Waters™

Nota applicativa

Using the Systematic Screening Protocol and MaxPeak™ Premier Columns to Separate Seven Janus Kinase Inhibitors

Kenneth D. Berthelette, Christopher Collins, Jamie Kalwood, Kim Haynes

Waters Corporation

Abstract

Developing analytical methods can be a challenging endeavor, particularly for structurally similar compounds. The similarities between the compounds means that the compounds will behave similarly when subjected to different method conditions, presenting added difficulty to the process of method development. Applying a systematic approach to method development, like the Systematic Screening Protocol coupled with MaxPeak™ High Performance Surfaces (HPS) Technology eliminates some of the challenges related to developing methods for structurally similar compounds.

This application note focuses on the development process for a separation of seven Janus Kinase Inhibitors (JKI). The final method conditions required the use of high pH mobile phases and methanol as the strong solvent on the ACQUITY™ Premier BEH™ C_{18} Column. This column not only takes advantage of the MaxPeak HPS hardware, but it has also been rated with an ACT Environmental Impact Factor label from MyGreenLab, to help scientists make informed choices about the environmental impact of the column.

Benefits

- Fast method development using the systematic screening protocol

- Baseline resolution of seven structurally similar Janus Kinase Inhibitors
- Use of hybrid particle technology to rapidly separate compounds

Introduction

A new analytical method can be challenging to create, especially when the compounds of interest have similar chemical structures and properties. The additional complexity of method development for these types of mixtures can be mitigated by implementing a structured, well defined, method development protocol. These protocols can be found in a variety of different laboratories, especially those that do method development regularly, as they have found that certain steps are critical to finding a successful method while others are less impactful. An example of a structured method development approach is the Systematic Screening Protocol (SSP). This approach uses a tiered screening approach, driven by experimental data, either interpreted manually or by the chromatographic data software.[1–4] This approach relies heavily on two main steps of method development which is pH scouting and column/solvent screening. The SSP has been shown to provide appropriate separation conditions for a variety of compounds and can be further improved by using columns with MaxPeak High Performance Surfaces (HPS) Technology.

MaxPeak Premier Columns employ a unique column hardware which mitigates secondary interactions between the analyte and metal. This technology can improve recovery and reproducibility for a variety of compounds, particularly for compounds containing acidic moieties like phosphates or sulfates.[5–7] This technology has also proven to help with kinase inhibitors in terms of both improved recovery, as well eliminating the need for specialized mobile phases during analysis.[8–11]

Janus Kinase (JAK) is a family of intracellular non-receptor tyrosine kinases that transduce cytokine-mediated signals. There are four different families of JAK called JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). JAK Inhibitors (JKI) work by interfering with the phosphorylation of signal transducers and activators, thereby preventing them from being transcribed. As of August 2024, there are ten JKI on the market approved by the FDA.[12] The first was approved in 2011, with the most recent being approved in September of 2023. Several peer review papers have been written examining the synthetic pathways of these compounds, along with their use in treating various diseases and conditions.[13–15] JKIs all contain similar chemical structures which act on the proteins in similar ways. Due to these structural similarities, the chemical properties of the compounds are also similar, making chromatographic separation difficult.

In this application note, seven different JKIs were combined. and a single analytical method was developed using MaxPeak Premier Columns and the SSP. The final method conditions were created in under just two days, highlighting the efficiency of the SSP in developing new methods for structurally similar compounds.

Experimental

Sample Description

1 mg/mL stock solutions of each Janus Kinase Inhibitor (JKI) were created. From those solutions a mixture containing 0.1 mg/mL of each JKI was created with a final sample composition of 20.5% acetonitrile in water.

LC Conditions

LC system:	ACQUITY UPLC™ H-Class Plus System with Quaternary Solvent Manager (QSM) with optional solvent select valve, Sample Manager Flow Through Needle (SM-FTN), Column Manager, Two Column Manager Auxs, and QDa mass detector
Detection:	MS full scan (ESI+)
Columns:	ACQUITY Premier BEH C_{18}, 2.1 x 50 mm, 1.7 µm (p/n: 186009452) ACQUITY Premier CSH™ Phenyl-Hexyl, 2.1 x 50 mm, 1.7 µm (p/n: 186009474) ACQUITY Premier BEH Shield RP_{18}, 2.1 x 50 mm, 1.7 µm (p/n: 186009497) Atlantis™ Premier BEH C_{18} AX, 2.1 x 50 mm, 1.7 µm (p/n: 186009366)

Column temperature:	30 °C
Sample temperature:	10 °C
Injection volume:	1.0 µL
Flow rate:	0.50 mL/min
Mobile phase A:	Milli-Q Water
Mobile phase B:	Acetonitrile
Mobile phase C:	Methanol
Mobile phase D1:	2% Formic acid in water
Mobile phase D6:	200 mM Ammonium hydroxide in water
Screening gradient conditions:	Constant 5% Dx maintained throughout gradient. Linear gradient of 5–95% B/C in 6.86 minutes, hold for 1.14

Data Management

Chromatography software:	Empower™ 3 Feature Release 4

Results and Discussion

Method development of Janus Kinase Inhibitors (JKI) was performed using the Systematic Screening Protocol (SSP) along with MaxPeak Premier Columns on an ACQUITY H-Class Plus System with both PDA and QDa™ mass detection. As outlined in the literature, the SSP has four well-defined steps to streamline method

development. The first step is determining the sample and separation criteria. While most analysts will want to find the absolute best separation possible, sometimes knowing when to stop during method development is a valuable skill. Knowing when a method has been developed that meets all the desired needs means that less time is wasted optimizing an already acceptable separation. For the analysis of JKIs, only two criteria will be needed. First, achieving baseline separation as measured by USP resolution value of >1.5. Second, adequate retention and elution of the compounds within the gradient. This means that no extra hold at high organic would be needed, while also ensuring that the compounds are retained outside of the void where unretained analytes can confound quantitation and integration of the peaks. With separation criteria defined, the sample can be determined. Figure 1 shows chemical structures for the seven JKIs analyzed in this application.

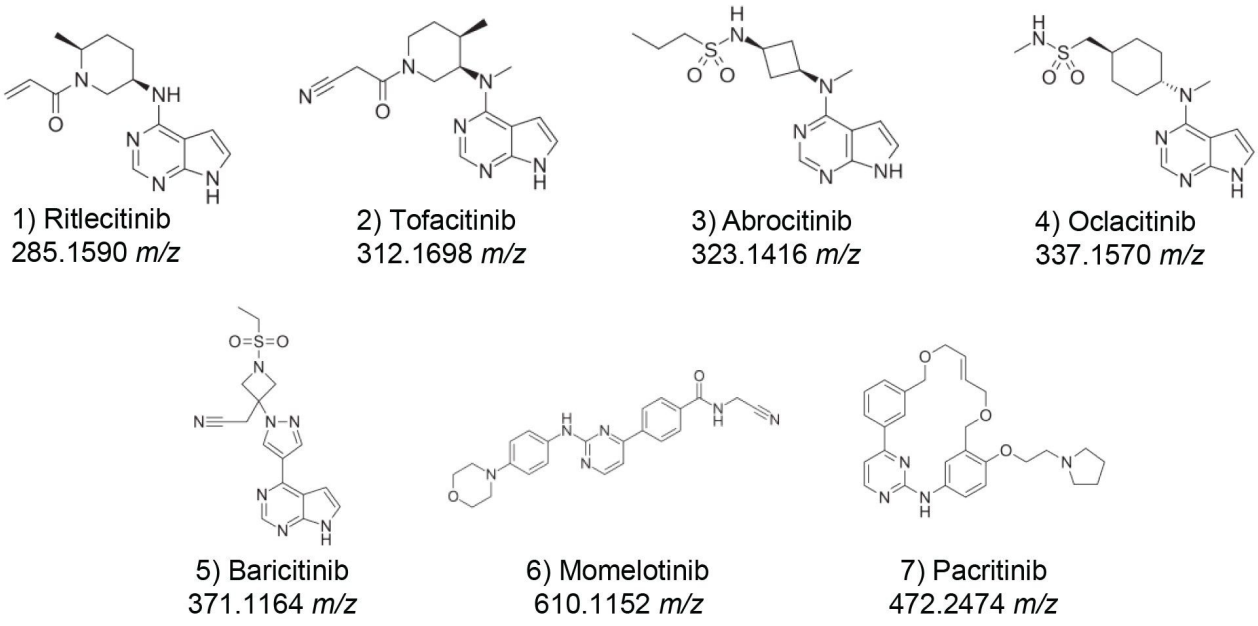

Figure 1. Chemical structures, including exact masses for the seven JKIs analyzed in this application note.

The next step is pH scouting, which requires the use of both high and low pH mobile additives, as well as a stationary phase that is stable at those conditions. For this reason, the ACQUITY Premier BEH C_{18} Column was selected for pH scouting. This column contains the fully porous bridged-ethyl hybrid (BEH) particle, which is well known for being stable at elevated pHs. The inclusion of the ethyl groups in the silica particle boosts the stability of the column, not only from a chemical standpoint, but also from a mechanical standpoint. During pH scouting, an analyst is checking for the conditions which produce the highest retention for the compounds. This step can

theoretically be predicted by examining the chemical properties of the compounds and determining their charge state at both high and low pH, however, running the actual experiments often provides additional insights, and is needed if the compounds of interest are unknown. Figure 2 shows the results of pH scouting using the ACQUITY Premier BEH C_{18} Column at both high and low pHs using ammonium hydroxide and formic acid additives, respectively.

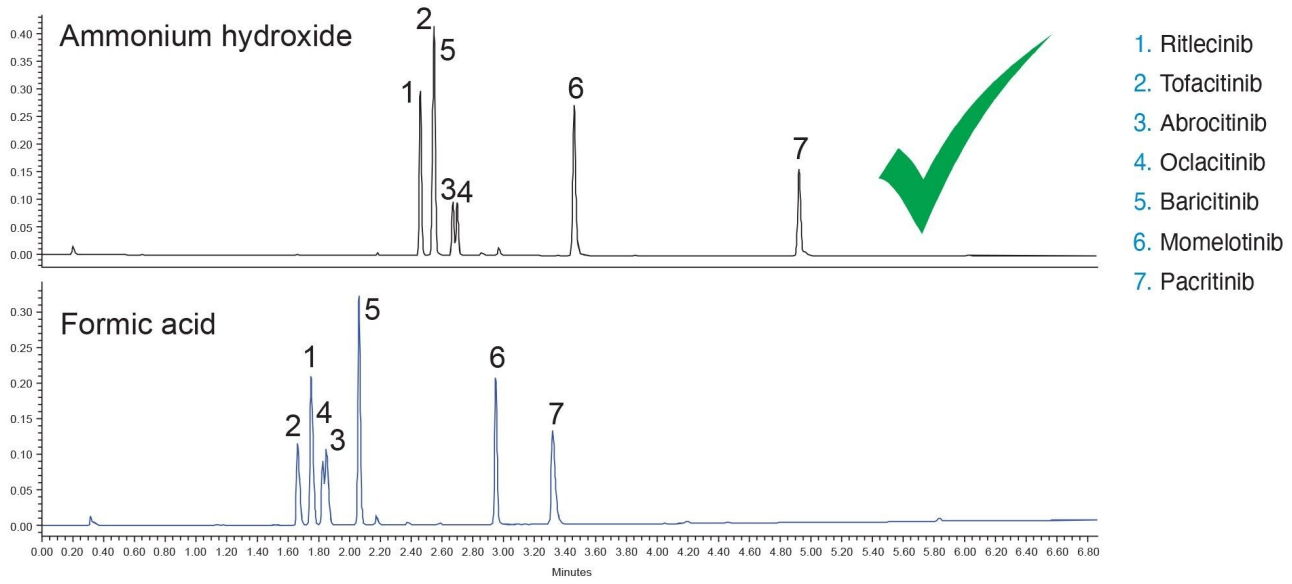

Figure 2. Chromatograms of pH scouting using the ACQUITY Premier BEH C_{18} Column. 1) ritlecitinib, 2) tofacitinib, 3) abrocitinib, 4) oclacitinib, 5) baricitinib, 6) momelotinib, 7) pacritinib.

For the JKIs tested, the use of ammonium hydroxide modified mobile phases produced higher retention. This was the expected outcome given that the JKIs all contain basic moieties which would be charged at low pH, effectively making them more polar and therefore less retentive in a reversed-phase separation. Additionally, comparing the two additives shows some selectivity differences as well. Tofacitinib elutes first at low pH, while ritlecitinib is the earliest eluter at high pH. Additionally, at high pH, baricitinib elutes before both abrocitinib and oclacitinib, but not at low pH. This change in selectivity may not have been realized if this step was skipped. For this reason, even though retention can be predicted using different modeling software, it is always prudent to run the experiments as additional information can often be seen. Given that high pH modified mobile phases yielded the highest retention for the analytes, it will be used going forward in the next step, which is column and solvent screening.

Solvent screening is an important thing to consider and include in any method development protocol. Acetonitrile and methanol behave differently in reversed-phase separations depending on the stationary phase being used. For a C_{18} column, for instance, the two solvents behave similarly with methanol being a weaker elution solvent thereby producing higher retention for the analytes. However, when alternative stationary phases like phenyl-hexyl bonded phases are used, the use of methanol can promote secondary interactions that acetonitrile actually mitigates. For instance, pi-pi bonding interactions are possible when methanol is being used as the strong solvent, as methanol does not contain any pi electrons. Acetonitrile, which does have pi electrons, reduce this secondary interaction. Because different selectivity can be obtained with the two solvents, it is important to screen both during method development.

Column selection is also an important aspect of method development. For this example, high pH additive mobile phases are needed, so the use of silica based stationary phases is not possible. At high pH, these stationary phases are subject to silica dissolution causing premature column degradation and performance loss. Four stationary phases were selected for this application, all based on either BEH or CSH, charged surface hybrid, technology.

The first column selected is the ACQUITY Premier BEH C_{18} Column. This column is used in pH scouting and provides a good starting point for developing methods. Additionally, this column has been widely accepted as a "go-to" column for new methods. The next column selected was the ACQUITY Premier CSH Phenyl-Hexyl Column. The CSH particle is designed to improve peak shape for basic analytes at low pH, which is not helpful in this case; however, the particle is high pH stable and structurally different than the BEH particle. Additionally, the use of the Phenyl-Hexyl (PH) ligand should provide some interesting selectivity differences, especially when comparing acetonitrile and methanol. Next, the ACQUITY Premier BEH Shield RP_{18} Column was selected. The BEH Shield RP_{18} stationary phase contains an embedded polar group in the ligand. This draws additional water towards the surface of the column, providing slightly different selectivity when compared to a traditional C_{18} ligand. Lastly, the Atlantis Premier BEH C_{18} AX Column was selected. This column contains a mid-coverage C_{18} ligand along with an anion exchange functional group. At elevated pHs, the ionic exchange functionality of the column is not charged, so retention will be driven purely by reversed-phase mechanisms. Figure 3 shows the separation of JKIs on the four columns selected using acetonitrile mobile phases.

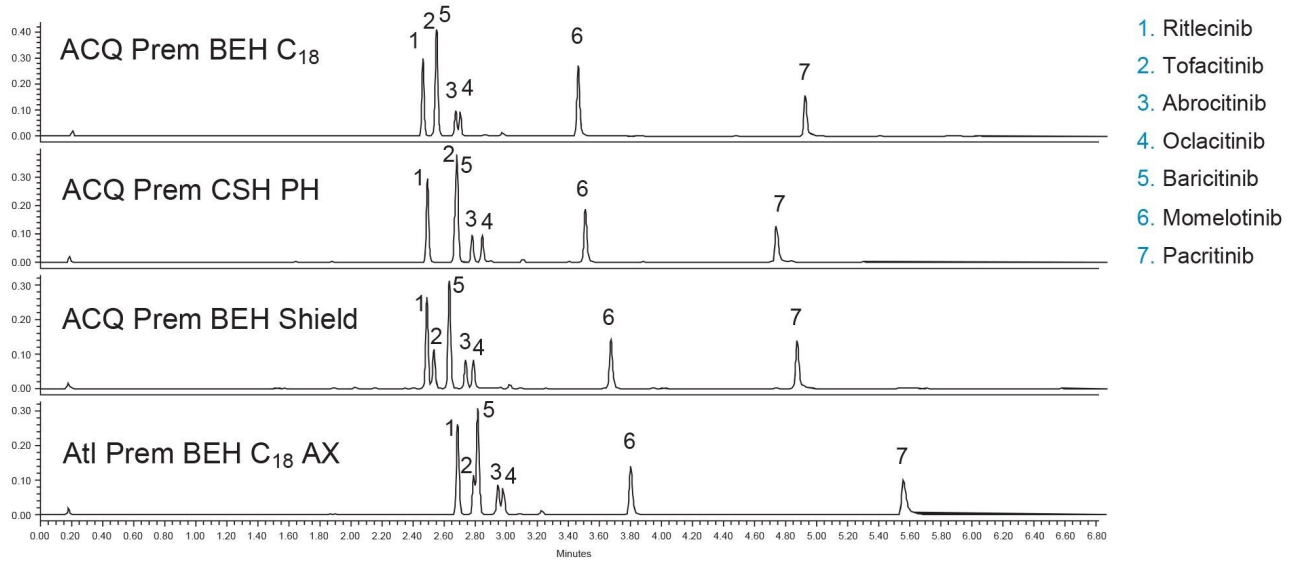

Figure 3. Separation of seven JKIs using four columns and acetonitrile mobile phases. 1) ritlecitinib, 2) tofacitinib, 3) abrocitinib, 4) oclacitinib, 5) baricitinib, 6) momelotinib, 7) pacritinib. ACQUITY Premier abbreviated to ACQ Prem, and Atlantis Premier abbreviated to Atl Prem to save space in the figure.

Using acetonitrile as the strong solvent, some selectivity differences are seen across the columns. Notably the retention of compound 2, tofacitinib, moves across the selected stationary phases. Using the ACQUITY Premier BEH C_{18} and ACQUITY Premier CSH Phenyl Columns, tofacitinib co-elutes completely with baricitinib. Meanwhile partial separation of the compounds is seen using the Atlantis Premier BEH C_{18} AX Column. The ACQUITY Premier BEH Shield Column provides the best overall separation using acetonitrile mobile phases. However, with a USP resolution of 1.3 for tofacitinib, our separation criteria have not been fully met. These conditions could in theory be optimized further to improve the resolution, however, a better separation was realized using methanol mobile phases, Figure 4.

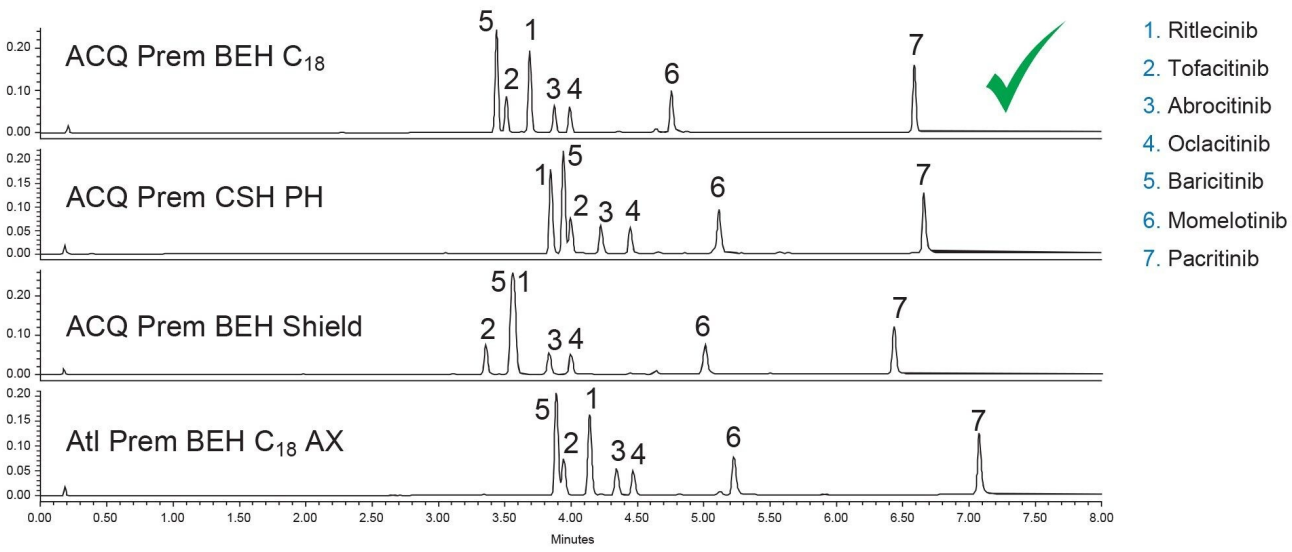

Figure 4. Separation of seven JKIs using four columns and methanol mobile phases. 1) ritlecitinib, 2) tofacitinib, 3) abrocitinib, 4) oclacitinib, 5) baricitinib, 6) momelotinib, 7) pacritinib. ACQUITY Premier abbreviated to ACQ Prem, and Atlantis Premier abbreviated to Atl Prem to save space in the figure.

The use of methanol mobile phases provides alternative selectivity for all columns tested, not just the PH Column. With acetonitrile, ritlecitinib was the first compound eluting, however, with methanol that is only true when the ACQUITY Premier CSH PH Column is used. The other three columns see either baricitnib or tofacitinib as the first eluter. Of the four columns tested with methanol, the ACQUITY Premier BEH C_{18} columns meet all testing criteria with a USP resolution for tofacitinib of 1.6. By using methanol mobile phases, a suitable separation was found without the need to optimize the conditions. This not only cuts down on development time, but also ensures that the entire method development process is traceable and the reasoning behind each step is well documented.

The use of the SSP along with MaxPeak Premier Columns streamlined the method development process for the analysis of seven JKIs. The final method conditions could now be used in various scenarios ranging from bioanalytical monitoring, DMPK studies, or even batch testing for product release.

Conclusion

Fast method development of structurally similar compounds can be challenging, especially for novice analysts. However, by implementing a structured method development protocol, like the Systematic Screening Protocol (SSP), methods can be developed quickly and with minimal need for decision making, which often relies on the experience of the analyst. Coupling the SSP with MaxPeak Premier High-Performance Surfaces (HPS) Technology ensures that not only will method development happen rapidly, but that the data collected is of the highest quality, minimizing possible rework.

To demonstrate how the SSP and MaxPeak Premier Columns can be used in method development, a mixture of seven Janus Kinase Inhibitors (JKI) was created and separated. The final method conditions were realized after just 10 injections and did not require optimization. From here, the method can be applied to sample analysis or optimized further to reduce run time.

References

1. Maziarz M, McCarthy S, Wrona M. Improving Effectiveness in Method Development by Using a Systematic Screening Protocol. Waters Application Note. 720005026 < https://www.waters.com/content/dam/waters/en/app-notes/2014/720005026/720005026-en.pdf> . April 2014.

2. 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 < https://www.waters.com/content/dam/waters/en/app-notes/2021/720007352/720007352-en.pdf> . August 2021.

3. Berthelette KD, Turner JE, Walter TH, Haynes K. Using a Systematic Screening Protocol and MaxPeak HPS Technology to Develop a UHPLC Method for the Analysis of Deferoxamine and its Forced Degradation Products. Waters Application note. 720007834. December 2022.

4. Hong P, McConville P. A Complete Solution to Perform a Systematic Screening Protocol for LC Method Development. Wates White Paper. 720005268 < https://www.waters.com/webassets/cms/library/docs/720005268en.pdf> . December 2022.

5. Clements B, Rainville P. Improved Chromatographic Analysis of Non-Steroidal Anti-inflammatory Drugs

(NSAIDs) Using CORTECS Premier Columns that Feature MaxPeak High Performance Surfaces HPS Technology. Waters Application note. 720007751. January 2023.

6. 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.

7. Walter TH, Alden BA, Belanger J, Berthelette K, 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 Supplements (2022) 28–34.

8. Chen P, Alden B, Lauber M. Modernized Impurity Analysis of the Kinase Inhibitor Imatinib by High-Resolution LC with MS-Compatible Mobile Phases. Waters Application note. 720007340. August 2021.

9. Clements B, Maziarz M, Rainville P. High-Speed Sensitive Analysis of Sunitinib and Related Metabolites Through UPLC-MS/MS Featuring CORTECS Premier C18 Columns with MaxPeak High Performance Surfaces (HPS) Technology. Waters Application note. 720007845. January 2023.

10. Layton C, Rainville P. Advantages of Using MaxPeak HPS Technology for the Analysis of Targeted Cancer Growth Inhibitor Therapies. Waters Application note. 720007565. March 2021.

11. Berthelette K, DeLoffi M, Kalwood J, Haynes K. Forced Degradation Study of Janus Kinase Inhibitor, Baricitinib, Using MaxPeak Premier HPLC Columns on an Alliance HPLC System. Waters Application note. 720008113. October 2023.

12. Anderson, Leigh Ann. Which JAK inhibitors are approved in the U.S.? https://www.drugs.com/medical-answers/jak-inhibitors-approved-3575793/#:~:text=Official%20answer&text=There%20are%20currently%2010%20Janus,Jakafi%20(ruxolitinib)%20on%20No . Accessed 16-Aug-2024.

13. Damsky W et al. The emerging role of Janus kinase inhibitors in the treatment of autoimmune and inflammatory diseases. *Journal of Allergy and Clinical Immunology*. (2021) 814–826.

14. Hosseini A, Gharibi T, Marofi F, Javadian M, Babaloo Z, Baradaran B. Janus kinase inhibitors: A therapeutic strategy for cancer and autoimmune diseases. *Journal of Cellular Physiology*. (2020) 5903–5924.

15. Shu L, Chen C, Huan X, Huang H, Wang M, Zhang J, Yan Y, Liu J, Zhang T, Zhang D. Design, synthesis, and

pharmacological evaluation of 4- or 6-phenyl-pyrimidine derivatives as novel and selective Janus kinase 3 inhibitors. *European Journal of Medicinal Chemistry*. (2020).

Featured Products

ACQUITY UPLC H-Class PLUS System <https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-uplc-h-class-plus-system.html>

ACQUITY QDa II Mass Detector <https://www.waters.com/nextgen/global/products/mass-spectrometry/mass-spectrometry-systems/acquity-qda-ii-mass-detector.html>

Empower Chromatography Data System <https://www.waters.com/nextgen/global/products/informatics-and-software/chromatography-software/empower-software-solutions/empower-cds.html>

720008528, September 2024