IMPURITIES INVESTIGATION OF ARV-825 PROTEOLYSIS TARGETING CHIMERA (PROTAC) COMPOUND THROUGH FRACTION COLLECTION

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INTRODUCTION

Proteolysis targeting chimeras (PROTACs) are drug modalities that bind to a target protein and a ubiquitin ligase to induce degradation of a protein of interest[1]. These heterobifunctional molecules contain E3 ubiquitin ligase ligand connected via a chemical linker to a ligand that recognizes the target protein. This configuration enables PROTACs to form a stable ternary complex, leading to ubiquitination and degradation of the target protein. The PROTACs molecules offer novel approaches for the treatment of cancer and other diseases through selective degradation of target proteins.

The ARV-825 is a newly developed molecule using PROTAC technology that has shown effectiveness for the treatment of pancreatic cancer, melanoma, cholangiocarcinoma, thyroid carcinoma, and acute myeloid leukemia[2]. The ARV-825 induces degradation of bromodomain-containing protein 4 (BRD4), a protein marker in many cancer types.

Currently, little work has been reported in the literature related to the stability and impurity characterization of PROTACs drug molecules. In this work, the degradation products of ARV-825 compound formed through forced degradation with acid were successfully isolated using an analytical scale fraction manager.

METHODS

Forced degradation sample

The ARV-825 sample was dissolved in a 45:55 acetonitrile/water diluent at 1 mg/mL. Forced degradation sample was prepared by adding 100 µL of 0.5 M hydrochloric acid (HCl) to 1 mL of ARV-825 solution. Stressed sample solution was stored at room temperature for 2 hours and subsequently neutralized with 100 µL of 0.5 M of sodium (NaOH).

LC System	• Arc™ Premier System with Binary Solvent Manager (BSM-R) and Flow-through Needle (FTN-R) • 2998 PDA and ACQUITY™ QDa™ II Detectors • Isocratic Solvent Manager (ISM) • Waters Fraction Manager-Analytical (WFM-A)
Mobile Phase	Solvent A: 0.1% Formic acid in water Solvent B: 0.1% Formic acid in acetonitrile
Column	XSelect™ Premier CSH™ C_{18} Column, 4.6 mm ID x 100 mm, 2.5 µm (p/n: 186009873)
Flow Rate	1.0 mL/min
Injection Vol.	40.0 µL
Sample Temp.	10°C
Gradient	Time (min) / Flow (mL/min) / %A / %B / Curve 1 Initial 1.000 95.0 5.0 Initial 2 10.00 1.000 5.0 95.0 6 3 10.50 1.000 5.0 95.0 6 4 10.60 1.000 95.0 5.0 6 5 13.00 1.000 95.0 5.0 6
Wash solvents	Purge/sample wash: 60/40 water/acetontrile Seal wash: 90:10 water/acetonitrile
MS Detection	Ionization mode: Electrospray negative (ESI+) MS Acquisition: range: 200 – 1000 m/z Probe temperature: 600°C Capillary Voltage: 0.5 kV Cone Voltage: 15 V
Isocratic solvent manager (ISM)	Makeup solvent: 50:50 water/acetonitrile with 0.1% formic acid Flow rate: 0.4 mL/min, with 5:1 split and dilute ratio
Data Management	Chromatography data system: MassLynx™ Software, version 4.2 SCN1049 Application manager: FractionLynx™ Application Manager

Table 1. Method conditions.

RESULTS

The forced degradation study of ARV-825 sample was performed based on a previously published application note[3]. Degradation with 0.5 M HCl acid for two hours at room temperature produced several degradation products (Figure 1).

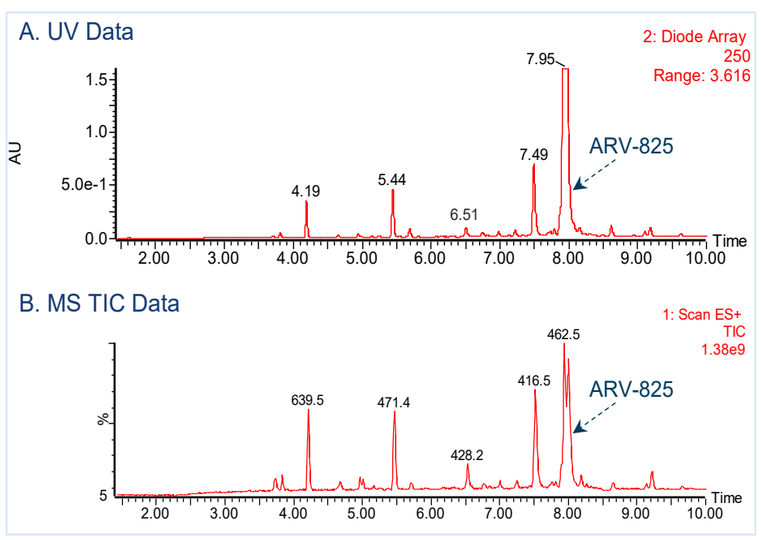

Figure 1. ARV-825 sample degraded with acid resulted in the formation of several degradation products. Analysis run on an Arc Premier System composed of WFM-A, PDA and ACQUITY QDa II Detectors using injection mode only, collection off. UV data at 250 nm (A) and MS TIC data with m/z (B).

Mass-directed and time-based fraction collection triggers were applied to isolate the degradation products of ARV-825 compound.

Mass-directed fraction collection

Fractions were collected based on the molecular mass. The Isocratic Solvent Manager (ISM) was used to split and dilute the flow entering the ACQUITY QDa II Detector. The ISM makeup (dilution) solvent was added post-column and mixed with the flow entering the source, allowing for the addition of a modifier to enhance ionization.

Various ISM makeup solvents were screened to enhance the MS signal for the degradation products. The makeup solvents included different compositions of acetonitrile and methanol in water, all containing 0.1% formic acid (Figure 2).

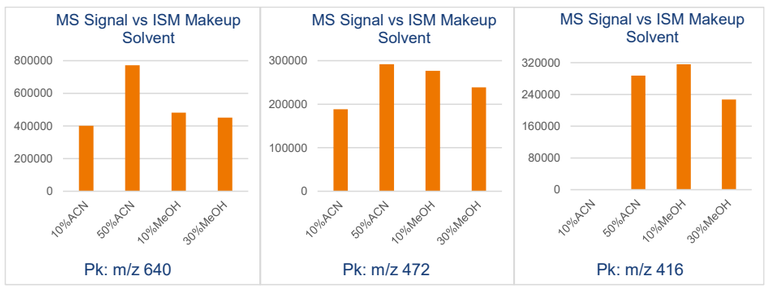

Figure 2. ISM makeup solvents screening to enhance MS signal for the degradation products. All solvents contained 0.1% formic acid. Injection mode only analysis, collection off. Solvent with 50:50 acetonitrile/water and 0.1% formic acid produced robust signal for the degradation products. MS TIC data.

A mass-directed fraction collection was applied to isolate and purify degradation products of ARV-825. Using the target masses (m/z 640, 472, 428 and 416) fractions were successfully collected and multiple fractions pooled into one vial, allowing for collection of larger volumes (Figure 3).

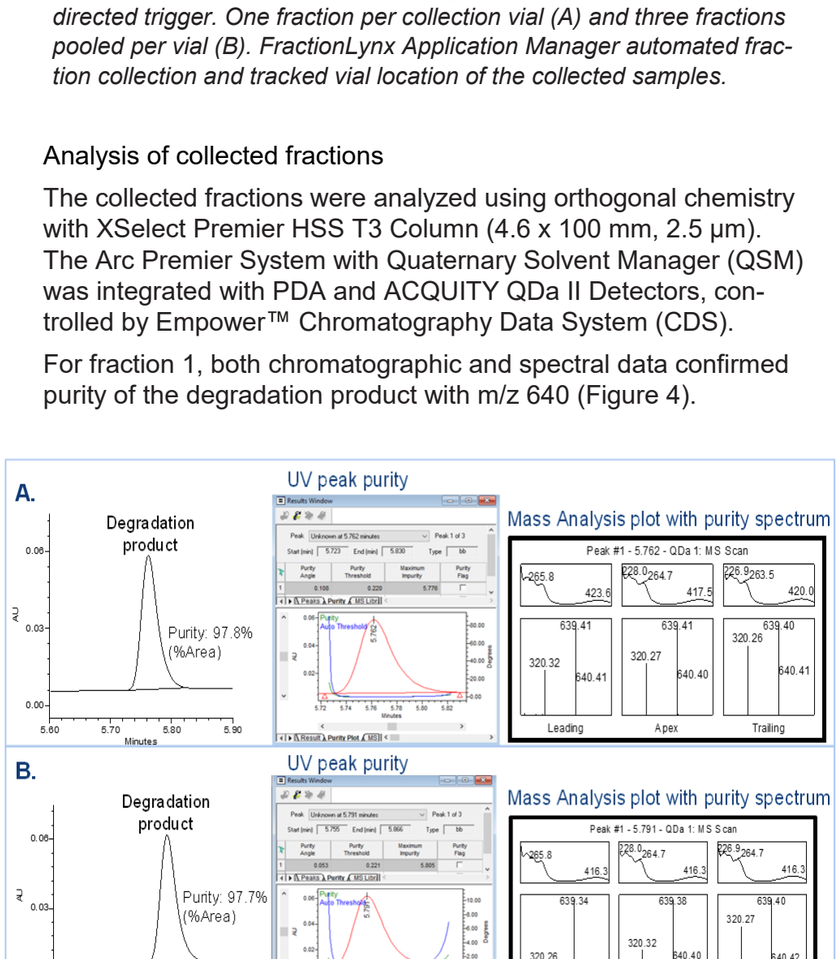

Figure 3. Fraction collection of the degradation products by mass-directed trigger. One fraction per collection vial (A) and three fractions pooled per vial (B). FractionLynx Application Manager automated fraction collection and tracked vial location of the collected samples.

Analysis of collected fractions

The collected fractions were analyzed using orthogonal chemistry with XSelect Premier HSS T3 Column (4.6 x 100 mm, 2.5 µm). The Arc Premier System with Quaternary Solvent Manager (QSM) was integrated with PDA and ACQUITY QDa II Detectors, controlled by Empower™ Chromatography Data System (CDS).

For fraction 1, both chromatographic and spectral data confirmed purity of the degradation product with m/z 640 (Figure 4).

Figure 4. Chromatographic and spectral purity verification of fraction 1 collected by mass-directed trigger with m/z 640. One fraction per collection vial (A) and three fractions pooled per vial (B). Analysis performed using orthogonal chemistry with XSelect Premier HSS T3 Column.

Fraction 2 was collected using a target mass of m/z 472. The orthogonal analysis revealed the presence of an additional peak with m/z 463 (Figure 5). Additionally, concentration of the additional peak increased over time, indicating degradation of the target compound in the collected fraction solution.

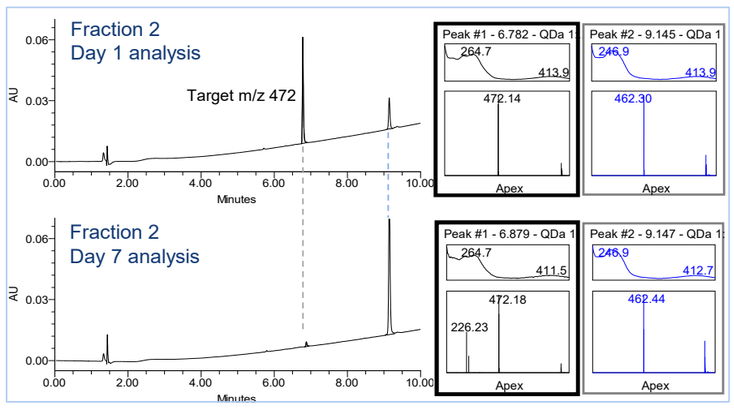

Figure 5. Analysis of fraction 2 collected by mass-directed trigger of m/z 472 run using orthogonal chemistry with XSelect Premier HSS T3 column. Fraction injected on day 1 (A) and day 7 (B) indicated degradation of the target compound over time.

Time-based fraction collection

The time-based collection mode was applied to fraction the chromatographic region near the ARV-825 peak (Figure 6). The collected fractions were re-injected on the WFM-A System with XSelect Premier CSH C_{18} column using a focused gradient to produce fast and efficient separation (Figure 7).

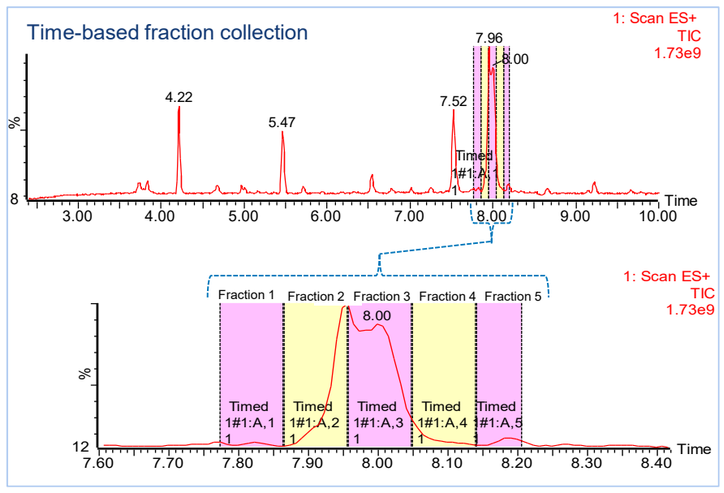

Figure 6. Time-based fraction collection near the ARV-825 PROTAC peak.

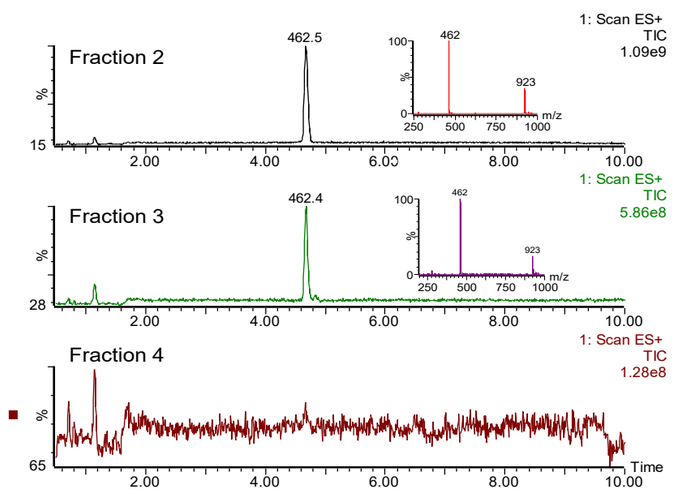

Figure 7. Analysis of time-based collected fraction on the WFM-A System using focused gradient, injection mode with collection off.

CONCLUSION

- Isolation of the degradation products of ARV-825 was successfully performed using the WFM-A integrated with an Arc Premier System.
- Mass-directed trigger enabled efficient fraction collection of the target degradation products using the ACQUITY QDa II Detector.
- The time-based fraction collection combined with the low peak dispersion design of the WFM-A enabled precise collection of closely eluting peaks.
- Automated fraction collection and different collection modes achieved using MassLynx Software with FractionLynx Application Manager.

References

1. Rahman M, Marzullo B, Holman SW, Barrow M, Ray AD. Advancing PROTAC Characterization: Structural Insights through Adducts and Multimodal Tandem-MS Strategies. Journal of the American Society for Mass Spectrometry (2024) 35:285-299
2. Liao X, Qian X, Zhang Z, Tao Y, Li Z, Zhang Q, Liang H, Li X, Xie Y, Zhuo R, Chen Y, Jiang Y, Cao H, Niu J, Xue C, Ni J, Pan J, Cui D. ARV-825 Demonstrates Antitumor Activity in Gastric Cancer via MYC-Targets and G2M-Checkpoint Signaling Pathways. Frontiers in Oncology, (2021) 11:753119.
3. Berthelette KD, Collins C, Haynes K. Method Development of Proteolysis Targeting Chimera (PROTAC) Compound ARV-825 Forced Degradation Sample Using the Systematic Screening Protocol. Waters Application Note, 720008328, 2024.

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