Nota applicativa

Using a Systematic Screening Protocol and MaxPeak™ HPS Technology to Develop a UHPLC Method for the Analysis of Deferoxamine and its Forced Degradation Products

Kenneth D. Berthelette, Jonathan E. Turner, Thomas H. Walter, Kim Haynes

Waters Corporation

Abstract

Often method development activities are performed “on the fly” with tight timelines to achieve the goal of developing robust methods quickly. Unfortunately, the approach to method development is an often-overlooked aspect of the process. The use of method development software with AQbD (analytical quality by design) approaches can provide robust methods but may take a while to implement and requires not only the software, but also training to use it correctly. Other approaches require more manual intervention, both to select the correct conditions and to interpret the results and make decisions based on the data. Employing a systematic screening protocol, which utilizes a structured decision-making process, alleviates the pressure on the analyst to select the best set of conditions, and streamlines the method development process.

In the work shown, a systematic screening protocol was used to develop a method for the analysis of deferoxamine mesylate after forced degradation under acidic and basic conditions. The final method conditions were realized after only two days of development and optimization. The final method conditions utilized an XSelect™ Premier HSS PFP Column and a methanol gradient with 0.1% formic acid. Good separation and detection were achieved for the known analytes of interest as well as unknown degradation products.

Benefits

- Baseline separation of eight analytes using an XSelect Premier HSS PFP Column
- Method development of a forced degradation sample in two days
- Systematic screening protocol reduces decision making by streamlining the process

Introduction

Method development can be a challenging endeavor, even for expert chromatographers. Not only must an analyst find appropriate method conditions for separating their analytes, which can be challenging on its own, but often they need to track how those conditions were determined and selected for future reference and documentation purposes. For bigger laboratories, where multiple method development activities happen concurrently, having different analysts develop methods can lead to different overall approaches. This makes tracking the activities more difficult. It can be beneficial to implement a standardized protocol for all new method development work. This may include screening certain column chemistries, as well as certain mobile phase conditions. By standardizing method development across a lab, the process that yielded the final method conditions is better understood and more easily documented. Additionally, having a standardized protocol allows less experienced users to perform method development, freeing up the experts to handle more challenging separations.

A variety of method development approaches exist, including software assisted AQbD and full factorial screening. Full factorial screening is designed to test each combination of the three critical parameters for HPLC methods: stationary phase chemistry, strong solvent, and mobile phase pH. This is the most comprehensive approach but does require not only creation of all the different mobile phases, but also considerable analyst and instrument time. Given a panel of four stationary phases, a full factorial approach would require 16 runs at a minimum, not including running replicates of each condition. This can take a significant amount of time to analyze and interpret once the data has been collected. AQbD approaches are generally faster than full factorials as only some of the critical parameters are tested. Typically employing DOE (design of experiments) the software assisted AQbD approach models the critical parameters for the separation and suggests the most robust area for method development. While this may be good for robustness testing or validation, AQbD does not account for different stationary phases, and often requires specialized training to run the software.

A more streamlined approach is the systematic screening protocol. Unlike full factorial, the systematic protocol first assesses the effects of mobile phase pH on the retention of the analytes. After at least two analytical runs, an analyst can easily determine the pH that provides the best retention for their analytes and then proceed to column and strong solvent screening. For a typical panel of four stationary phases, the systematic screening protocol can provide the best method conditions after ten injections, not including replicates, saving time over the full factorial approach. In this application, the iron-chelating drug deferoxamine mesylate was subjected to forced degradation via acidic and basic hydrolysis at an elevated temperature. After degradation, the systematic screening protocol was applied to develop a method to separate the known peaks of interest as well as any unknown degradants. The final method achieves baseline separation of all eight components, including deferoxamine and the counter-ion mesylate, with good detection using a QDa mass detector. MaxPeak Premier HPS Technology was also employed for this analysis to minimize any analyte adsorption, as deferoxamine is known to adsorb to LC systems and columns[1].

Experimental

Sample Description

A 1 mg/mL stock solution of deferoxamine mesylate was made using Milli-Q water. Two separate 1 mL portions of the stock solution were removed and placed in separate vials. 100 µL of 0.1 N NaOH was added to one vial, while 100 µL of 0.1 N HCl was added to the other. Both vials were heated at 70 °C for one hour. After heating the contents of the vials were combined to quench the degradation. The combined sample was then diluted 1:10 with Milli-Q water and placed on the system for analysis. The final concentration of deferoxamine mesylate after dilution was 0.1 mg/mL, not accounting for concentration changes due to degradation.

LC Conditions

LC system:	ACQUITY™ Premier Quaternary Solvent Manager (QSM) System with Sample Manager Flow Through Needle (SM-FTN), Column Manager, Column Manager Aux, and QDa mass detector

Detection:	SIRs of degradation products and API (table 1)
Columns:	All columns are 2.1 x 50 mm with 2.5 µm particles XBridge™ Premier BEH C18 (p/n: 186009827) XSelect Premier CSH Phenyl-Hexyl (p/n: 186009879) XSelect Premier HSS PFP (p/n: 186010043) Atlantis™ Premier BEH C18 AX (p/n: 186009390)
Column temperature:	30°C
Sample temperature:	10°C
Injection volume:	1.0 µL
Flow rate:	0.7 mL/min
Mobile phase A:	Milli-Q Water
Mobile phase B:	Acetonitrile
Mobile phase C:	Methanol
Mobile phase D:	0.1% Formic Acid (D1) or 200 mM Ammonium Hydroxide (D2)
Gradient conditions:	Constant 5% D to ensure consistent additive concentration was maintained throughout the gradient. Linear gradient of 5–95% B/C over 4.90 minutes. Hold at 95% B/C for 0.82 minutes. Return to starting conditions of 5% B/C and hold for 1.6

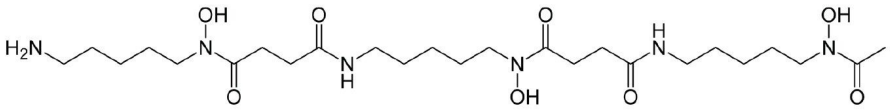
minutes. Total run time 7.37 minutes.

Compound identifier	Monoisotopic mass (*m/z*)	ESI mode
Deferoxamine	561.29	Pos
Mesylate	94.96	Neg
160	160.12	Pos
161	161.07	Pos
219	219.14	Pos
260	260.24	Pos
361	361.27	Pos
259	259.24	Neg

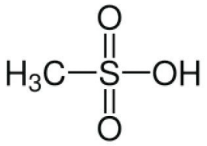
Table 1. SIR detection and compound identifiers for the detected degradation products of deferoxamine mesylate tracked during method development.

Data Management

Chromatography software:	Empower™ 3 Feature Release 4



Deferoxamine
(560.69 da)



Methanesulfonic acid (mesylate)
(96.10 da)

Figure 1. Chemical structures of the known components of the sample. The active ingredient Deferoxamine and counter-ion mesylate along with unknowns were monitored by electrospray ionization mass spectrometry.

Results and Discussion

Using a systematic screening approach to method development starts with setting minimum allowable criteria for the final method and defining the system that will be used. The criteria can be as broad or as specific as the laboratory requires. Typically, criteria such as USP resolution or tailing factors are used, however some analyses may require other parameters to be met. These criteria are not only a goal for the method but are also a "stopping point" to let the analyst know when a method has been created that meets the needs of the analysis. Over-development of a method can waste time when an already sufficient method exists. Worse yet, without specific criteria a method may be developed for weeks only to find that the final version yields similar results as the first few attempts. In the work shown, USP resolution values must be >1.5 for all compounds, and USP tailing factors must be between 0.8 and 1.2. For this work, an ACQUITY Premier Quaternary System was used to minimize potential interactions between the analytes and the metal components present in the flow path of the HPLC system.

Keeping these criteria in mind, we employed the systematic screening protocol outlined in previous applications.[2–3] It should be noted that different columns were selected for this work compared to previously published

applications. When selecting columns for a method development project it is important to cover a range of selectivities while also keeping the analytes of interest in mind. A good starting point is to select columns with varying particle technologies, such as hybrid organic/inorganic *vs* silica, as well as different bonding chemistries. As such the columns selected for this work incorporate three different particle technologies and four different bonding chemistries.

The XBridge Premier BEH C_{18} Column is a rugged, durable C_{18} phase bonded to hybrid-organic/inorganic particles.[4] This is a good starting point for method development as the column is suitable for both high pH and low pH mobile phase additives. Next, the XSelect Premier CSH Phenyl-Hexyl Column was selected. This column, which also uses hybrid particles, has weak anion-exchange functionality due to the slight positive charge applied during manufacturing.[5–6] This base particle when coupled to the phenyl-hexyl bonded ligand can provide unique selectivity, especially when using methanol as the organic mobile phase. The third column selected, an XSelect Premier HSS PFP Column, is bonded to high purity silica particles. The PFP (pentafluorophenyl) bonded phase can provide not only pi-pi interactions, but also dipole and hydrogen bond interactions. The last column, the Atlantis Premier BEH C_{18} AX Column, was selected to address the retention of the counter-ion mesylate. The mixed mode reversed phase/anion exchange bonded chemistry attached to the previously described BEH particle, employs an intermediate coverage C_{18} bonding and a proprietary anion exchange functional group ideal for retaining acidic species.[7–8] Images of the four stationary phases used in this application note are shown in Figure 2.

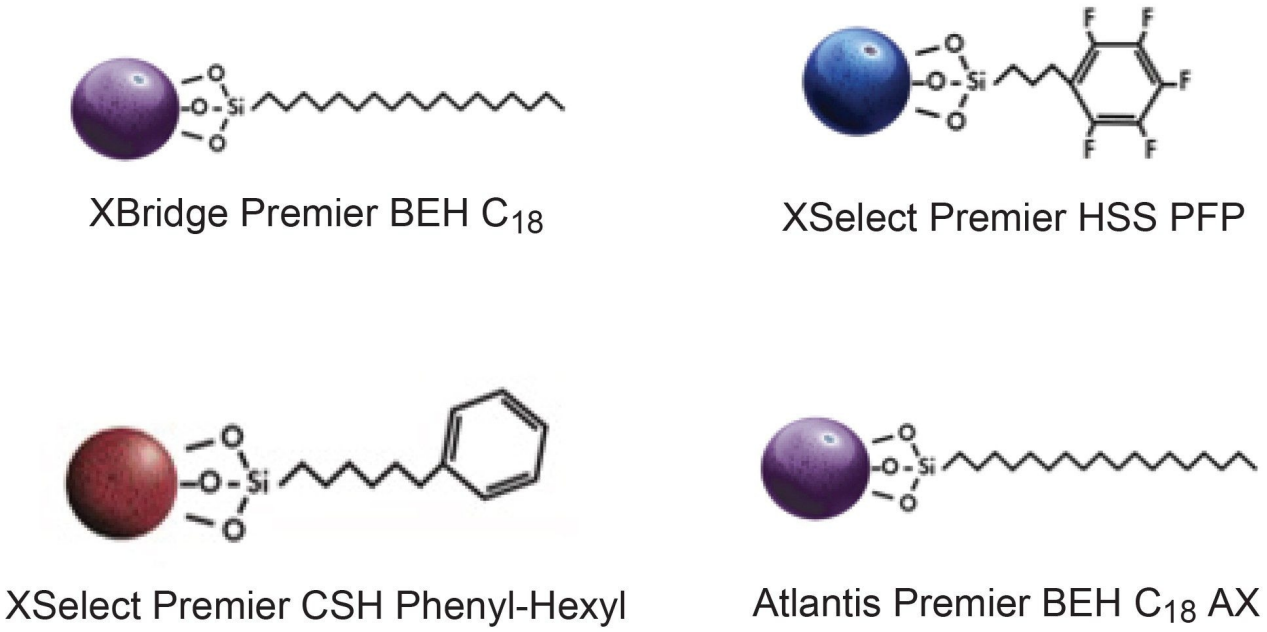


Figure 2. Bonded chemical structure of the selected columns. Base particles shown are either fully porous hybrid, BEH/CSH, or pure silica, HSS.

Additionally, the use of appropriate column and system hardware is needed to ensure optimal performance for the method. As previously discovered, some compounds like nucleotides and small acidic compounds can interact with the metal hardware used in many LC columns and systems. MaxPeak Premier HPS hardware, used in both ACQUITY Premier LC Systems as well as many columns, mitigates these interactions by employing a hybrid organic/inorganic layer over the metal surface.[9–10] While some of the compounds analyzed in this work may not be metal sensitive, the API deferoxamine has been shown to be affected by stainless steel columns.[1] As such, all columns and systems selected employ MaxPeak HPS Technology to reduce the potential analyte loss to not only the API but also the degradants should they be metal sensitive.

The first step of the systematic screening protocol involves assessing the retention of the analytes at high pH and low pH and determining the conditions that provide the best retention. For this step it is important to select a column that is stable at high pH, like the XBridge Premier BEH C_{18} Column. Unlike the final method requirements set earlier, in this step, only retention is assessed. The conditions which provide the best overall retention for the compounds of interest will be selected for the next step. Figure 3 shows the chromatography from the pH scouting.

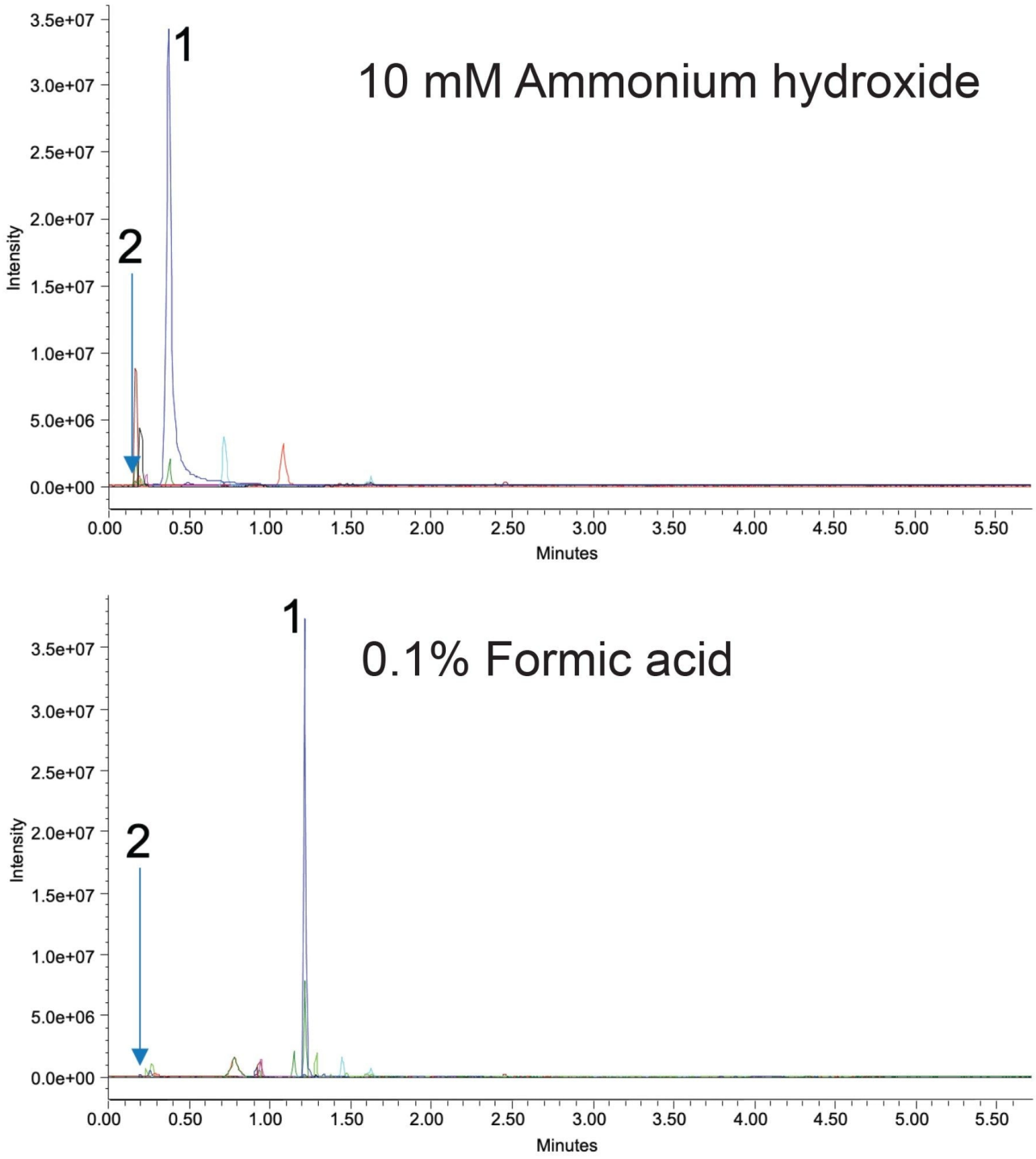


Figure 3. Rapid pH scouting results using the XBridge Premier BEH C_{18} Column. 1) Deferoxamine, and 2) Mesylate. Other analytes are not labelled but were tracked via SIRs.

The use of ammonium hydroxide mobile phase additive leads to lower retention for the analytes. Both deferoxamine (1) and mesylate (2) are less retained at high pH than at low pH. Additionally, more of the degradants are retained at low pH, with most of them co-eluting in the void under high pH conditions. The decision to use low pH can be made easily in this case. Moving forward, only low pH mobile phases will be used. This opens some possibilities in terms of column selectivity, which would have been hindered if a high pH mobile phase was chosen.

Step two of the systematic screening protocol is column and mobile phase screening. In this step, the four columns selected previously are screened at the chosen pH, with both acetonitrile and methanol as the strong solvent. The purpose of this step is to determine the best combination of mobile phase and stationary phase to use for the separation. At this step, all method development criteria come into play, with the best conditions being selected and optimized, if necessary. Figure 4 shows the separation of the sample with the four columns using an acetonitrile mobile phase.

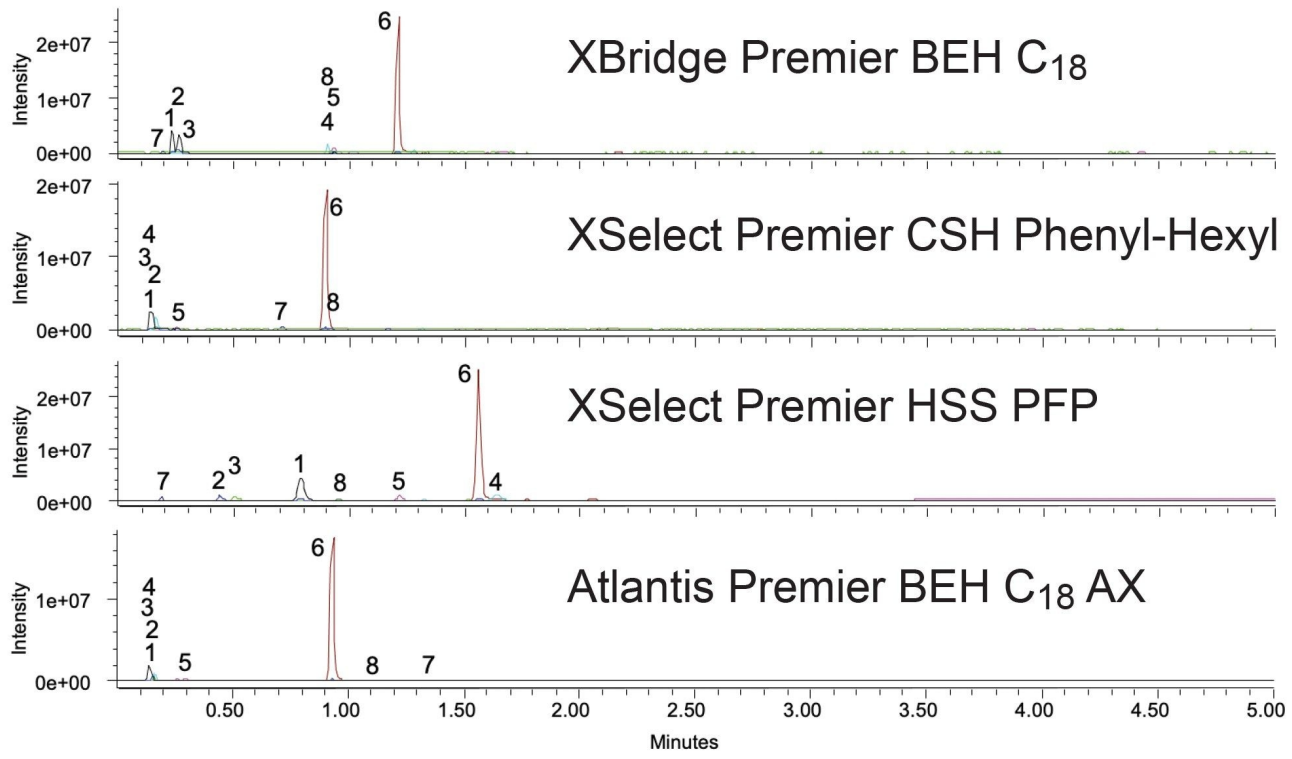


Figure 4. Separation of the forced degradation sample with the four selected columns using acetonitrile as the strong solvent. 1) m/z 160, 2) m/z 161, 3) m/z 219, 4) m/z 260, 5) m/z 361, 6) Deferoxamine, 7) Mesylate, 8) m/z 259. Low intensity analytes may not show; however, the number indicator is placed where analyte elutes.

For most column/mobile phase combinations, at least two analytes co-elute. Components 1,2,3,4 co-elute on both the XSelect Premier CSH Phenyl-Hexyl and Atlantis Premier BEH C_{18} AX Columns. Components 1-3 also co-elute on the XBridge Premier BEH C_{18} Column. The XSelect Premier HSS PFP Column separated all the components, with only two sets of critical peaks. It should be noted, that both the XSelect Premier CSH Phenyl-Hexyl Column and the Atlantis Premier BEH C_{18} AX Column provided good retention for component 7, mesylate. This is attributed to the anion exchange functionality of both columns. While the CSH particle provides low anion exchange retention, the Atlantis Premier BEH C_{18} AX Column is designed to have greater anion exchange character. This is confirmed by the fact that mesylate is better retained on the latter column compared to the former. Of the four conditions tested, only the HSS PFP Column came close to meeting all the set criteria. It falls short for resolution of the critical pairs 2/3 and 6/4 with USP resolutions of 1.3 and 1.4 respectively. Figure 5 shows the separation of the sample with the four columns using methanol as the strong mobile phase.

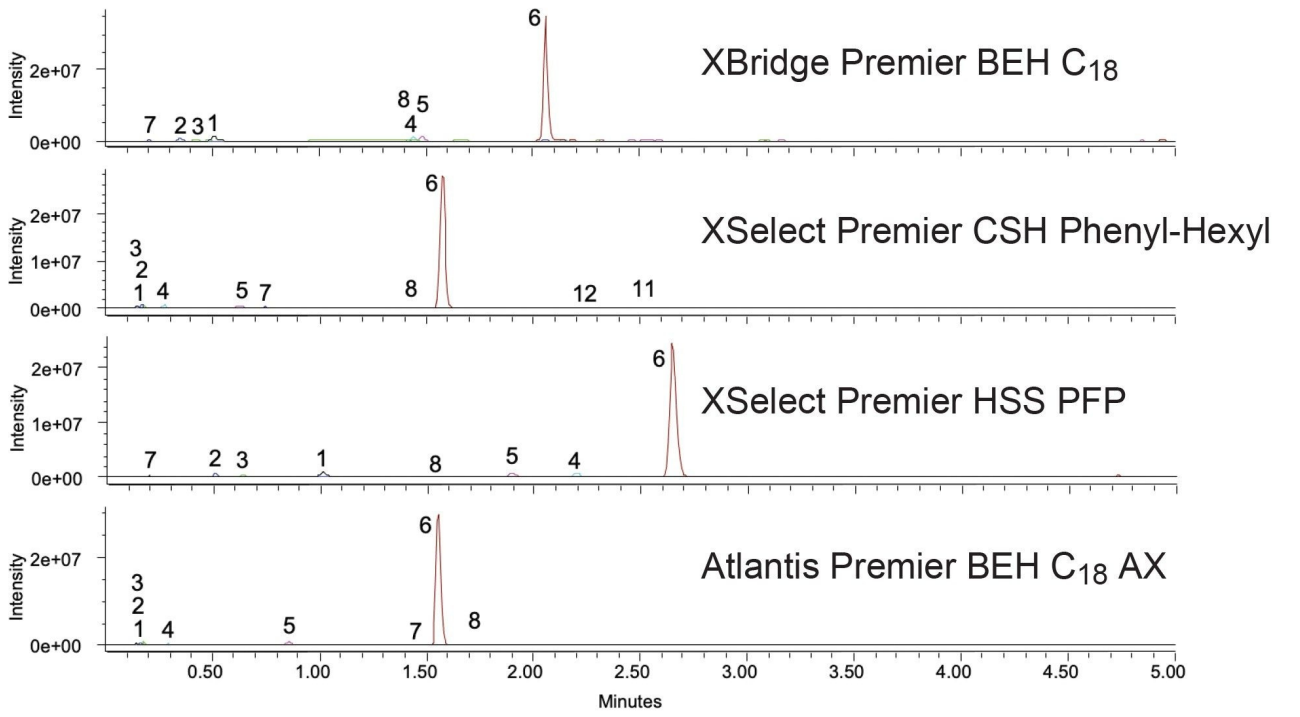


Figure 5. Separation of the forced degradation sample with the four selected columns using methanol as the strong solvent. 1) m/z 160, 2) m/z 161, 3) m/z 219, 4) m/z 260, 5) m/z 361, 6) Deferoxamine, 7) Mesylate, 8) m/z 259. Low intensity analytes may not show; however, the number indicator is placed where the analyte elutes.

The use of methanol mobile phases provides different selectivity compared to the acetonitrile conditions. For instance, on the XBridge Premier BEH C_{18} Column, the early eluting components 7, 1–3 are better resolved with methanol compared to acetonitrile. One thing to note is that the retention of mesylate[7] is almost entirely governed by the anion exchange functionality of the stationary phase and its retention is only marginally affected using methanol compared to acetonitrile. The only column that separated all components is the XSelect Premier HSS PFP Column. Methanol as the strong mobile phase provided a better separation for these components compared to acetonitrile. USP resolutions are >1.5 for all components with methanol, and tailing values are better with methanol as well. Using the XSelect Premier HSS PFP Column with methanol mobile phases meets all required criteria but is not as successful at retaining mesylate as other columns. However, mesylate is not eluting in the void of the column, allowing for quantitative measurements if desired.

While optimization was not required for this sample, the gradient slope was adjusted to shorten the analytical

run time. Figure 6 shows the final method conditions for this separation using scheduled SIRs on the ACQUITY QDa mass detector to improve the cycle time and increase sensitivity. The y-axis was normalized to better show the lower intensity peaks and confirm acceptable peak symmetry.

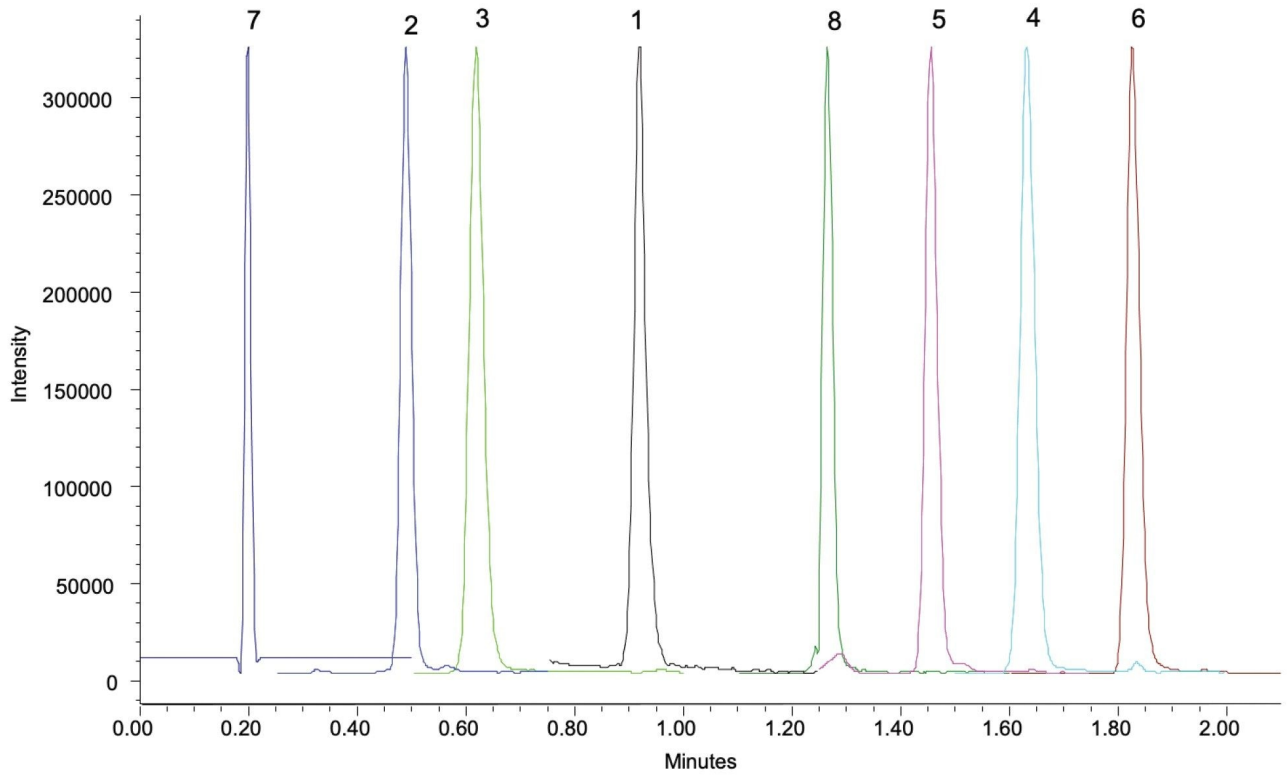


Figure 6. Final method conditions using an XSelect Premier HSS PFP, 2.1 x 50 mm 2.5 µm Column. A methanol mobile phase gradient was used with 0.1% formic acid and a gradient slope of 6.0 %/V_c. SIRs were scheduled via Empower software to improve sensitivity of the QDa. 1) m/z 160, 2) m/z 161, 3) m/z 219, 4) m/z 260, 5) m/z 361, 6) Deferoxamine, 7) Mesylate, 8) m/z 259.

For all analytes, USP tailing factors were within 0.8–1.2, and the USP resolutions were all greater than 1.5. This satisfies our required criteria for the separation. By implementing a systematic screening protocol, the method for analysis of a deferoxamine forced degradation sample was developed in just under two days, including optional optimization to reduce run time. Structured method development approaches like the one shown eliminate the uncertainty that can arise from different analysts developing methods. If a laboratory standardizes to a method development approach, then regardless of analyst the final method will have been developed in a systematic,

traceable, and easily justified way.

Conclusion

A step-by-step approach can be a critical asset for method development activities. Not only does having a standardized protocol ensure that all analysts are developing methods in the same fashion, but it can also reduce overall method development time if designed appropriately. While some method development approaches, like AQbD, have clear advantages in terms of statistical modeling, a more manual approach like a systematic screening protocol is much more accessible and does not require specialized software or training. A systematic screening protocol first examines the effects of pH on retention, before moving on to a column and strong solvent screen. By first assessing pH, the selected attribute can be locked in eliminating it from further activities. This also can help guide which columns to select in the screening portion as not all columns are suitable for high pH conditions. Additional column and mobile phase screening optimization can occur, if needed.

The systematic screening protocol was used to develop a method for the analysis of a forced degradation sample of the iron chelating drug deferoxamine mesylate. Both the active ingredient, deferoxamine, and the counter-ion mesylate were tracked along with six degradants. Using the systematic protocol, a final method using methanol, formic acid, and an XSelect Premier HSS PFP Column was developed after just two days of running the system. Further activities such as robustness testing, and validation would still need to be performed however, but those activities were out of scope for this work. The use of MaxPeak Premier HPS Technology in both the column hardware and the system allowed for reliable analysis of the metal sensitive compound deferoxamine, without sacrificing the chromatographic performance for the other compounds present in the sample.

References

1. Boissel C, Walter TH, Shiner SJ. ACQUITY Premier Solution Improves the UPLC-MS Analysis of Deferoxamine – an Iron Chelating Drug. Waters Application Note. 720007239.
2. Maziarz M, McCarthy S, Wrona M. Improving Effectiveness in Method Development by Using a Systematic

Screening Protocol. Waters Application Note. 720005026.

3. Berthelette KD, Nguyen JM, Turner, J. Method Development of Ten Antibiotic Compounds Using a Systematic Screening Protocol on an ACQUITY ARC With UV and QDa Detection. Waters Application Note. 720007352.

4. Wyndham KD, Walter TH, Iraneta PC, Neue UD, McDonald PD, Morrison D, Baynham M. A Review of Waters Hybrid Particle Technology: Ethylene-Bridged [BEH Technology] Hybrids and Their Use in Liquid Chromatography. Waters White Paper 720001159 < https://www.waters.com/webassets/cms/library/docs/720001159en.pdf> .

5. Iraneta PC, Wyndham KD, McCabe DR, Walter TH. A Review of Waters Hybrid Particle Technology. Part 3. Charged Surface Hybrid (CSH) Technology and Its Use in Liquid Chromatography. Waters white paper 720003929 <https://www.waters.com/webassets/cms/library/docs/720003929en.pdf> .

6. Smith K, Rainville P. Improved Analytical Sensitivity and Chromatographic Peak Shape for the Quantification of TCA Cycle Analytes in Human Plasma Using the ACQUITY Premier System Solution. Waters Application Note. 720007107.

7. Kadlecova Z, Kalikova K, Folprechtova D, Tesarova E, Gilar M. Method for evaluation of ionic interactions in liquid chromatography. *J. Chromatogr. A.* 1625 (2020) 461301.

8. Walter TH, Alden BA, Field J, Lawrence N, Osterman D, Patel AV, DeLoffi M. Characterization of a Highly Stable Mixed-Mode Reversed-Phase/Weak Anion-Exchange Stationary Phase Based on Hybrid Organic/Inorganic Particles. *J. Sep. Sci.* 44 (2021) 1005–1014.

9. Delano M, Walter TH, Lauber M, Gilar M, Jung MC, Nguyen JM, Boissel C, Patel A, Bates-Harrison A, Wyndham K. Using Hybrid Organic-Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC. *Anal. Chem.* 93 (2021) 5773–5781.

10. Walter TH, Alden BA, Belanger J, Berthelette KD, Boissel C, Delano M, Kizekai L, Nguyen JM, Shiner S. Modifying the Metal Surfaces in HPLC Systems and Columns to Prevent Analyte Adsorption and Other Deleterious Effects. *LCGC Supplement*, June (2022) 28–34.

Featured Products

ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

Empower Chromatography Data System <https://www.waters.com/10190669>

720007834, December 2022

© 2023 Waters Corporation. All Rights Reserved.
Terms of Use Privacy Trademarks Sitemap Careers Cookie Preferenze cookie