Application Note

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Analysis of PROTAC Molecule Bavdegalutamide (ARV-110) Using LC/UV and LC/MS

Suitable for Agilent
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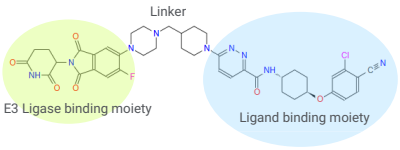

Bavdegalutamide (ARV-110)

Agilent Revident LC/Q-TOF

Author

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Abstract

PROteolysis TArgeting Chimeras (PROTAC) are bifunctional, complex molecules with unique treatment modality, necessitating advanced analytical techniques to characterize their intricate structures, identify synthesis-related impurities, and monitor their stability. As a rapidly emerging therapeutic modality, PROTACs enable targeted degradation of disease-relevant proteins, offering new opportunities to address traditionally "undruggable" targets and overcome limitations of occupancy-based small-molecule inhibitors. Their growing pipeline has intensified the need for robust analytical workflows that support development, quality, and regulatory readiness. This application note details a forced degradation study and impurity profiling of bavdegalutamide (ARV-110), a PROTAC molecule. Structural confirmation of ARV-110, along with the detection of impurity and degradation products, was accomplished using LC/UV and LC/MS techniques.

Introduction

PROteolysis TArgeting Chimera (PROTAC) technology has become a promising method for eliminating specific proteins linked to illness leveraging the cell's endogenous protein degradation pathways. Several PROTAC molecules are in clinical trials for cancer treatment.[1] This rapid clinical adoption has heightened interest in robust analytical and characterization strategies capable of supporting discovery, development, and regulatory submission of these structurally complex molecules. These bifunctional molecules identify target proteins to the cell degradation machinery through the ubiquitin–proteasome degradation pathway. Bavdegalutamide (ARV-110), a PROTAC in clinical trials for prostate cancer, targets the androgen receptor (AR) for degradation.[2] It consists of a cyclohexyl moiety that binds to the AR and an E3 ligase recognition moiety to enable polyubiquitination.

The complex, multi-step synthesis of ARV-110 involves multiple components that can generate various impurities. These impurities and intermediates may interact with unintended proteins, potentially leading to adverse side effects. Therefore, it is essential to conduct thorough impurity profiling studies during development and manufacturing to ensure product quality. Impurities can arise from production processes, degradation, storage, or delivery, and they pose significant risks to efficacy, safety, and immunogenicity.[3] Identifying these impurities is critical to ensure that the therapeutic performs as intended without causing adverse effects. In this context, forced degradation tests are conducted to understand the chemical and physical stability of the molecule by producing degradation-related impurities. From a regulatory perspective, forced degradation studies provide for the identification of possible degradants or impurities.[4] As a result, it is essential to have analytical techniques that can detect and identify the degradation products.

This application note describes a forced degradation study of ARV-110 under various stress conditions. Multiple LC columns were evaluated to achieve high-resolution separation of ARV-110 from its impurities. Advanced LC/UV and high-resolution LC/MS workflows were applied to enable confident detection, separation, and characterization of both synthesis-related and degradation-induced impurities. The major impurity was characterized using high-resolution quadrupole time-of-flight (Q-TOF) mass spectrometry, with structural elucidation aided by molecular structure correlator (MSC) software.

Experimental

Reagents and chemicals

Bavdegalutamide was obtained from MedChemExpress (Monmouth Junction, NJ) and stored according to the manufacturer's instructions. DMSO, hydrogen peroxide (H_2O_2), sodium hydroxide, and hydrochloric acid were from Sigma (St. Louis, MO). LC/MS grade acetonitrile (ACN) was from Fisher (Waltham, MA). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Billerica, MA).

Analytical equipment

The LC consisted of an Agilent 1290 Infinity II LC system including

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117B)
- Agilent Revident LC/Q-TOF (G6575A)

Software and data processing

- Agilent OpenLab CDS Version 2.8
- Agilent MassHunter data acquisition software 12.1
- Agilent MassHunter Qualitative Analysis software 12.0
- Agilent Molecular structure correlator software 8.3

Sample preparation

A stock solution of ARV-110 was prepared at 5.0 mg/mL in DMSO. For LC/UV and LC/MS analysis, samples were diluted to 1 mg/ml and 0.5 mg/mL in 50% can, respectively. For forced degradation studies, samples were subjected to the following conditions:

- Oxidative stress: incubation with H_2O_2 (10%) at room temperature for 3 hours.
- Acidic and basic hydrolysis: incubation with 1 N HCl at 80 °C for 1 hour and 1 N NaOH at room temperature for 1 hour.
- Thermal stress: dry heat at 70 °C for 3 hours.

LC/MS analysis

The LC and MS parameters are provided in Tables 1 and 2.

Results and discussion

Analyzing impurities in pharmaceuticals is a vital and increasingly complex task. New, unknown impurities with various structural modifications/chemical nature can form during the manufacturing process or storage conditions, making it essential to select the right column to separate these impurities from the active pharmaceutical ingredient effectively. Among the numerous parameters influencing chromatographic performance, the selectivity of the stationary phase is one of the most important factors to consider. For PROTAC molecules in particular, chromatographic selectivity plays a critical role in resolving closely related species arising from linker chemistry, stereochemistry, or partial degradation. Initially, various column chemistries were evaluated to assess their chromatographic performance for ARV-110.

Agilent InfinityLab Poroshell 120 superficially porous columns for reversed-phase liquid chromatography (LC) separations offer exceptional efficiency and reliability. The Poroshell 120 PFP (pentafluorophenyl) stationary phase can give extra retention and selectivity for positional isomers. It can separate analytes based on small differences in structure, substitution, and steric access to polar moieties. InfinityLab Poroshell 120 EC-C18 is best suited for separating a wide range of compounds, including acids, neutrals, and bases. InfinityLab Poroshell 120 Aq-C18 enhances the retention of polar molecules and can withstand 100% aqueous conditions without phase collapse. It is ideal for method development of polar and non-polar compounds together.

Table 1. Liquid chromatography parameters.

Parameter	Agilent 1290 Infinity II LC System
Columns	– AdvanceBio Peptide Plus, 2.1 × 50 mm, 2.7 µm (p/n 699775-949) – Poroshell 120 PFP, 2.1 × 50 mm, 1.9 µm (p/n 699675-408) – Poroshell 120 EC-C18, 2.1 × 50 mm, 1.9 µm (p/n 699675-902) – Poroshell 120 Aq-C18 2.1 × 50 mm, 2.7 µm (p/n 699775-742) – Poroshell 120 Phenyl-Hexyl, 2.1 × 50 mm, 1.9 µm (p/n 699675-912)
Sample Thermostat	10 °C
Mobile Phase A	0.1% FA
Mobile Phase B	0.1% FA in ACN
Gradient	Time (min) %A %B 0.00 85 15 10.00 10 90 11.00 10 90 11.10 85 15 12.00 85 15
Stop Time	12 min
Column Temperature	50 °C
Injection Volume	0.5 µL (MS)
Flow Rate	0.4 mL/min
UV Detection	250 nm

Table 2. MS data acquisition parameters.

Parameter	Agilent Revident LC/Q-TOF (G6575A)
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	300 °C
Drying Gas Flow	11 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Nebulizer	30 psi
Capillary Voltage	3,500 V
Nozzle Voltage	1,000 V
Fragmentor Voltage	175 V
Skimmer Voltage	45 V
Oct RF Vpp	750 V
Reference Mass	922.009798
MS Range	100–1,700 *m/z*
MS/MS Range	50–1,700 *m/z*
MS Acquisition Rate	5 spectra/sec
MS/MS Acquisition Rate	3 spectra/sec
Isolation Width	Narrow (~ 1.3 *m/z*)
Collision Energy	3.6*(*m/z*)/100–4.8
Maximum Precursors per Cycle	5
Precursor Threshold	3,000 count
Active Exclusion	Enabled
Isotope Model	Common organic molecules

Poroshell Phenyl-Hexyl phase provides alternative selectivity to C18 phases, particularly for analytes containing aromatic groups. AdvanceBio Peptide Plus uses a unique charge hybrid/C18 bonded phase to provide improved peak shape.

Figure 1 shows the overlay of ARV-110 chromatograms obtained with five different columns. The comparison clearly identifies the AdvanceBio Peptide Plus column as the superior choice among the five columns tested for ARV-110, exhibiting the best performance across all key metrics. With a positively charged surface in the bonding chemistry, the AdvanceBio Peptide Plus column offers the greatest separating power, showing excellent peak shape and minimal band broadening.

The diversity of stationary phases evaluated reflects the need for flexible method development strategies capable of accommodating the structural heterogeneity typical of PROTAC-related impurities. Figure 2 presents an expanded view of the elution window for the different columns, illustrating the separation of impurity peaks from the main ARV-110 peak. The chromatograms reveal that the choice of the stationary phase significantly impacts the ability to resolve the complex impurity profile of ARV-110. The results demonstrate optimal selectivity with the AdvanceBio Peptide Plus column for the analysis of ARV-110 and its related substances, with maximum impurity detection and superior resolution.

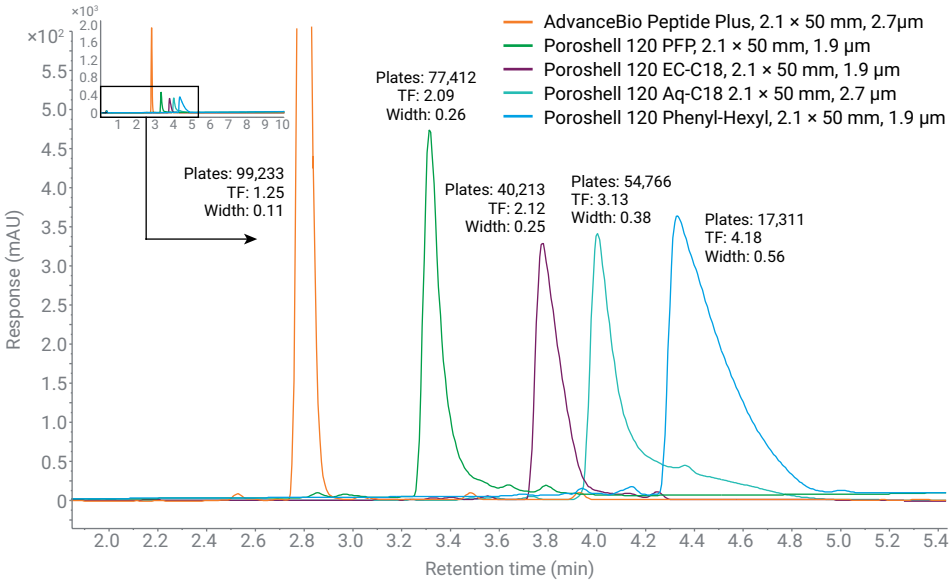


Figure 1. Comparison of LC/UV chromatograms of ARV-110 with various column chemistries.

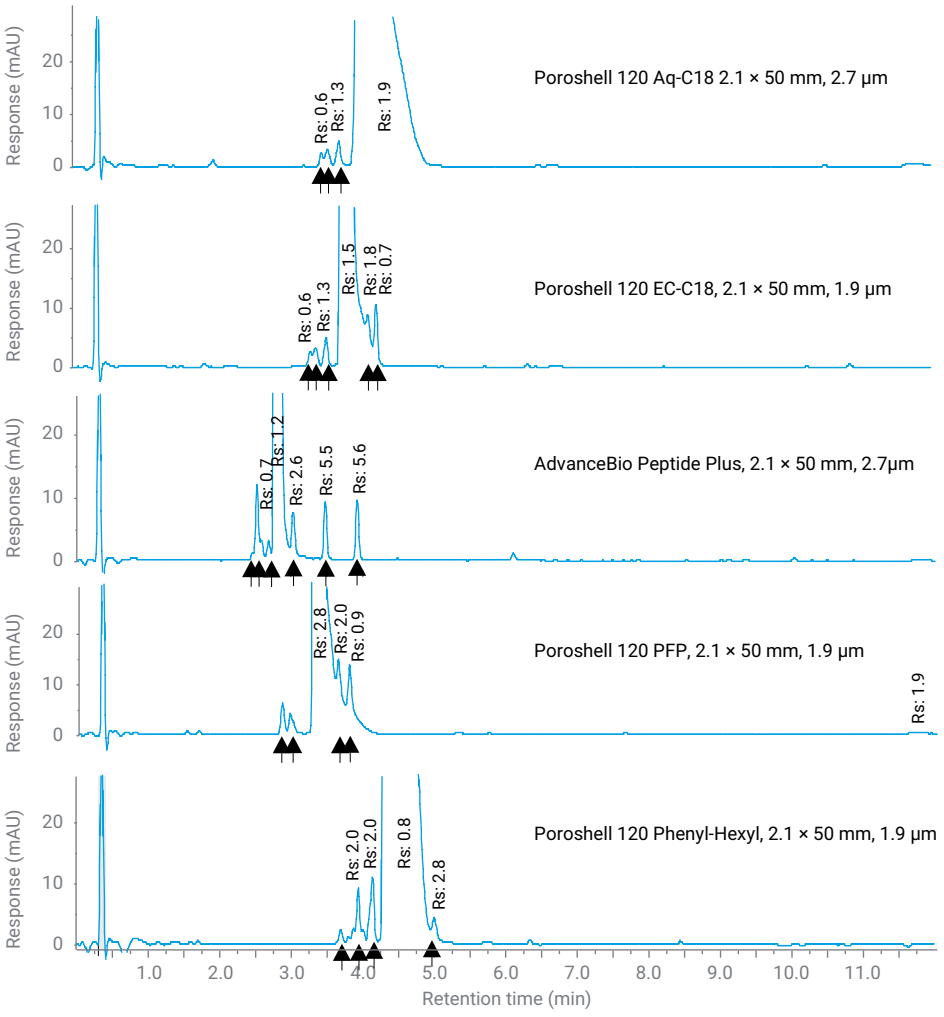


Figure 2. Zoom view of chromatograms shown in Figure 1.

To assess the forced chemical degradation products, the ARV-110 was subjected to H_2O_2, acid hydrolysis, base hydrolysis, and thermal stress conditions. Figure 3 compares the chromatographic profile of the untreated control sample against the forced degradation sample. The various stress conditions yielded a distinct set of breakdown products. Oxidative stress produced eight degradation peaks, likely resulting from modifications to susceptible functional groups like piperazine or cyclohexylamine moieties. Thermal stress did not result in any new breakdown products, confirming that ARV-110 is stable at high temperature. Acid hydrolysis showed fewer degradation products compared to the thermal or peroxide stress. Under base hydrolysis conditions, the complete absence of the main ARV-110 peak was observed, suggesting total degradation of ARV-110. This stress condition generated a unique profile featuring distinct, early-eluting peaks indicative of highly polar hydrolysis products.

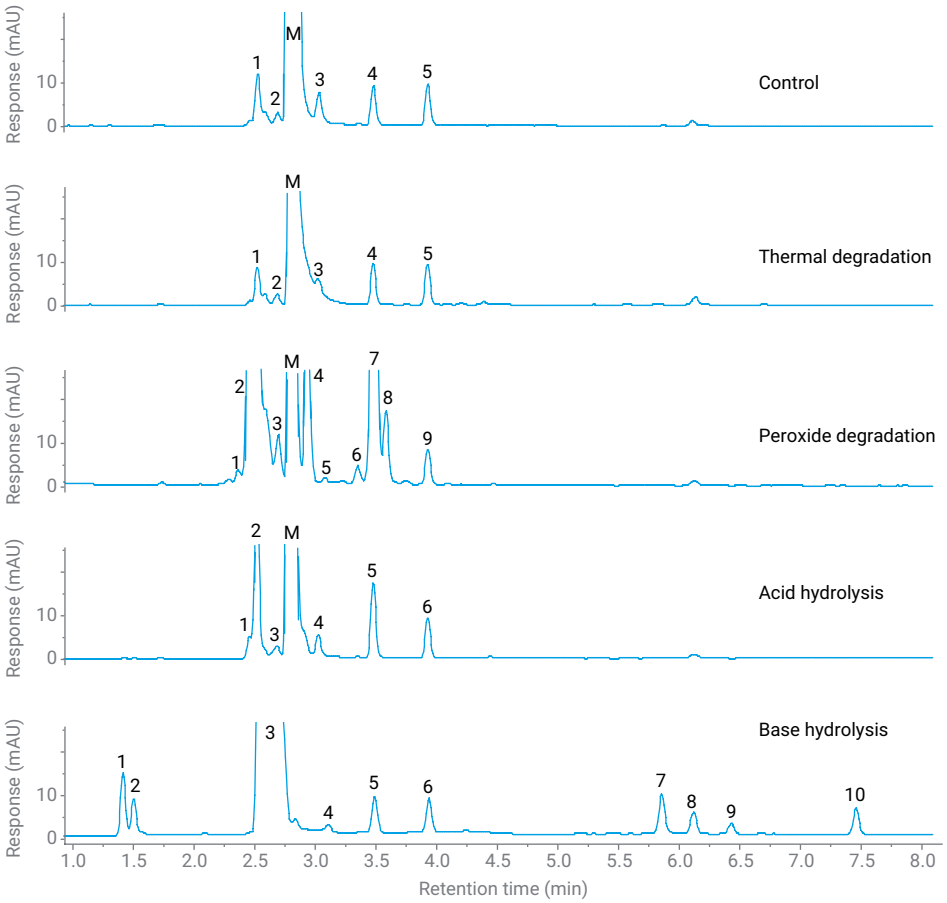


Figure 3. Stability study: LC/UV chromatograms of forced degradation samples. M: ARV-110 peak.

ARV-110 was further analyzed using LC/MS. The AdvanceBio Peptide Plus column provided excellent total-ion chromatogram (TIC) peak shape under formic acid conditions (Figure 4A), demonstrating efficient separation. The MS spectrum (Figure 4B) shows singly and doubly charged species, confirming its molecular weight and charge state. To elucidate the fragmentation pattern, ARV-110 (*m/z* 812.3084) was subjected to MS/MS; Figure 4C depicts the resulting fragment pattern with structure assignments to all major *m/z* signals. High-confidence structural confirmation is essential for PROTAC molecules, where accurate mass and fragmentation data support both identity verification and impurity interpretation. This fragmentation map is crucial for confirming the compound's identity. The mass accuracy was within ±1 ppm for all measured ions (Table 3), highlighting the high precision of the Q-TOF instrument.

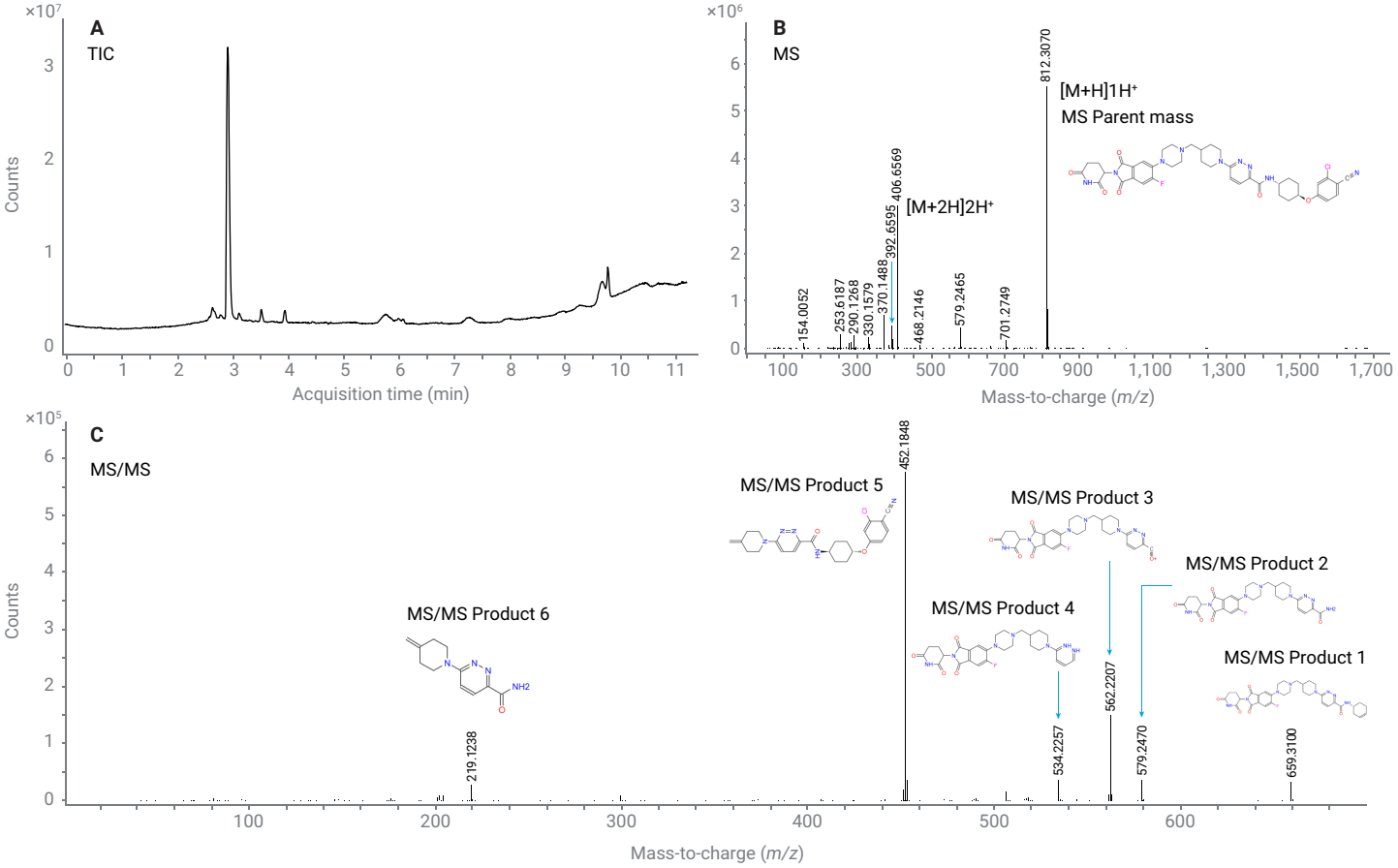


Figure 4. Mass spectrometry analysis of ARV-110.

Table 3. Mass accuracy for all measured ions.

Ion	Molecular Formula	Exact Mass	Experimental Mass	Mass Error (ppm)
MS Parent Mass	$C_{41}H_{44}ClFN_9O_6$	812.3082	812.3082	0
MS/MS Product 1	$C_{34}H_{39}FN_8O_5$	659.3100	659.3100	0
MS/MS Product 2	$C_{28}H_{31}FN_8O_5$	579.2474	579.2470	−0.69
MS/MS Product 3	$C_{28}H_{29}FN_7O_5$	562.2209	562.2207	−0.35
MS/MS Product 4	$C_{27}H_{29}FN_7O_4$	534.2260	534.2257	−0.56
MS/MS Product 5	$C_{24}H_{27}ClN_5O_2$	452.1848	452.1848	0
MS/MS Product 6	$C_{11}H_{14}N_4O$	219.1240	219.1238	−0.91

The method was also able to separate and detect the impurities present in the ARV-110 sample. Extracted ion chromatogram (EIC) of the major impurity peak is presented in Figure 5. The major impurity peak corresponds to the *m/z* 470.1947. To elucidate the structure of this major impurity, the MS/MS spectrum was acquired and processed using MSC software. Figure 6 displays the mass spectrum obtained by subjecting the *m/z* 470.1947 ion to MS/MS fragmentation.

Advanced data analysis tools are increasingly important for interpreting high-resolution MS/MS data generated during impurity profiling of complex therapeutics. This fragmentation pattern is vital for structural elucidation, providing a fingerprint that can be compared to known compounds within a database. To expedite this process, MSC software was employed. MSC correlates the accurate mass of MS/MS fragment ions of a compound of interest with one or more proposed molecular structures for that compound. MSC accomplishes this by trying to explain each observed fragment ion into the proposed structure using a "systematic bond-breaking" approach. As shown in Figure 6, the MSC software aided in the identification of the *m/z* 470.1947 impurity. The accurate masses of the precursor ion and fragment ions were used to calculate the molecular formula, which was then searched against the ChemSpider database to retrieve all possible structures. Multiple candidate structures were proposed by MSC software with their calculated correlation scores. The primary MSC proposed structure with the highest correlation score of 97.7% matches an impurity previously reported in the literature, confirming its identity.[5] The additional unknown impurity peaks can be evaluated using MSC software to suggest possible molecular formulas and structural features, effectively narrowing the list of candidate structures, although unambiguous identification of unknowns cannot be guaranteed. This approach of structural identification highlights the value of integrating intelligent data analysis tools with high-resolution MS/MS data.

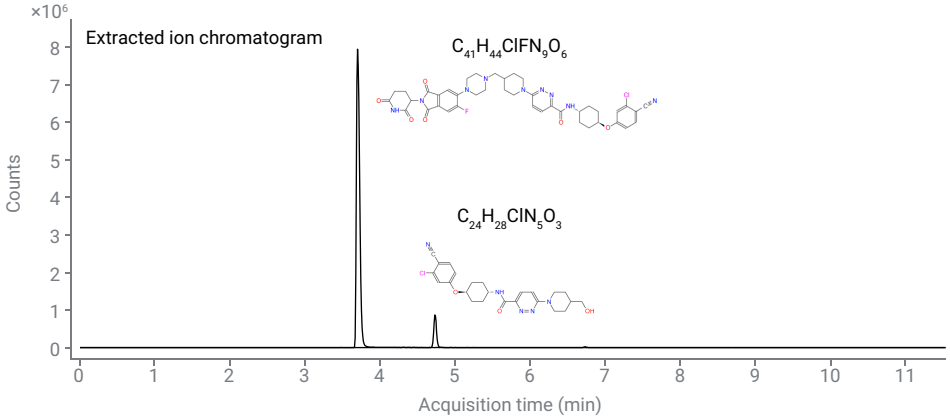
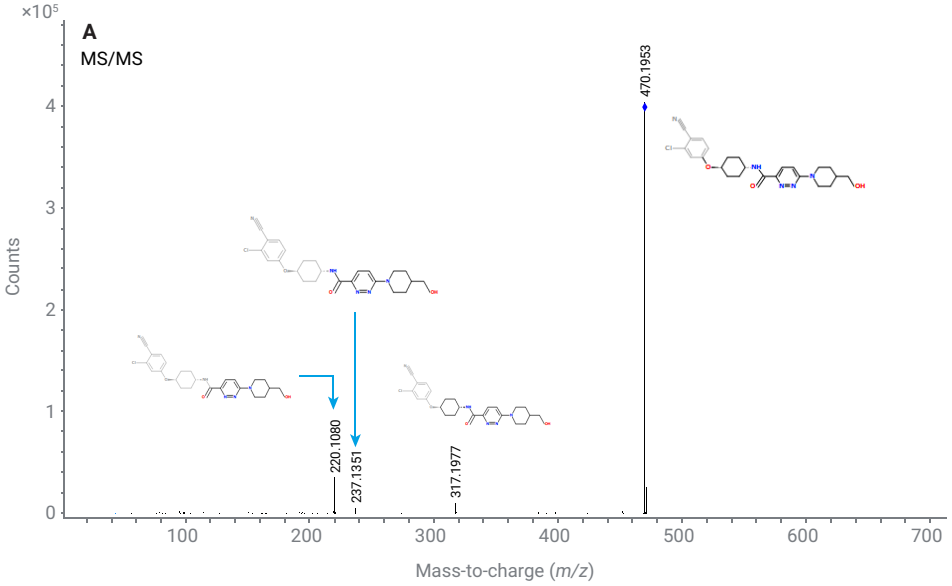


Figure 5. Extracted ion chromatograms of ARV-110 and the impurity peak.



B Molecular Structure Correlator (MSC) results

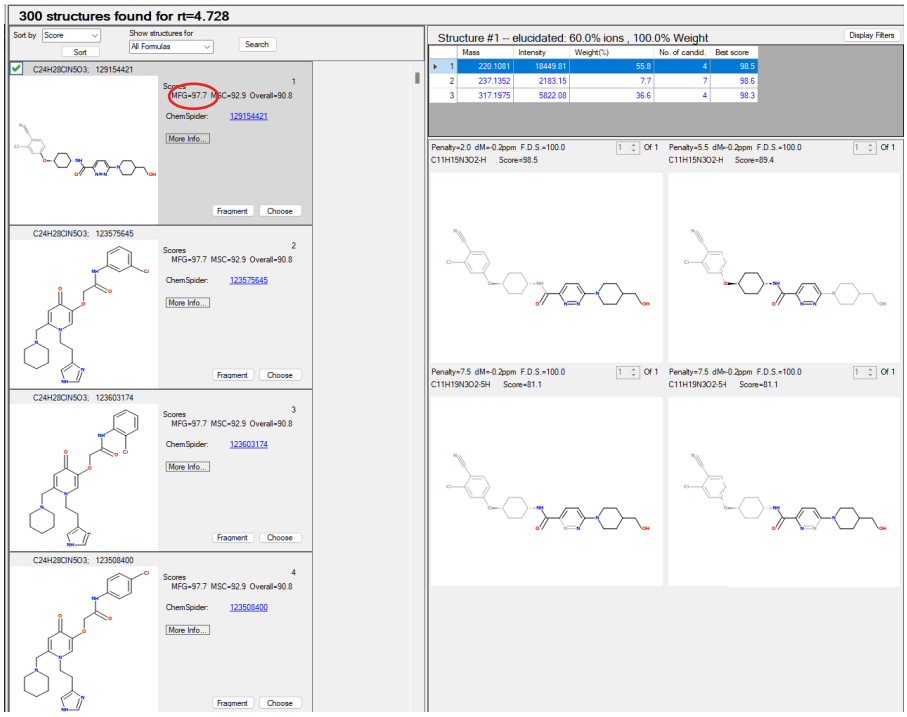


Figure 6. Structure elucidation of impurity *m/z* 470.1947 assisted by MSC software. MS/MS spectra and screenshot of MSC result.

Conclusion

The study examines the forced degradation of PROTAC compound ARV 110 using LC/UV and LC/MS. The LC/UV analysis evaluated the number of degradation peaks and resolution for various columns. The AdvanceBio Peptide Plus column was found to be the most effective, as it separated more degradation peaks with better resolution. These findings highlight the importance of stationary phase selection when addressing the structural complexity and impurity burden associated with PROTAC molecules. LC/MS analysis was used to identify the molecular formulas and exact masses of the parent ARV-110 compound and its fragmentation products. LC/MS/MS and MSC software analysis identified a major impurity peak by correlating accurate mass MS/MS fragment ions with proposed molecular structures. The combination of optimized chromatography and high-resolution MS enables detailed characterization of PROTAC molecules, supporting informed decision making during development and manufacturing, crucial for the development of new pharmaceutical compounds.

References

1. Anaya, Y. A.; *et al.* Proteolysis-Targeting Chimeras in Cancer Therapy: Targeted Protein Degradation for Next-Generation Treatment. *Cancer* **2025**, *131(21):e70132*, 1–16.
2. Snyder, L. B.; *et al.* Preclinical Evaluation of Bavdegalutamide (ARV-110), a Novel PROteolysis TArgeting Chimera Androgen Receptor Degrader. *Mol. Cancer Ther*. **2025**, *24(4)*, 511–522.
3. Moreau, K.; *et al.* Proteolysis-Targeting Chimeras in Drug Development: A Safety Perspective. *Br. J. Pharmacol*. **2020**, *177(8)*, 1709–1718.
4. ICH Expert Working Group. Quality of Biotechnological Products: Stability Testing of Biotechnological/ Biological Products Q5C. ICH Harmonized Tripartite Guideline (1995).
5. Methods of Manufacturing a Bifunctional Compound, Ultrapure Forms of the Bifunctional Compound, and Dosage Forms Comprising the Same. Patent number: US 12,043,612 B2, July 23 (2024).

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