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Developing Analytical Chromatographic Methods for Pharmaceutical Stability Investigations

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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.

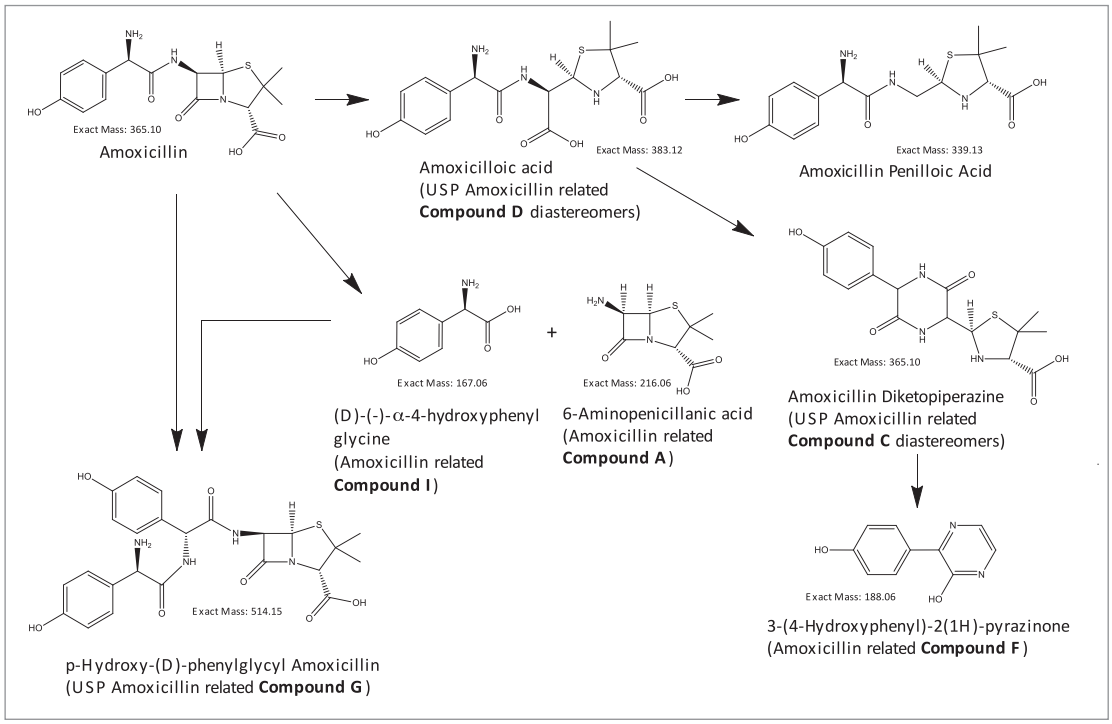

Figure 1. Amoxicillin and some degradation pathways.

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.

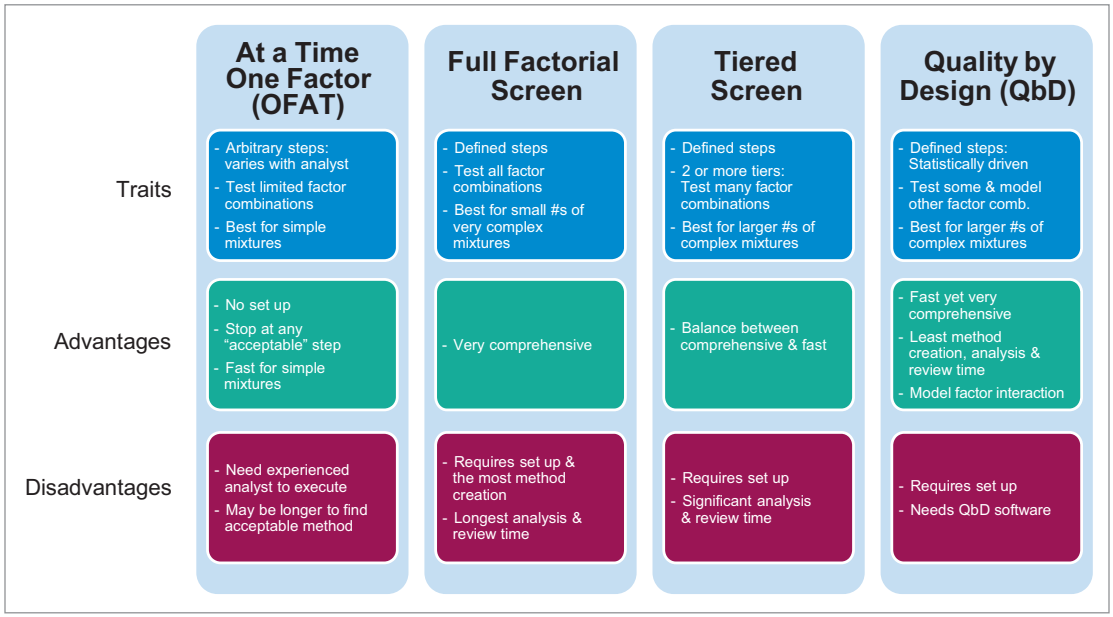

Figure 2. Comparison of different method development strategies.

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.

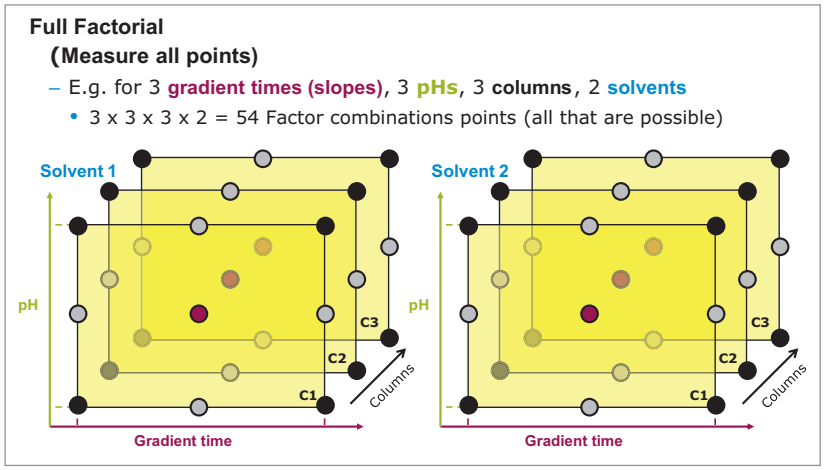

Figure 3. Generic schematic example for Full Factorial measurements at all factor combination points.

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.

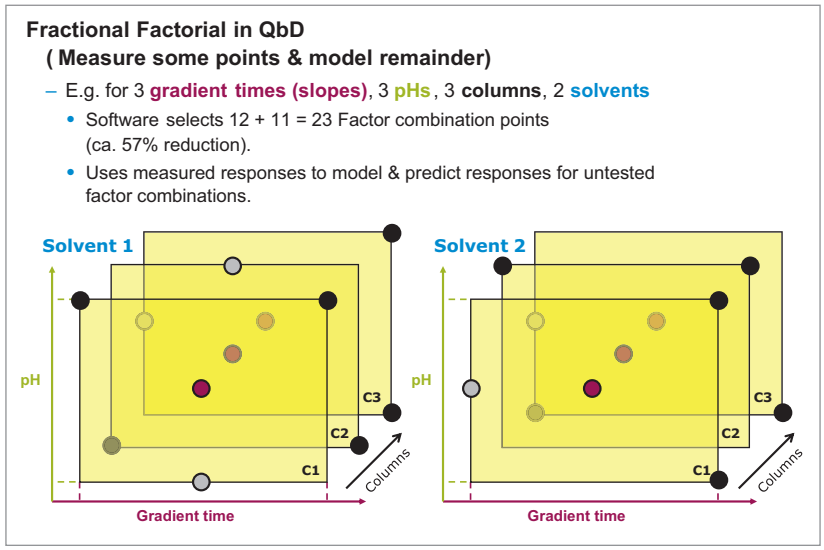

Figure 4. Generic schematic example for Fractional Factorial measurements at balanced and orthogonally selected factor combination points.

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.

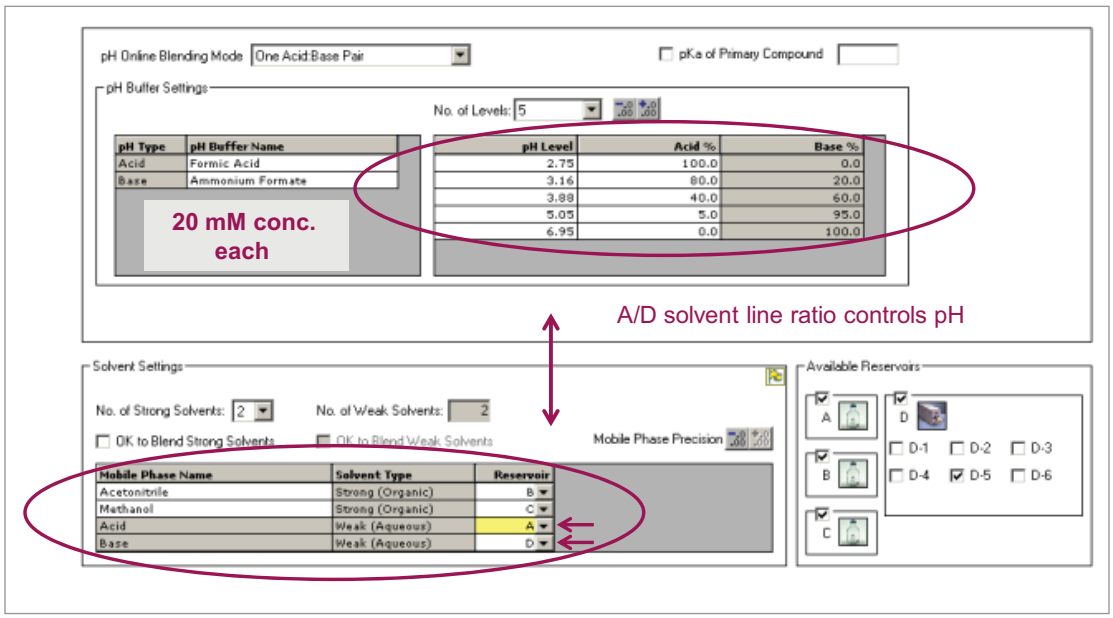

Figure 5. Fusion QbD set up of solvents and pH on-line blending.

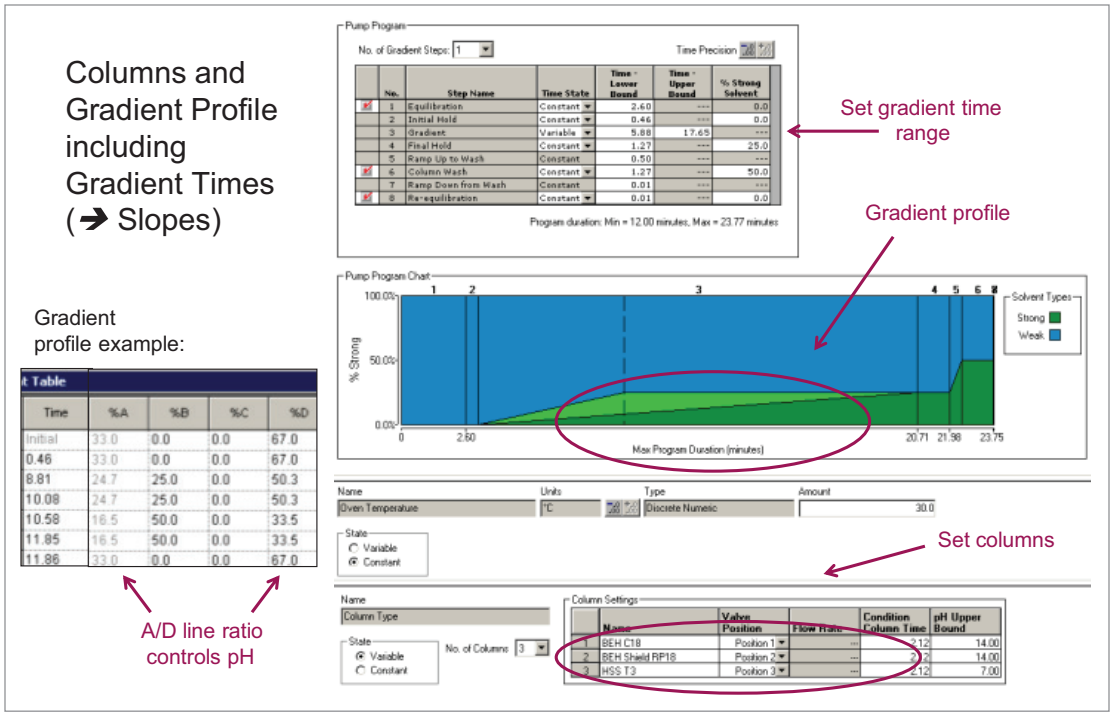

Figure 6. Fusion QbD set up of gradient slopes and columns.

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).

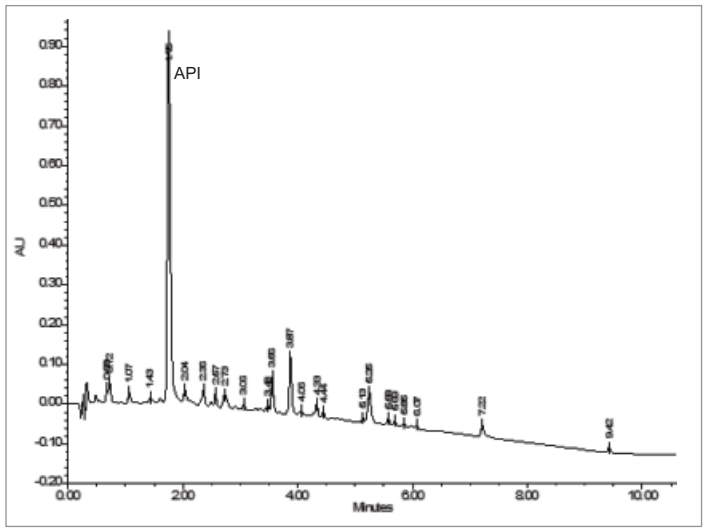

Figure 7. Representative APOS stress sample chromatogram.

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

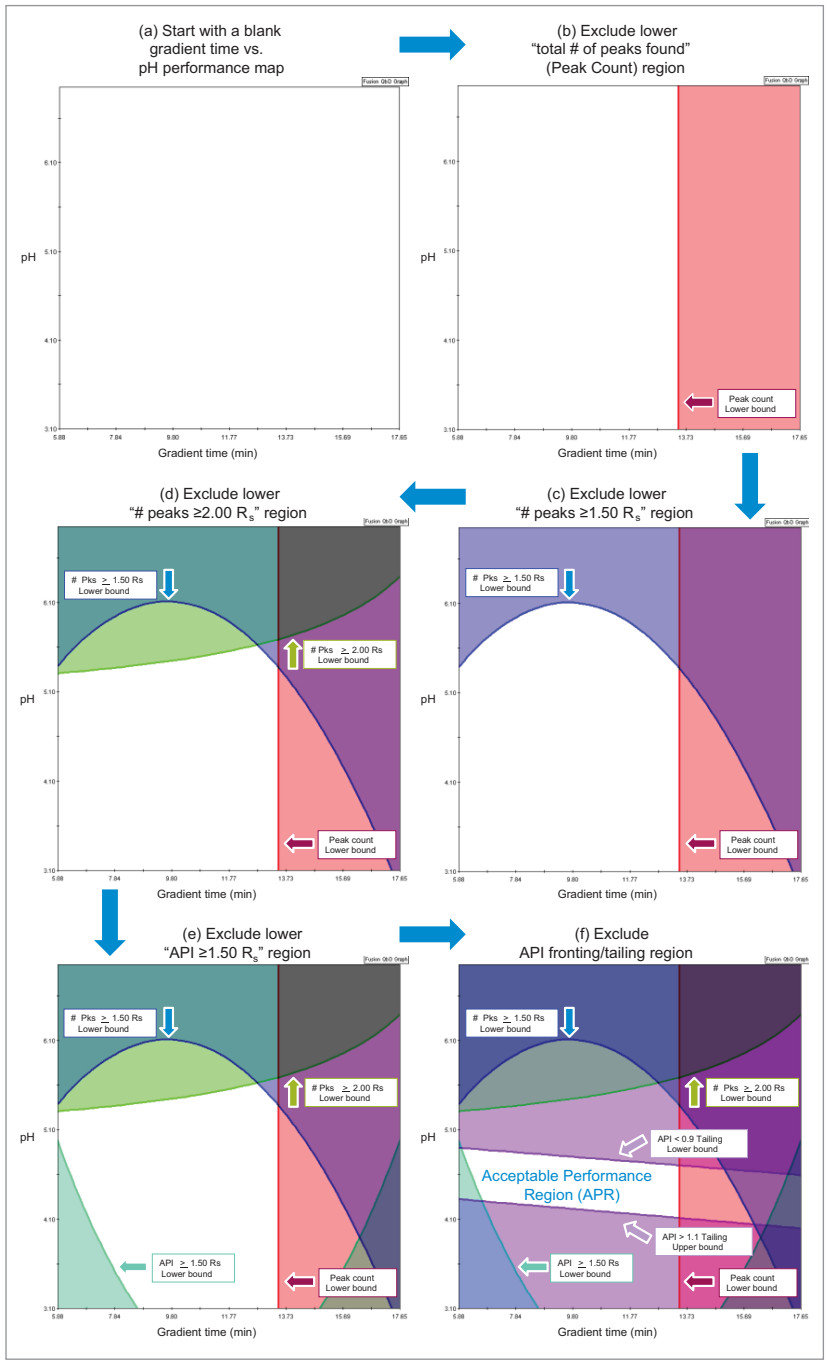

Figure 8. Construction of the Fusion QbD performance map.

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.

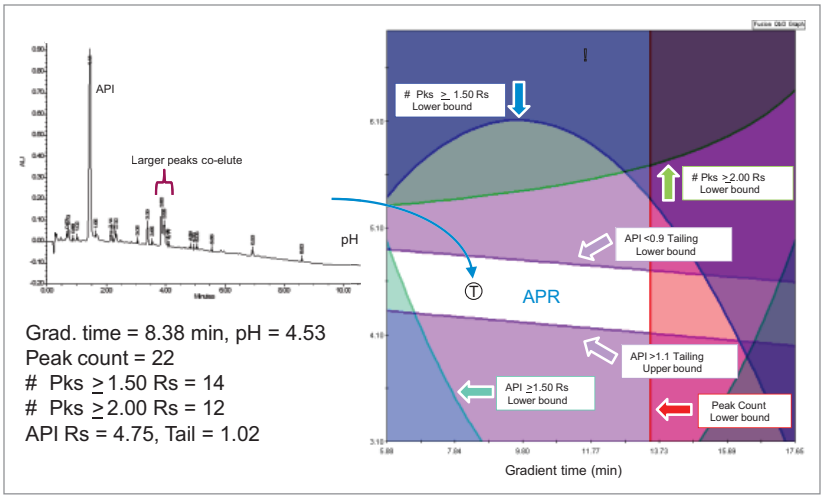

Figure 9. Example chromatogram within the modeled Acceptable Performance Region (APR).

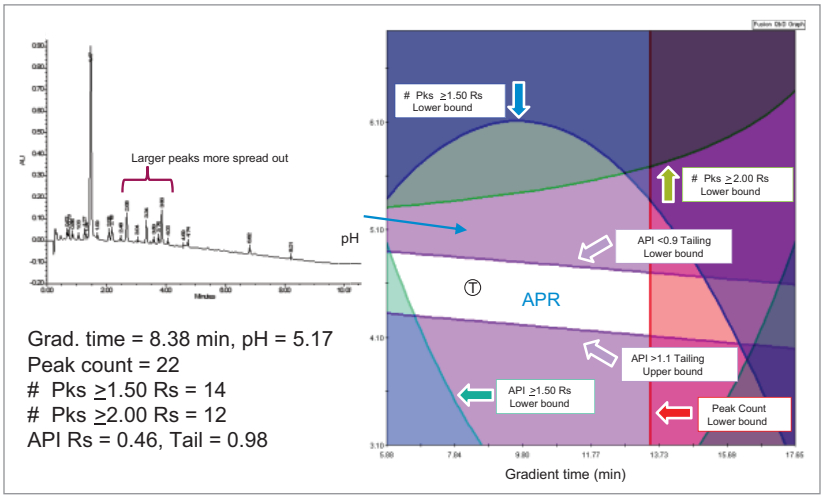

Figure 10. Example chromatogram above the modeled Acceptable Performance Region (APR).

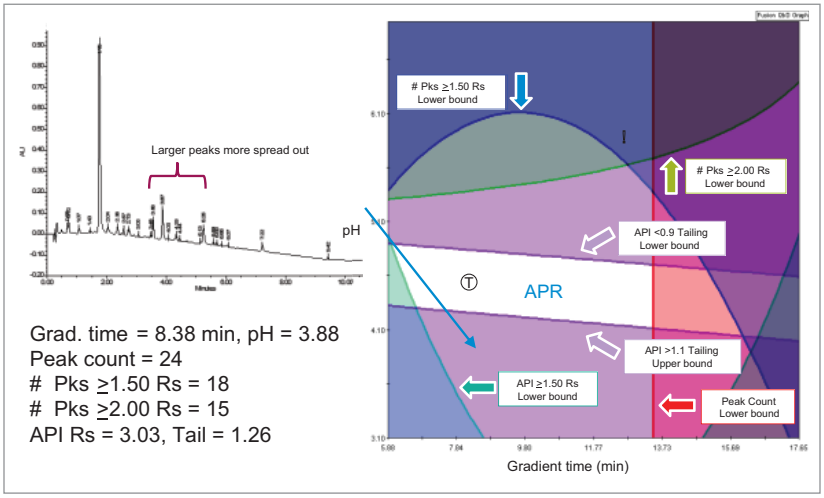

Figure 11. Example chromatogram below the modeled Acceptable Performance Region (APR).

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.

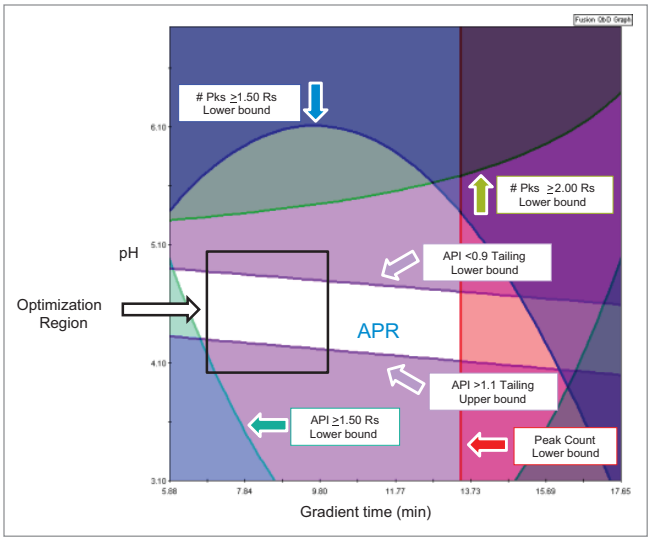

Figure 12. Fusion QbD Performance Map with the optimization region.

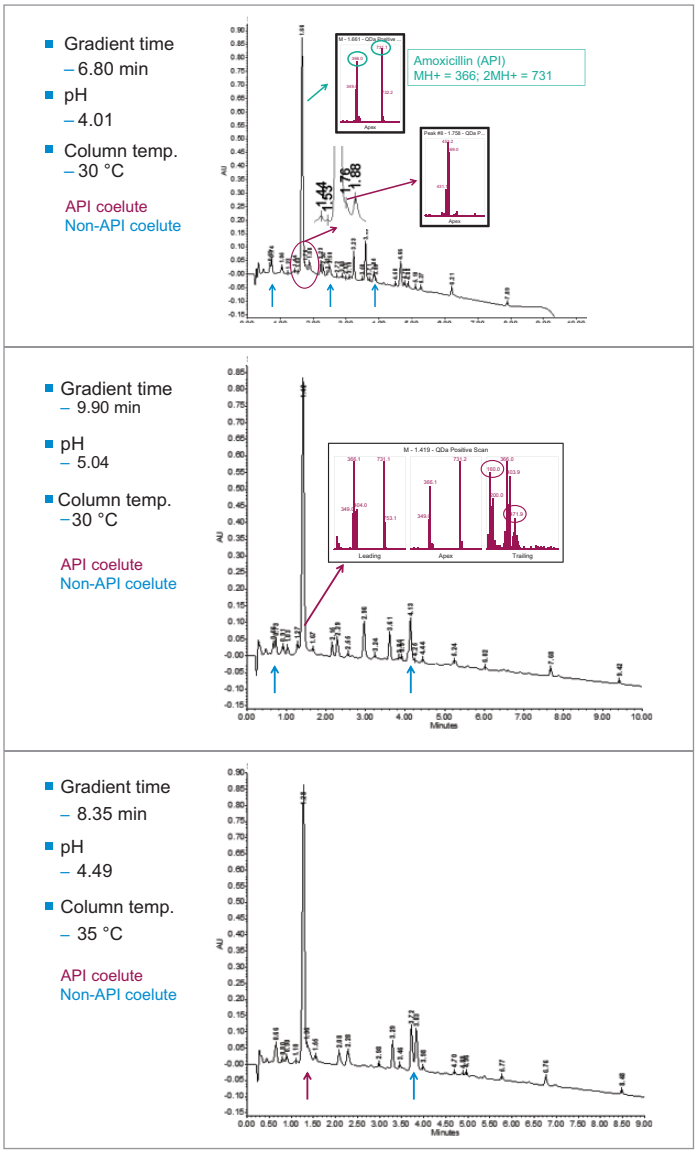

Figure 13. Example optimization chromatograms.

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.

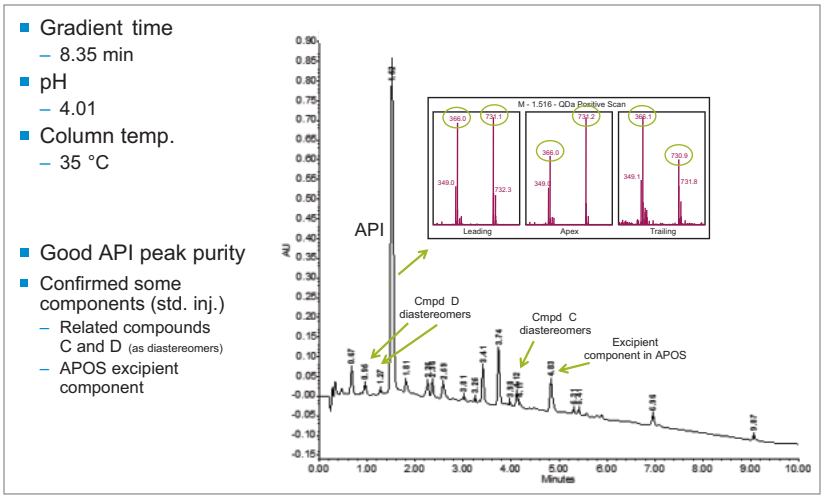

Figure 14. Chromatogram using best optimization conditions.

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.

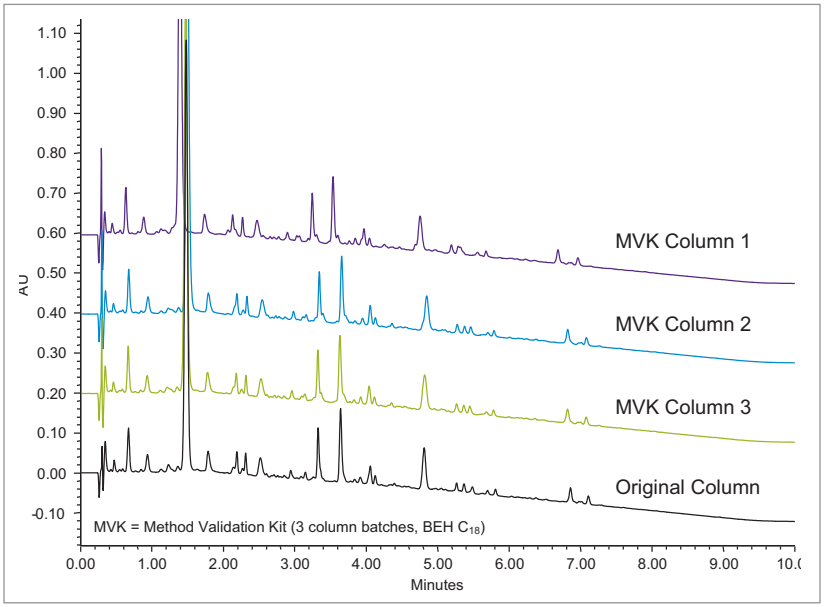

*Figure 16. Final SIM using a Method Validation Kit compared to the original XBridge BEH C_{18} **XP** Column.*

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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