.

This application note will first compare the USP method for levonorgestrel and ethinyl estradiol tablets using two different L7 HPLC columns. The method is then modified to UPLC using a UPLC column with the same stationary phase. The use of UPLC and sub-2µm particle columns allows for a significantly faster analysis while still meeting the system suitability criteria specified in the USP monograph. Finally, suggestions are offered to alleviate the increased backpressure that may be observed when repeatedly analyzing formulated levonorgestrel-ethinyl estradiol tablet samples in a typical quality control laboratory.

EXPERIMENTAL

Alliance 2695 HPLC Conditions

Mobile Phase:	7:3:9 acetonitrile: methanol:water
Separation Mode:	Isocratic
Detection:	UV at 215 nm
USP Column:	Zorbax® C_8, 4.6 x 150 mm, 5 µm (USP designation: L7); XBridge C_8, 4.6 x 150 mm, 5 µm (USP designation: L7), part number 186003017
Needle Wash:	acetonitrile
Sample Purge:	acetonitrile
Seal Wash:	50:50 methanol:water
Flow Rate:	1 mL/min
Injection Volume:	50 µL

ACQUITY UPLC H-Class Conditions

Mobile Phase:	7:3:9 acetonitrile: methanol:water
Separation Mode:	Isocratic
Detection:	UV at 215 nm
USP Column:	ACQUITY UPLC BEH C_8, 2.1 x 50 mm, 1.7 µm (USP designation: L7), part number 186002877
Needle Wash:	acetonitrile
Sample Purge:	acetonitrile
Seal Wash:	50:50 methanol:water
Flow Rate:	0.61 mL/min
Injection Volume:	3.5 µL

Data Management

Empower 2 CDS

USP System Suitability Criteria

USP Resolution:	NLT 2.5
Peak Area RSD:	NMT 2.0%

Sample Preparation

Standard:
Levonorgestrel, 15 µg/mL and ethinyl estradiol, 3 µg/mL in mobile phase (Waters Analytical Standard).

Sample:
Dissolve levonorgestrel and ethinyl estradiol commercially-available tablets in mobile phase to a final concentration of 15 µg/mL levonorgestrel and 3 µg/mL ethinyl estradiol. Sonicate for 5 minutes, shake mechanically for 20 minutes. Centrifuge at 4000 rpm for 10 minutes. Collect supernatant and re-centrifuge at 12,000 rpm for 30 minutes, pipet clear supernatant for injection.

RESULTS AND DISCUSSION

Samples were prepared according to the USP compendial assay method for levonorgestrel and ethinyl estradiol tablets.[1] Samples were first centrifuged at 4000 rpm for 10 minutes and yielded a pale cloudy solution. Next, an aliquot of sample was filtered through a 0.2 µm PTFE filter, but the filtrate remained cloudy due to the extremely fine nature of the particulates in the sample and further filtration was not pursued. Samples were instead centrifuged at 12,000 rpm for 30 minutes and the supernatant was collected, yielding a clear solution for injection.

The USP method for levonorgestrel and ethinyl estradiol tablets requires the use of an L7 column and suggests an Agilent Zorbax C_8 column. This column was tested per the USP assay method on an Alliance HPLC system, with five replicate injections of both levonorgestrel-ethinyl estradiol standard and tablets. The samples were also run on the HPLC system using a Waters XBridge C_8 column. This column was chosen since it has similar selectivity to the Zorbax C_8 column and it has an equivalent UPLC column chemistry (ACQUITY UPLC BEH C_8), facilitating direct method transfer to UPLC. Chromatograms comparing the USP method using the Zorbax and XBridge C_8 HPLC columns are compared in Figure 1. The columns show similar selectivity, but the Zorbax C_8 column shows greater overall retention of the two active ingredients compared to the XBridge C_8 column. However, the faster elution of analytes using the XBridge column allows for a much shorter analysis time, while sacrificing only a small amount of resolution. All of the USP assay suitability results were well within the specified criteria limits for both columns (Table 1). The effect of retentivity on productivity in the high throughput analysis of generic drugs is important to consider, even within the same USP column designation categories.

Next, the USP assay method was scaled from HPLC to UPLC using the ACQUITY UPLC Columns Calculator.[2] Scaling calculations were performed accounting for particle size and the method was scaled from the XBridge C_8 HPLC column to an ACQUITY UPLC BEH C_8, 1.7 µm column. Both columns have the same stationary phase chemistry and only differ in particle size. Five replicate injections of both levonorgestrel and ethinyl estradiol tablets and standard were analyzed separately. Assay suitability criteria including %RSD for peak area, and USP resolution between ethinyl estradiol and levonorgestrel peaks were compared between HPLC and UPLC. A comparison of both systems is shown in Table 1, where the UPLC transferred method clearly passes all system suitability criteria. The run time of the UPLC method is 1.5 minutes compared to a 10-minute HPLC method, resulting in an approximate 85% savings in analysis time and 92% savings in solvent consumption and sample injected (Figure 1).

System	USP Method	Column (L7 designation)	Sample	Peak Area % RSD		USP Resolution	Runtime (min)
				ethinyl estradiol	levonorgestrel		
HPLC	Original	Zorbax C_8	standard tablet	0.22 0.58	0.60 0.65	8.5 8.6	20
HPLC	Original	XBridge C_8	standard tablet	0.24 0.31	0.19 0.05	6.1 6.1	10
UPLC	UPLC Transferred	ACQUITY UPLC BEH C_8	standard tablet	0.16 0.09	0.39 1.12	5.3 5.3	1.5

Assay Suitability Criteria

USP Resolution (between 2 peaks): NLT 2.5

Peak Area RSD: NMT 2.0%

Table 1. Assay suitability results comparing HPLC to UPLC for five replicate injections of levonorgestrel and ethinyl estradiol standard and tablet samples.

ROUTINE USE STUDY

In order to evaluate the performance of the UPLC modified USP method with high-throughput analysis of formulated tablet samples, a routine use study was performed using the ACQUITY UPLC BEH C_8, 1.7 µm column.

Levonorgestrel and ethinyl estradiol tablet samples were injected along with a standard mixture of levonorgestrel and ethinyl estradiol as a bracketing standard to simulate routine use in a typical quality control (QC) laboratory. Five replicate injections of standard were followed by twenty replicate injections of formulated tablet sample and this cycle of injections was repeated continuously until assay suitability criteria no longer passed. Pressure, peak area, retention time and USP resolution between the two peaks (levonorgestrel and ethinyl estradiol) were monitored throughout the study.

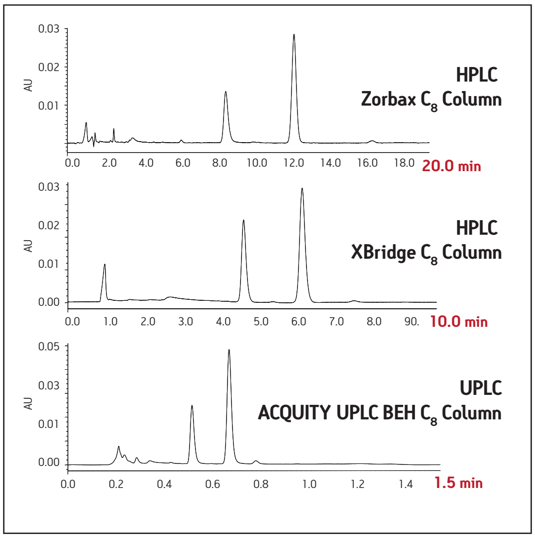

Figure 1. Chromatograms of levonorgestrel and ethinyl estradiol tablet sample comparing HPLC to UPLC.

Peak area RSD remained within 2.0% for levonorgestrel and ethinyl estradiol standard injections, and USP resolution remained greater than 2.5 throughout the study, which is within assay specifications (Figure 2). Backpressure increased steadily throughout the study (Figure 3a), and increased approximately 36% from 7200 psi to 9800 psi over the first 1000 injections. The overall pressure trend for the UPLC routine use study was compared to a routine use evaluation for the original USP method run on an Alliance HPLC system using the Zorbax C_8 column. On the HPLC system (Figure 3b), the pressure began near 1350 psi and rose to approximately 2350 psi over 1000 injections, a 74% increase. Both the original USP method on HPLC, and the UPLC method for levonorgestrel and ethinyl estradiol exhibited a gradual increase in pressure using different columns and different systems. For this reason, the pressure increase is attributed to the sample, likely due to poor aqueous solubility of the hydrophobic steroids and sample formulation components in the aqueous mobile phase, which contains only about 50% organic solvent. While the pressure increase on the ACQUITY UPLC system is noticeable, the relative pressure increase is far below that of the HPLC system using the Zorbax column. Despite the observed pressure increases throughout the study, both systems were well within the running pressure limits of the instrumentation and all system suitability criteria were met.

The routine use evaluation on the ACQUITY UPLC system was stopped at 2200 injections. The assay suitability criteria still passed all requirements, as shown in Table 2, demonstrating robust column performance for levonorgestrel and ethinyl estradiol on the ACQUITY UPLC BEH C_8 column, even after 2000 injections.

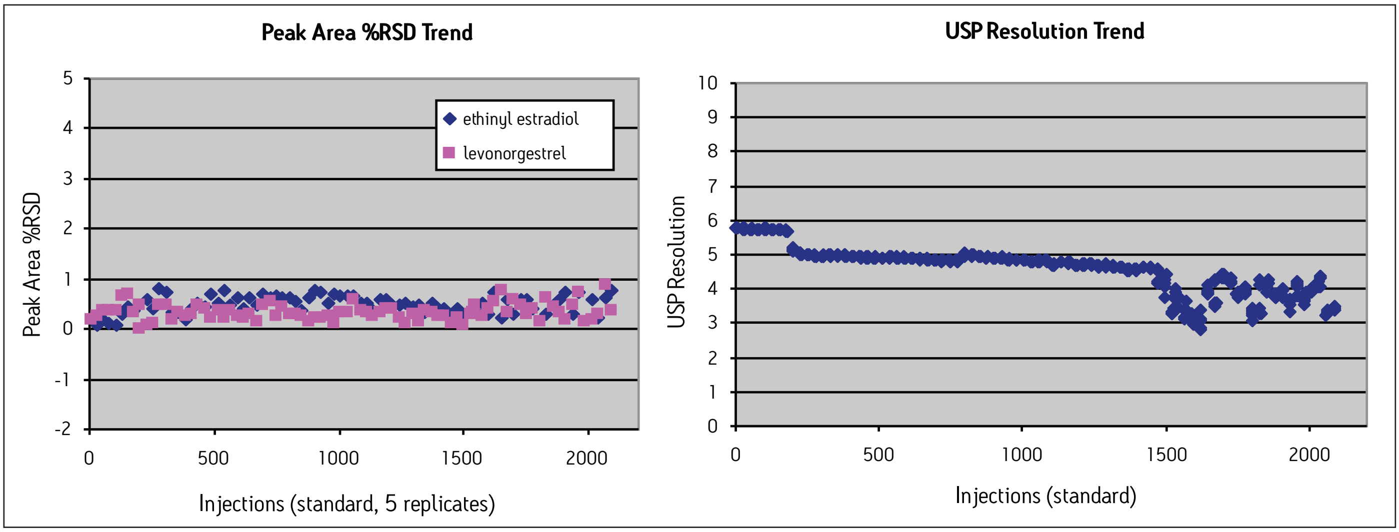

Figure 2. USP assay suitability trend plots from the routine use evaluation for levonorgestrel and ethinyl estradiol using the UPLC transferred method.

Levonorgestrel and Ethinyl Estradiol Standard

	%RSD Peak Area* (NMT 2.0%)		USP Resolution (NLT 2.5)
	ethinyl estradiol	levonorgestrel	
Routine Use Study: start	0.21	0.20	5.7
Routine Use Study: 2200 injections	0.57	0.22	3.3

*from 5 replicate standard injections

Table 2. USP assay suitability results before and after 2200 injections from the routine use evaluation using the UPLC transferred method.

Although the UPLC method meets assay suitability criteria for more than 2000 injections, an additional modification to the method was made in an attempt to alleviate the pressure increase over time, which is thought to be caused by sample build–up on the column bed. A second routine use evaluation was performed on a new ACQUITY UPLC BEH C_8 column, this time adding a gradient wash at the end of the isocratic USP method after each sample injection. After the 1.5 minute isocratic portion of the separation for the UPLC method, a gradient to 100% acetonitrile in 0.3 minutes was added, with a 1-minute hold at 100% acetonitrile (wash step) and then re-equilibration at 100% mobile-phase A. While the addition of a gradient wash lengthened the total cycle time to 4 minutes (including re-equilibration), this high organic washing step after each injection helps to elute the hydrophobic sample components and prevent build-up of sample on the column, thereby stabilizing the pressure throughout repeated injections from routine use (Figure 3c).

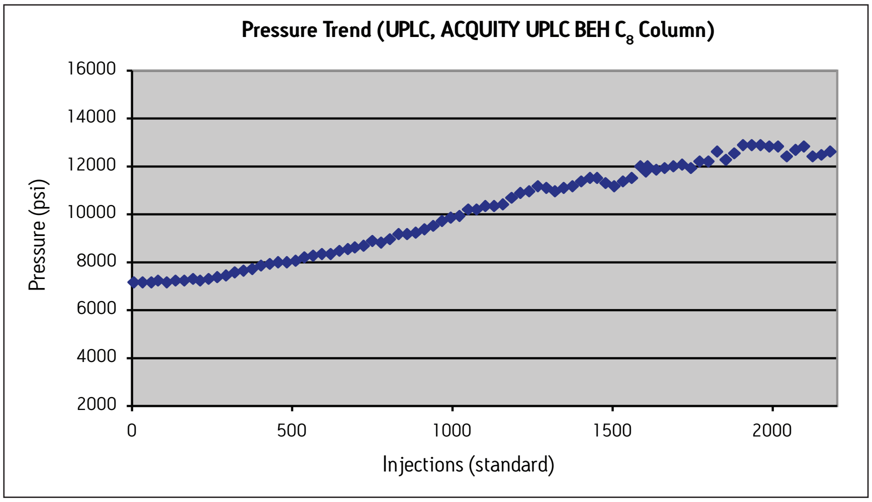

Figure 3a. Pressure trend plot from the routine use evaluation on a UPLC system with an ACQUITY UPLC BEH C_8 column (UPLC transferred USP method).

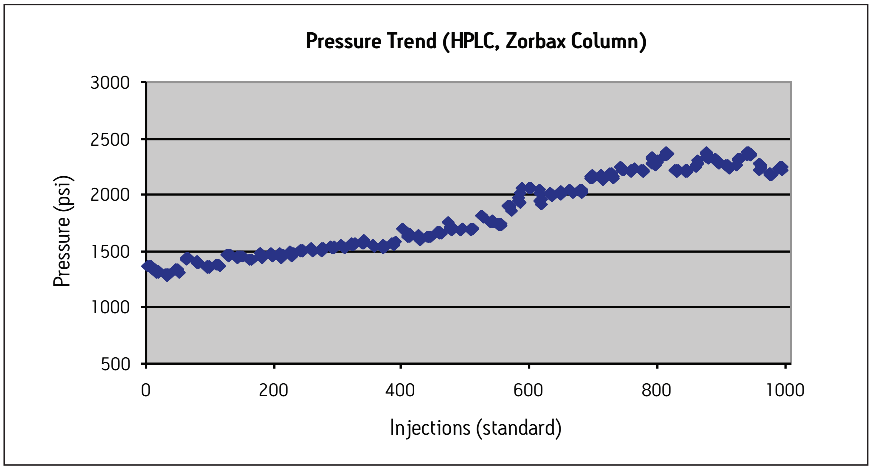

Figure 3b. Pressure trend plot from the routine use evaluation on an HPLC system with a Zorbax C_8 column (original USP method).

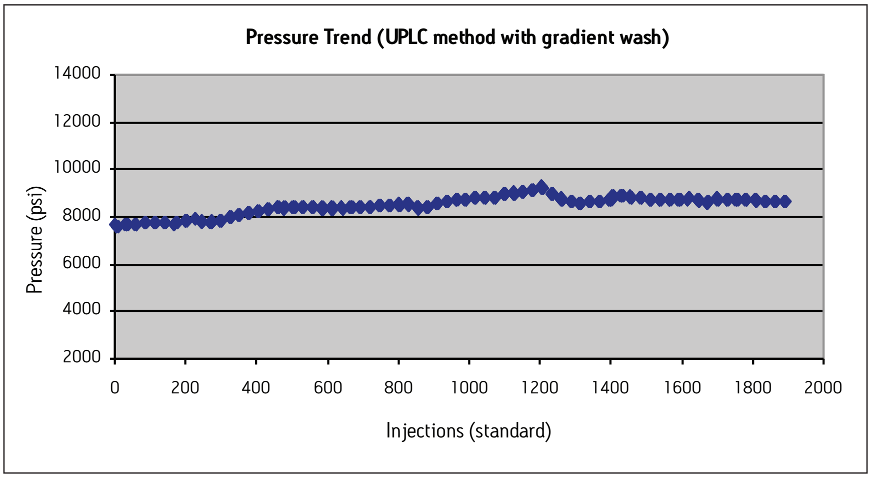

Figure 3c. Pressure trend plot from the routine use evaluation on an UPLC system with an ACQUITY UPLC BEH C_8 column (UPLC transferred method with gradient wash added).

CONCLUSIONS

The USP compendial method for levonorgestrel and ethinyl estradiol tablets was successfully scaled from HPLC to UPLC using scalable column chemistries and the ACQUITY UPLC Columns Calculator. The UPLC method is approximately 85% faster than the HPLC method and results in a 92% savings in sample amount injected and mobile-phase solvent consumption. While extended centrifugation of the tablet sample was helpful in preparing a better quality sample for injection, it did not fully alleviate the increased pressure seen in both HPLC and UPLC during a routine use study. Instead, incorporation of a gradient wash to the isocratic method aided in preventing sample build-up on column, thereby stabilizing the pressure during routine use evaluations. Routine use of the UPLC modified USP method was evaluated using formulated tablet samples on an ACQUITY UPLC BEH C_8, 1.7 µm column. After 2200 injections, the column still passed all USP assay suitability specifications for levonorgestrel and ethinyl estradiol tablets, demonstrating that extended column performance is achievable for high throughput analysis of generic tablet formulations using an isocratic USP monograph method.

References

1. USP Monograph. *Levonorgestrel and Ethinyl Estradiol Tablets*, USP34-NF29, 3299. The United States Pharmacopeial Convention, official from August 1, 2011.
2. Jones M.D., Alden P., Fountain K.J., Aubin A. *Implementation of Methods Translation between Liquid Chromatography Instrumentation*, Waters Application Note [2010], Part Number 720003721EN.

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Scaling of a USP Assay for Quetiapine Fumarate Across Different Liquid Chromatographic Systems

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APPLICATION BENEFITS

- The Waters Column Calculator enables users to scale a method by calculating operating parameters that give equivalent chromatographic performance.
- Improved USP method throughput is obtained by scaling HPLC column dimensions to columns with smaller particle sizes and shorter column lengths.
- The Quetiapine Fumarate assay method run time was reduced by 57% and 87% when analyzed on a 3.5 µm column with a UHPLC system and on a 1.7 µm column on a UPLC system, respectively.
- System suitability requirements as stated in USP monograph for the quetiapine fumarate assay are met when scaling down the column dimensions of the method.

WATERS SOLUTIONS

Alliance™ HPLC System

ACQUITY™ Arc™ System

ACQUITY UPLC™ H-Class PLUS System

Waters Column Calculator

XBridge™ Columns

Empower™ 3 Chromatography Data Software

KEYWORDS

USP methods, column scaling, quetiapine fumarate, method transfer, dispersion, dwell volume

INTRODUCTION

Users of compendial chromatographic procedures increasingly look to achieve cost savings through analytically equivalent procedures that decrease analysis time and solvent consumption. The United States Pharmacopeia (USP) compendial methods are often written for high performance liquid chromatography (HPLC) columns which use >3 µm particles resulting in long runtimes at higher flow rates. Although USP methods prescribe specific conditions, isocratic methods are capable of being scaled to columns which have a smaller particle size. This results in faster run times and lower solvent consumption. The USP has outlined in the General Chapter <621>[1] acceptable column and method adjustments to allow scaling of isocratic methods to provide the same if not improved performance of a method. These allowable adjustments include scaling particle size and column dimensions to maintain the *L/dp* ratio, where *L* is the length of the column and *dp* is the diameter of the particle size of the column packing material, as well as adjusting the flow rate and injection volumes accordingly. For these scaled methods it is important to consider the impact of dwell volume and extra-column dispersion on the chromatographic separation, and whether or not a lower dispersion, higher pressure system such as a UHPLC or a UPLC system is preferred.

In this study, the Waters Columns Calculator will be used to scale the USP monograph assay method for quetiapine fumarate,[2] an anti-psychotic drug, to smaller particle sized columns. These resulting methods will then be run on a variety of LC systems. The LC system platforms that will be included are an Alliance HPLC System, an ACQUITY Arc UHPLC System, and an ACQUITY UPLC H-Class PLUS System. We will demonstrate improvements to method performance, decreased run times and improved throughput while achieving the system suitability requirements as stated in the USP monograph for the quetiapine fumarate assay.

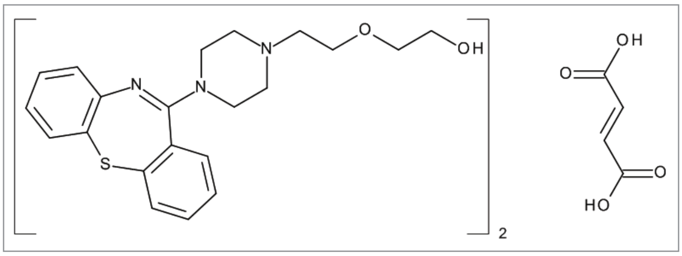


Figure 1. Structure of quetiapine fumarate.

EXPERIMENTAL

Sample description

Two reference standards were obtained from the USP: Quetiapine System Suitability (Catalog#: 1592715), and the Quetiapine Fumarate Standard (Catalog#: 1592704). The unknown sample was obtained from Alibaba.com. All samples were diluted in mobile phase to the following concentrations: 1.0 mg/mL for the system suitability solution and 0.08 mg/mL for the standard solution and sample solution.

Method conditions

LC conditions

Column temp.:	25 °C
Sample temp.:	4 °C
Mobile phase:	Methanol, acetonitrile, and buffer (54:7:39) Premixed and filtered using a 0.45 µm filter
Buffer:	2.6 g/L of dibasic ammonium phosphate adjusted to pH 6.5 with phosphoric acid
Gradient:	Isocratic (premixed mobile phase)
PDA wavelength:	230 nm at 4.8 nm resolution

Individual system conditions

LC system 1:	Alliance e2695 Separations Module with 100 µL syringe, 2998 PDA Detector and CH-30 equipped with a passive preheater
Column:	XBridge BEH C_8 Column, 5 µm, 4.6 mm × 250 mm (p/n: 186003018)
Injection volume:	50 µL
Flow rate:	1.3 mL/min
Run time:	15 minutes
Max. system pressure:	3850 psi
LC system 2:	ACQUITY Arc System with active solvent preheating (CH-30A) and 2998 PDA Detector (Path 2)
Column:	XBridge BEH C_8 Column, 3.5 µm, 3.0 mm × 150 mm (p/n: 186003052)
Injection volume:	12.8 µL
Flow rate:	0.8 mL/min
Run time:	7 minutes
Max. system pressure:	5200 psi
LC system 3:	ACQUITY UPLC H-Class PLUS System with active solvent preheating (CH-30A), 50 µL extension loop, and ACQUITY UPLC PDA Detector
Column:	ACQUITY UPLC BEH C_8 Column, 1.7 µm, 2.1 mm × 75 mm (p/n: 186005606)
Injection volume:	3.1 µL
Flow rate:	0.5 mL/min
Run time:	3 minutes
Max. system pressure:	14,000 psi

Data management

Empower 3 Chromatography Data Software, FR 3

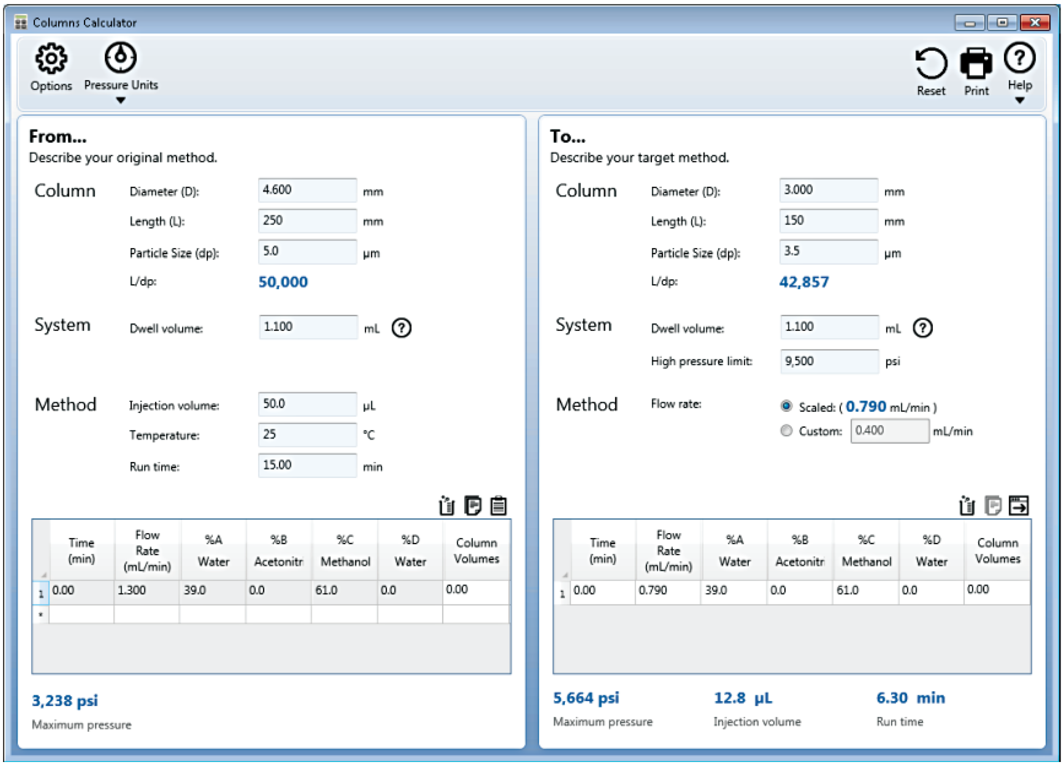
RESULTS AND DISCUSSION

The USP assay for quetiapine fumarate was initially run on the Alliance HPLC System following the column and method details described in the monograph. The column dimensions and method conditions were then scaled according to the USP guidelines[1] using the Waters Columns Calculator (Figure 2), and subsequently run on the ACQUITY Arc UHPLC System and the ACQUITY UPLC H-Class PLUS System. Both the column dimensions and the features of an LC system should be used to determine the optimal combination for running a method. Two key attributes of an LC system that should be considered are the dwell volume and the extra-column dispersion. The dwell volume of a system consists of the amount of volume from where the solvents are mixed until the mixed solvents contact the column inlet. While this may have a significant impact for gradient methods, dwell volume has very little effect on isocratic methods.[4] Therefore, the extra-column dispersion is the main factor in determining the optimum LC system for a column with an isocratic method. It is important to reduce the extra-column dispersion when using smaller columns since it will affect the resolution of the compounds, the broadening of peaks, and the efficiency of the column.[5] Therefore, to achieve optimal performance, it is important to keep the extra-column dispersion as minimal as possible in comparison to the column volume. For a more in depth breakdown of dwell volume and extra-column dispersion refer to the white paper listed in reference #5.

To maintain the *L/dp* ratio the column dimensions and particle size were scaled from the original column dimensions to a column with a 3.5 µm particle size and 3.0 mm × 150 mm column dimensions (Figure 2). In order to match the lower column volume with a lower dispersion system, the analysis of the quetiapine fumarate was performed on the ACQUITY Arc UHPLC System. The *L/dp* ratio decreased by 14% but is within the USP criteria of -25% to +50% of the original method. When the particle size and diameter of a column are changed, the following equation is used to calculate the change in flow rate:

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

where F_1 and F_2 are the flow rates for the original and scaled method, respectively; dc_1 and dc_2 are the column diameters for the original and scaled method, respectively, and dp_1 and dp_2 are the diameters of the particle sizes of the original and scaled methods, respectively.[1] The flow rate for this experiment was decreased from 1.3 mL/min in the original method to 0.8 mL/min for the 3.5 µm particle size column method. According to the USP general guidelines, "the injection volume for a method may be adjusted as long the value is within the accepted precision, linearity, and detection limits".[1] The injection volume was decreased from the original method of 50 µL to 12.8 µL for the scaled method conditions and was determined using the Waters Column Calculator (Figure 2).



From...
Describe your original method.

Column
Diameter (D): 4.600 mm
Length (L): 250 mm
Particle Size (dp): 5.0 µm
L/dp: 50,000

System
Dwell volume: 1.100 mL

Method
Injection volume: 50.0 µL
Temperature: 25 °C
Run time: 15.00 min

	Time (min)	Flow Rate (mL/min)	%A Water	%B Acetonitr	%C Methanol	%D Water	Column Volumes
1	0.00	1.300	39.0	0.0	61.0	0.0	0.00
*							

3,238 psi
Maximum pressure

To...
Describe your target method.

Column
Diameter (D): 3.000 mm
Length (L): 150 mm
Particle Size (dp): 3.5 µm
L/dp: 42,857

System
Dwell volume: 1.100 mL
High pressure limit: 9,500 psi

Method
Flow rate:
● Scaled: (0.790 mL/min)
○ Custom: 0.400 mL/min

	Time (min)	Flow Rate (mL/min)	%A Water	%B Acetonitr	%C Methanol	%D Water	Column Volumes
1	0.00	0.790	39.0	0.0	61.0	0.0	0.00

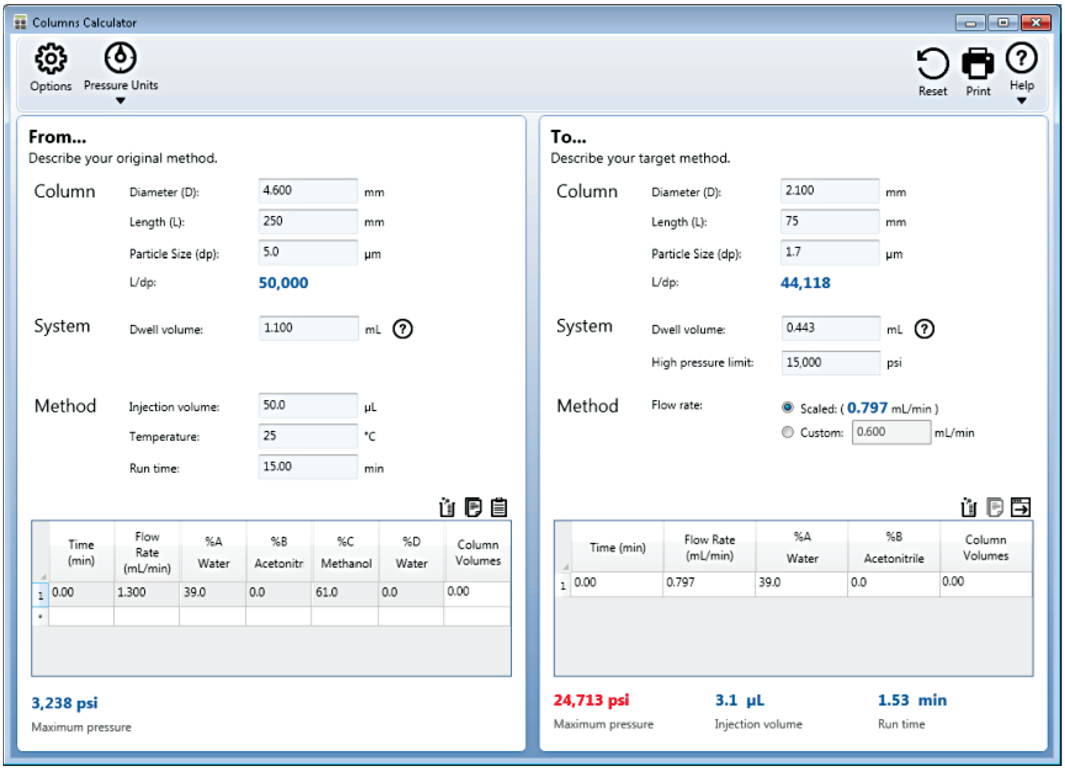
5,664 psi
Maximum pressure

12.8 µL
Injection volume

6.30 min
Run time

Figure 2. Waters Columns Calculator showing the original USP compendial method on the 5 µm particle size column method (left) scaled to the 3.5 µm particle size column method conditions (right).

In order to scale the quetiapine fumarate assay to a 1.7 µm particle column, the Waters Columns Calculator was utilized once again (Figure 3). The analysis of the quetiapine fumarate on the 2.1 mm × 75 mm column was performed on the ACQUITY UPLC H-Class PLUS System in order to decrease the extra-column dispersion that would be present on the Alliance HPLC System and ACQUITY Arc UHPLC System. The scaled column dimensions resulted in an *L/dp* ratio decrease of 12%, however, are within the USP criteria of -25% to +50% of the original method. The flow rate was decreased from 1.3 mL/min to 0.8 mL/min and the injection volume was decreased from 50 µL to 3.1 µL when scaling from the original method to the 1.7 µm particle size column. The flow rate was not run at 0.8 mL/min as stated in the Columns Calculator (Figure 3) because the ACQUITY UPLC H-Class PLUS System was over the 15,000 psi pressure limit at that flow rate. The final flow rate of 0.5 mL/min was used since the maximum system pressure achieved was 14,000 psi. This flow rate adjustment is acceptable under the USP conditions stated in the General Chapter <621> that once the flow rate is adjusted accordingly to the column, the flow rate can then be additionally adjusted within ± 50%.[1]



From...
Describe your original method.

Column
Diameter (D): 4.600 mm
Length (L): 250 mm
Particle Size (dp): 5.0 µm
L/dp: **50,000**

System
Dwell volume: 1.100 mL

Method
Injection volume: 50.0 µL
Temperature: 25 °C
Run time: 15.00 min

	Time (min)	Flow Rate (mL/min)	%A Water	%B Acetonitr	%C Methanol	%D Water	Column Volumes
1	0.00	1.300	39.0	0.0	61.0	0.0	0.00
*							

3,238 psi
Maximum pressure

To...
Describe your target method.

Column
Diameter (D): 2.100 mm
Length (L): 75 mm
Particle Size (dp): 1.7 µm
L/dp: **44,118**

System
Dwell volume: 0.443 mL
High pressure limit: 15,000 psi

Method
Flow rate:
● Scaled: (**0.797** mL/min)
○ Custom: 0.600 mL/min

	Time (min)	Flow Rate (mL/min)	%A Water	%B Acetonitrile	Column Volumes
1	0.00	0.797	39.0	0.0	0.00

24,713 psi
Maximum pressure

3.1 µL
Injection volume

1.53 min
Run time

Figure 3. Waters Columns Calculator showing the original USP compendial method on the 5 µm particle size column (left) scaled to the 1.7 µm particle size column method conditions (right).

All samples were prepared as described in the experimental conditions according to the USP assay with six replicate injections for each solution. In order to determine the successful performance of the scaled methods run on the appropriate chromatographic system, the system suitability requirements stated in the quetiapine fumarate USP monograph were evaluated. Additionally, an unknown sample was analyzed on each of the three systems to determine the reproducibility of the scaled methods.

System suitability tests are an important component of the USP monograph given that they determine whether or "not a chromatographic system is adequate for an intended analysis".[3] For the system suitability, the USP monograph requires that the resolution between the quetiapine desethoxy and the quetiapine peaks (Figure 4) must be not less than (NLT) 1.5, with peak identification based on relative retention times contained in the USP monograph. The standard solution requires the tailing factor be not more than (NMT) 2.0 and the relative standard deviation (RSD) be NMT 2.0%. The first peak of the chromatogram for all samples pertains to the fumaric acid and was not considered when evaluating the system suitability requirements for retention time, area, and tailing since it is not the main active pharmaceutical ingredient (API).

The USP assay method for quetiapine fumarate was evaluated using the original and scaled methods run on the prescribed LC systems. Example chromatograms obtained using the three methods are shown in Figure 4 (system suitability) and Figure 5 (sample solution). All methods met the system suitability requirements for both the system suitability solution and the standard solution with the results located in Table 1.

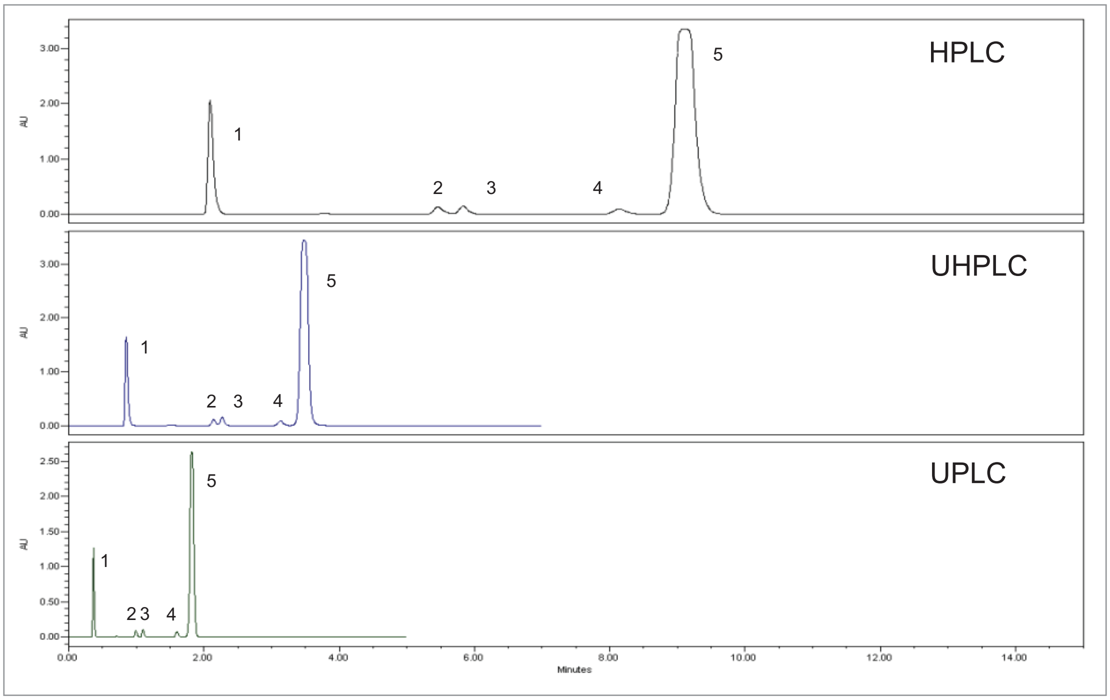


Figure 4. Comparison of the system suitability reference sample of quetiapine fumarate run on the 5 µm particle column on the Alliance HPLC System (top) and scaled to a 3.5 µm and a 1.7 µm particle columns analyzed on the ACQUITY Arc UHPLC (middle) and ACQUITY UPLC H-Class PLUS (bottom) systems, respectively. The API (quetiapine) is saturated in the system suitability solution in order to get a high enough sensitivity of the impurity compounds. The compounds are identified as follows: Peak 1: fumaric acid, Peak 2: quetiapine related compound G, Peak 3: quetiapine related compound B, Peak 4: quetiapine desethoxy, and Peak 5: quetiapine.

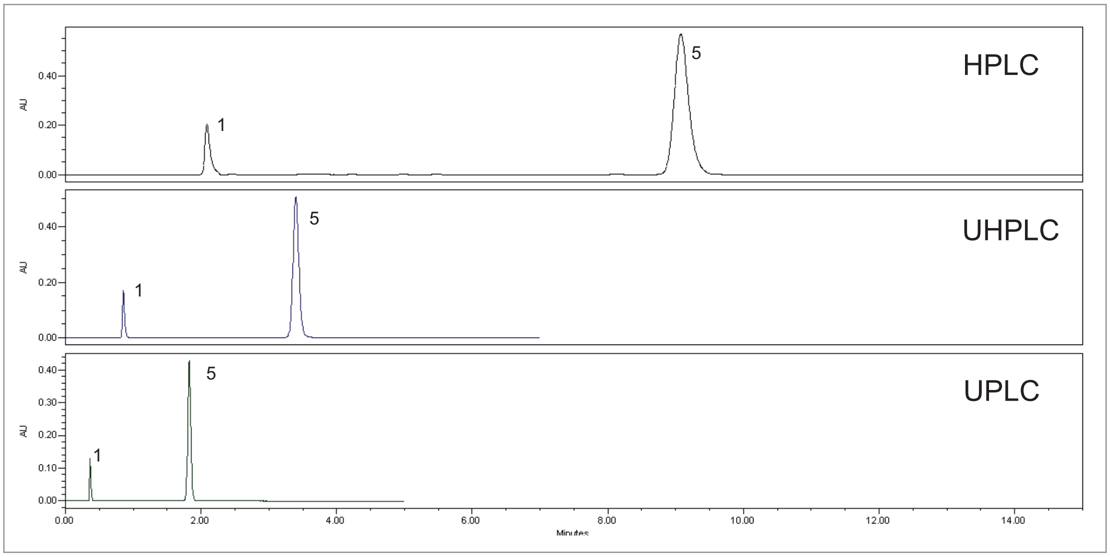


Figure 5. Comparison of the sample solution run on the 5 µm particle size column on the Alliance HPLC System (top), and scaled to a 3.5 µm and a 1.7 µm particle size columns analyzed on the ACQUITY Arc UHPLC System (middle), and the ACQUITY UPLC H-Class PLUS System (bottom), respectively. The compounds were identified based on the relative retention time provided in the USP method. The compounds are identified as follows: Peak 1: fumaric acid, Peak 5: quetiapine.

	Resolution (Peaks 4 & 5)	Quetiapine tailing	Quetiapine area %RSD	Quetiapine retention time %RSD	Run time (min)	Solvent consumption per sample (mL)
Alliance HPLC System	2.4	1.2	0.09	0.16	15	19.5
ACQUITY Arc UHPLC System	2.2	1.1	0.09	0.02	7	5.6
ACQUITY UPLC H-Class PLUS System	2.5	1.1	0.01	0.10	3	2.4
USP requirements	NLT 1.5 *System suitability*	NMT 2.0 *Standard solution*	NMT 2.0% *Standard solution*	NMT 2.0% *Standard solution*	N/A	N/A

Table 1. The USP quetiapine fumarate assay results for the system suitability and standard solutions on the Alliance HPLC System, the ACQUITY Arc UHPLC System, and the ACQUITY UPLC H-Class PLUS System compared to the USP requirements along with the run time and solvent consumption for each method.

In addition to providing similar or improved chromatographic performance, scaling the original method to a smaller particle column also significantly decreased the run time and solvent consumption. Scaling the original method to a 3.5 µm column decreased the run time from 15 minutes to 7 minutes, and decreased the solvent usage by 66%. Further scaling to a 1.7 µm column decreased the run time from 15 minutes to approximately 2 minutes and reduced the solvent usage by almost 90%. Scaling USP methods to run on smaller particle columns can drastically increase throughput while decreasing cost per sample.

Also, an unknown sample was analyzed in order to determine the percent of quetiapine fumarate present. The standard solution and the sample solution data were used to calculate the percent as follows:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

where r_u is the peak response from the sample solution, r_s is the peak response from the standard solution, C_s is the concentration of USP quetiapine fumarate standard in the standard solution (mg/mL), and C_u is the concentration of quetiapine fumarate in the sample solution (mg/mL).[2] The calculated percent of quetiapine fumarate generated using the different methods can be found in Table 2. These values do not fall within the USP acceptance criteria of 98.0% to 102.0%. If this sample were to be used for batch release, a further investigation would be required to determine the source of the over-estimation of the purity. This may include evaluation of peak purity or possibly analysis by an orthogonal detection mechanism such as mass spectrometry. However, since the aim of this application note is to demonstrate the scalability of the isocratic method, further evaluation is outside the scope of this work.

An important part of method scaling is the ability to generate the same results using either the original or the scaled method conditions. Scaling the USP quetiapine assay method across the different LC systems produced consistent results for the unknown sample (Table 2). This demonstrates that scaled methods can be used to generate reliable data.

	Sample result (%)
Alliance HPLC System	109.5
ACQUITY Arc UHPLC System	108.9
ACQUITY UPLC H-Class PLUS System	109.7

Table 2. The USP quetiapine fumarate assay calculated results for the percent of quetiapine fumarate in the sample solution on the 5 µm particle size column on the Alliance HPLC System, the 3.5 µm particle size column on the ACQUITY Arc UHPLC System and the 1.7 µm particle size column on the ACQUITY UPLC H-Class PLUS System.

CONCLUSIONS

Utilizing the method scaling guidelines within the USP General Chapter <621>,[1] traditional isocratic HPLC methods are capable of being scaled to columns with smaller particle sizes and shorter lengths. Scaling a USP method enables the use of modern chemistries and LC hardware to deliver improved throughput with decreased solvent consumption all while providing accurate and reproducible chromatographic data. The scaled modifications for the USP quetiapine fumarate assay method enabled the original method run time to be reduced by 57% for a 3 µm column on an ACQUITY Arc UHPLC System and 87% reduction for the 1.7 µm column on an ACQUITY UPLC H-Class PLUS System. The scaled methods maintained the same or improved chromatographic performance in terms of resolution, peak tailing, and retention time and peak area RSD.

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