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Application Note

Degradation Products of ARV-825 Proteolysis Targeting Chimera (PROTAC) Compound: Isolation and Purification Using Analytical Scale Fraction Collection

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Abstract

Proteolysis targeting chimeras (PROTACs) are new drug modalities applied for the treatment of various cancer-related diseases. Since their initial discovery, numerous PROTAC molecules were designed to target a wide range of diseased proteins. The ARV-825 is a novel PROTAC compound. In this application note, degradation products of ARV-825 generated through acid hydrolysis were investigated. The degradation products were isolated using Waters™ Fraction Manager - Analytical (WFM-A) configured with an Arc™ Premier System, 2998 PDA Detector, and ACQUITY™ QDa™ II Detector. The specificity of the mass-directed trigger enabled efficient fraction collection of the target degradation products, while the time-based trigger allowed collection of closely eluting peaks. The MassLynx™ Software with FractionLynx™ Application Manager automated the collection process, while tracking the location of the collected fractions.

Benefits

- The Waters Fraction Manager - Analytical (WFM-A) configured with an Arc Premier System, 2998 PDA Detector, and ACQUITY QDa II Detector enables fast and easy collection of the target compounds.
- The low dispersion system configuration together with the use of sub-5 µm particle-size analytical columns is designed to collect narrow peaks, delivering fast chromatography with enhanced resolution for fraction collection and in an off-collection analysis mode.
- MassLynx Software with the FractionLynx Application Manager provides automated fraction collection, while tracking the location of the collected fractions.

Introduction

PROTACs offer novel approaches for the treatment of cancer and other diseases through selective degradation of target proteins.[1,2] These heterobifunctional molecules contain E3 ubiquitin ligase ligand connected via a chemical linker to a ligand that recognizes and binds to the target protein. This configuration enables PROTACs to form a stable ternary complex, leading to ubiquitination and degradation of the target protein. The ARV-825 is a newly developed inhibitor using PROTAC technology that has shown effectiveness for the treatment of pancreatic cancer, melanoma, cholangiocarcinoma, thyroid carcinoma, and acute myeloid leukemia.[3-5] The ARV-825 induces degradation of bromodomain-containing protein 4 (BRD4), a target protein marker in many cancer types. Currently, little work has been reported in the literature related to the stability and impurity characterization of PROTAC drug molecules.

The forced degradation studies, also referred to as stress testing, are designed to generate degradation products or impurities that could potentially form during storage and use of drug substances and drug products. The studies provide data to support identification of possible degradants, degradation pathways and the development of stability-indicating methods.[6] While numerous techniques can be employed to identify the unknown degradants, an analytical-scale fraction collection approach isolates a pure target compound for further characterization and identification. The WFM-A is a low dispersion fraction collector for analytical scale isolation and purification when only small amounts of sample are available.[7] Multiple modes of fraction collection are available with WFM-A, based on the elution time, peak threshold, slope, or combinations of these parameters. When combined with mass detection, target compounds can be isolated based on a mass-directed trigger.

Work in this application note investigates degradation products of the ARV-825 molecule generated through a

forced degradation study with a 0.5 M hydrochloric (HCl) acid. The degradation products were isolated and collected by using the WFM-A configured with an Arc Premier System. Using the ACQUITY QDa II Mass Detector, mass-directed fraction collection was applied to isolate the target degradation products. To verify purity, the collected fractions were analyzed using orthogonal chemistry. Time-based mode and focused gradients were employed to effectively separate and identify closely eluting components. The FractionLynx Application Manager Software facilitated the collection process.

Experimental

Mass spectrometry grade solvents and formic acid were purchased from Sigma-Aldrich. ARV-825 PROTAC compound was obtained from Sigma-Aldrich.

Sample Description

Forced Degradation Sample

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

LC Conditions

LC system:	Arc Premier System with Binary Solvent Manager (BSM-R) and Flow-Through Needle (FTN-R) 2998 PDA and ACQUITY QDa II Mass Detectors Waters Fraction Manager - Analytical (WFM-A)
Vials:	LCMS Maximum Recovery 2 mL (p/n: 600000749CV)
Column(s):	XSelect™ Premier CSH™ C_{18}, 4.6 mm ID x 100 mm,

	2.5 µm (p/n: 186009873)
Column temperature:	30 °C
Sample temperature:	15 °C
Injection volume:	40 µL
Mobile phase:	A: water with 0.1% formic acid B: acetonitrile with 0.1% formic acid
Wash solvents:	Purge/sample wash: water/acetonitrile Seal wash: water/acetonitrile 90:10
UV detection:	210–450 nm, derived at 250 nm, sampling rate: 10 pts/sec

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.00	95.0	5.0	6
10.00	1.00	5.0	95.0	6
10.50	1.00	5.0	95.0	6
10.60	1.00	95.0	5.0	6
13.50	1.00	95.0	5.0	6

MS Conditions

MS system:	ACQUITY QDa II Mass Detector

Ionization mode:	ESI+
Acquisition range:	200–1000 m/z
Capillary voltage:	0.8 kV (+)
Cone voltage:	5 V
Probe temperature:	600 °C
Data:	Centroid

Isocratic Solvent Manager (ISM) Conditions

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 (WFM-A System)

Chromatography data software:	MassLynx version 4.2 SCN1049
Application manager:	FractionLynx

Results and Discussion

The forced degradation study of an ARV-825 compound was performed based on a previously published application note.[8] Degradation with 0.5 M HCl for two hours at room temperature produced several degradation products. The chromatographic separation was performed on the XSelect Premier CSH C_{18} Column using an Arc Premier System with WFM-A in injection mode only, collection off (Figure 1). The major degradation products

were successfully separated, allowing for effective collection of high purity fractions. The mass-directed and time-based fraction collection triggers were applied to isolate the degradation products of ARV-825.

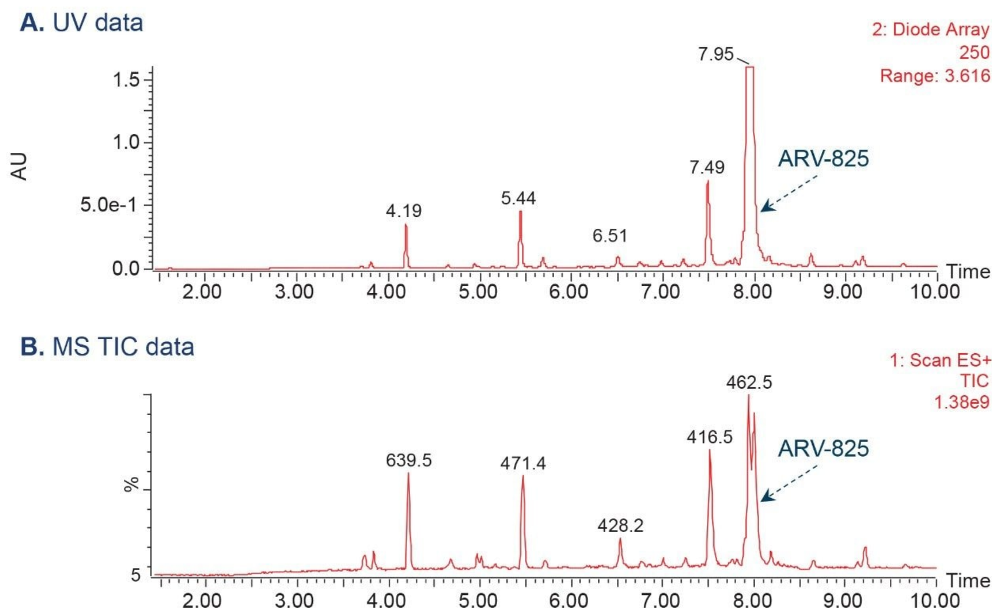

Figure 1. ARV-825 sample degraded with acid run on an Arc Premier System with WFM-A in injection mode, collection off. UV data at 250 nm (A) and MS TIC data with m/z values acquired on ACQUITY QDa II Mass Detector (B).

Mass-Directed Fraction Collection

In mass-directed mode, fractions were collected based on the molecular mass acquired on an ACQUITY QDa II Mass Detector. The ISM was used to split and dilute the flow entering the ACQUITY QDa II Mass 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.

In this study, 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). The makeup solvent with 50:50 acetonitrile/water with 0.1% formic acid produced a robust signal for the degradation products.

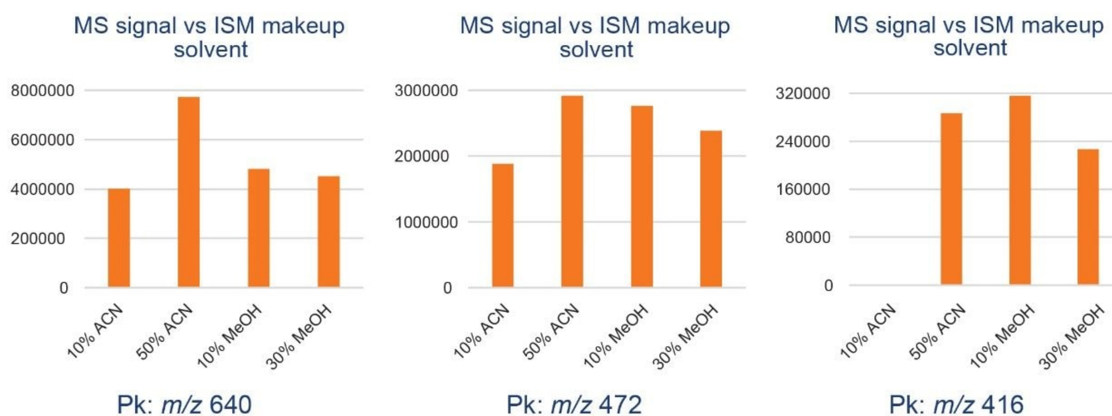

Figure 2. ISM makeup solvents screening to enhance MS signal for degradation products. All solvents contained 0.1% formic acid. Analysis run on an Arc Premier System with WFM-A in injection mode, collection off. MS TIC data, ACN: acetonitrile, MeOH: methanol.

A mass-directed fraction collection was applied to isolate degradation products of ARV-825. Using the target masses (*m/z* 640, 472, 428, and 416), fractions were successfully collected and multiple fractions pooled or combined into one vial (Figure 3). Pooling fractions allowed for collection of larger volumes. FractionLynx automated fraction collection and tracked vial location of the collected samples.

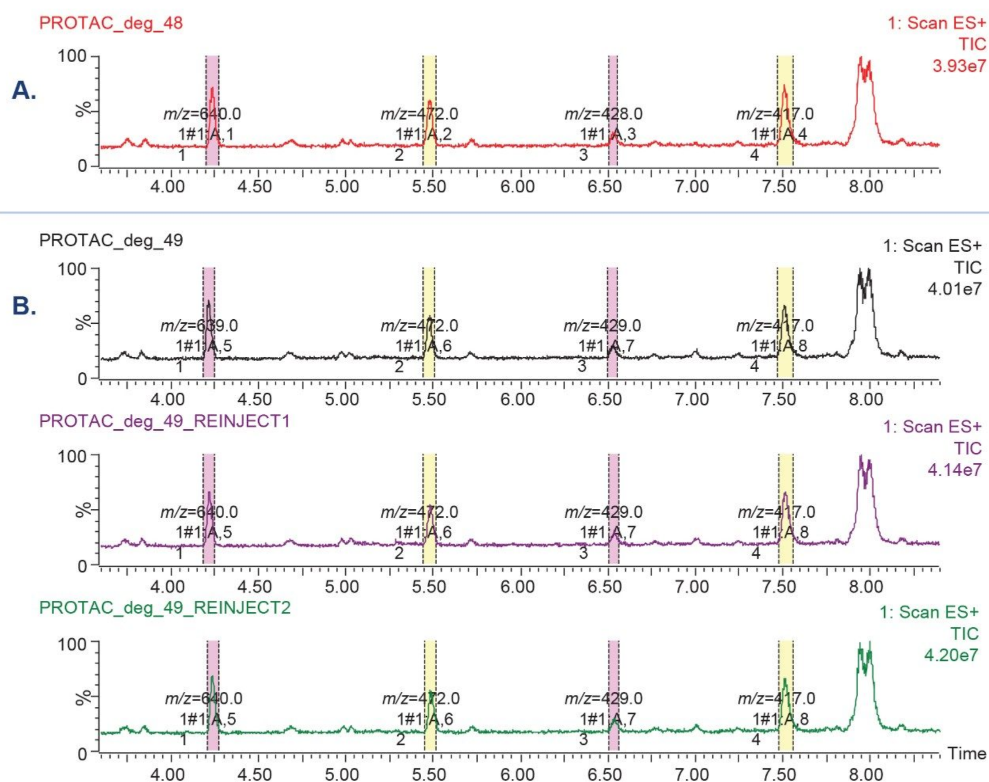

Figure 3. Fraction collection of degradation products by mass-directed trigger. One fraction per collection vial (A) and three fractions pooled per collection vial (B).

Analysis of the Collected Fractions

The fractions collected by mass-trigger were analyzed using orthogonal chemistry with XSelect HSS™ T3 Column (4.6 x 100 mm, 2.5 µm particle size, p/n 1860061510). The analysis was run on an Arc Premier System with Quaternary Solvent Manager (QSM) integrated with 2998 PDA Detector and ACQUITY QDa II Detector, controlled with Empower™ Chromatography Data System (CDS). To demonstrate purity of the collected fractions, both chromatographic and spectral purity of the degradation products were evaluated.

Analysis of fraction 1 with *m/z* 640 showed chromatographic purity or percentage area of 97.8% and 97.7% for single and pooled fractions, respectively (Figure 4). Both the UV and MS spectral data demonstrated spectral purity of the collected fractions, indicating target compounds are not coeluting with other components from the sample.

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, the concentration of this additional peak increased with time, as shown for the collected fraction reanalyzed a week later (Figure 5B). This indicated degradation of the target compound in the collected fraction solution.

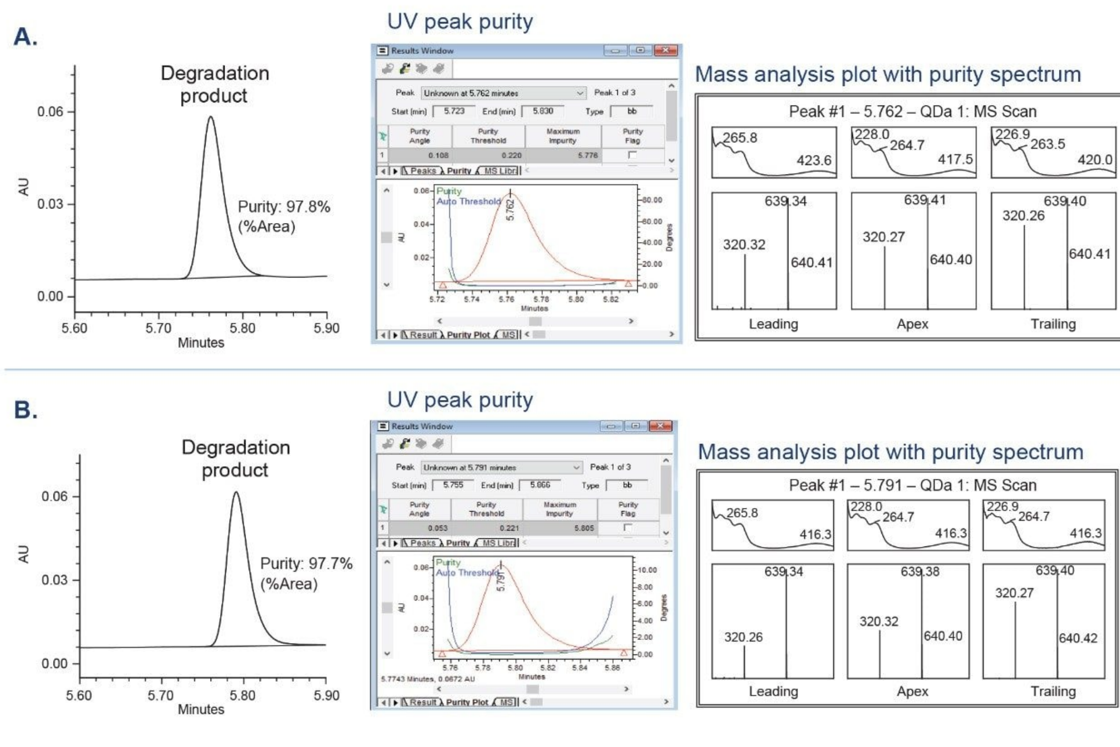

Figure 4. Analysis of fraction 1 collected by mass-directed trigger with m/z 640 performed using orthogonal chemistry with XSelect Premier HSS T3 Column. Chromatographic and spectral purity verification of a single fraction collected per vial (A) and three fractions pooled per vial (B).

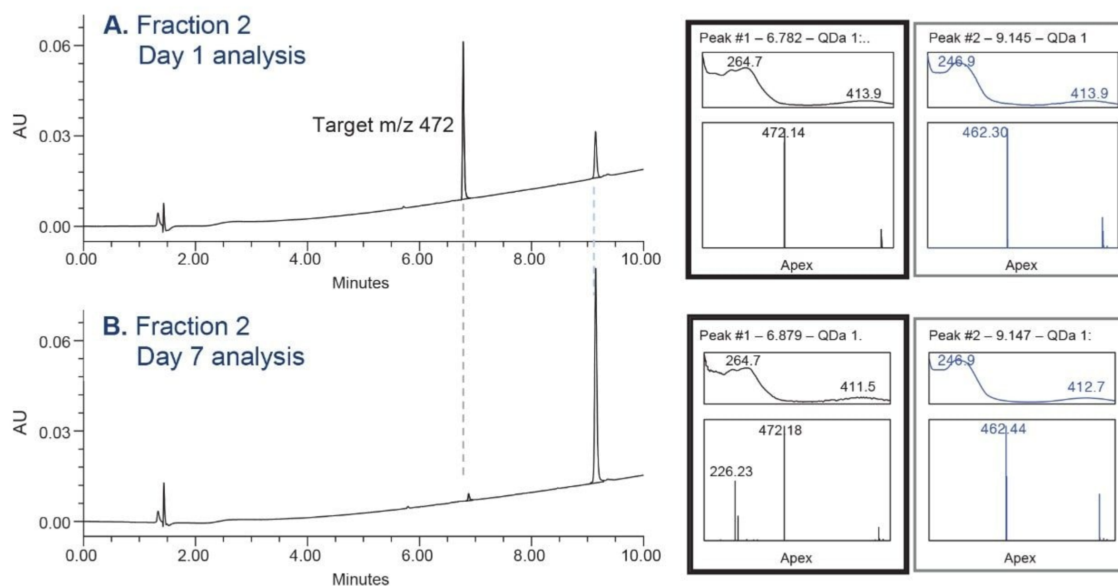

Figure 5. Analysis of fraction 2 collected by mass-directed trigger of m/z 472 performed using orthogonal chemistry with XSelect Premier HSS T3 Column. Fraction injected on day 1 (A) and day 7 (B) indicated degradation of the target compound over time.

Time-based Fraction Collection

Time-based fraction collection over a defined chromatographic region allows collection of closely eluting peaks through slicing into smaller sections.

The time-based collection mode was applied to fraction the chromatographic region near the ARV-825 peak into smaller sections (Figure 6). In this case, the collected fractions were re-injected on the WFM-A System with the same column using a focused gradient to produce a fast and efficient separation (Figure 7). Analysis of fractions 2 and 3 on an ACQUITY QDa II Mass Detector showed the presence of a peak with masses corresponding to *m/z* 923 and 462, specific to the ARV-825 compound.

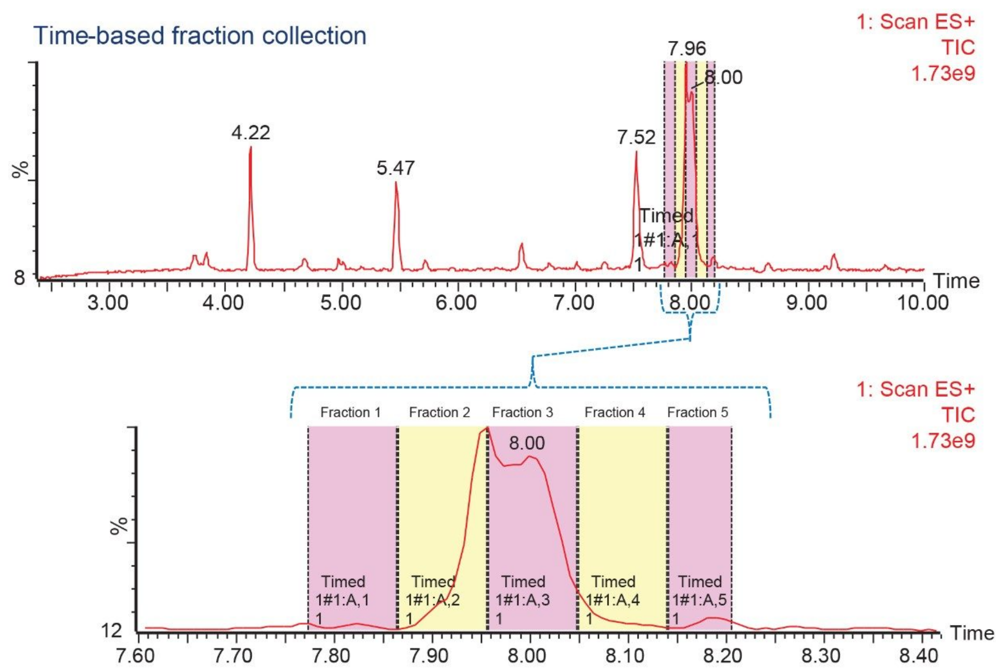

Figure 6. Time-based fraction collection near the ARV-825 peak.

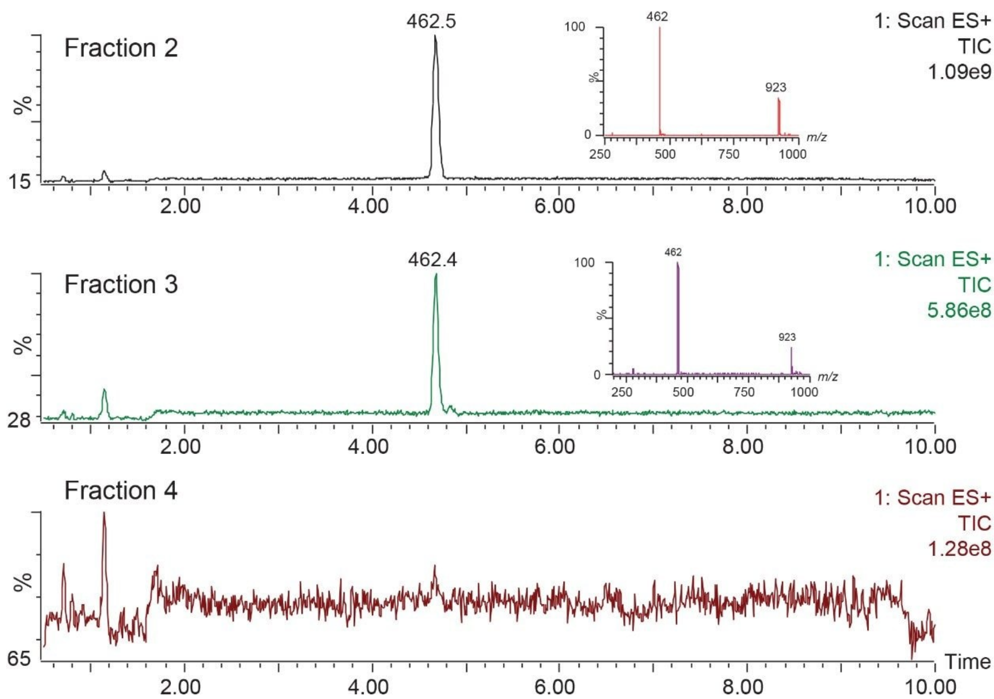

Figure 7. Analysis of the time-based collected fractions using a focused gradient in injection mode with collection off.

Conclusion

Isolation of the degradation products of the ARV-825 compound was successfully performed using the WFM-A integrated with an Arc Premier System. Combination with the ACQUITY QDa II Mass Detector facilitated the use of a mass-directed trigger, enabling efficient fraction collection of the target degradation products. The time-based fraction collection combined with the low peak dispersion design of the WFM-A, allowed precise collection of closely eluting peaks. Purity of the collected fractions was verified through analysis using an orthogonal chemistry. MassLynx Software with FractionLynx Application Manager facilitated the fraction collection process. The WFM-A configured with an Arc Premier System enabled fast and easy collection of the target compounds in PROTACs drug molecule.

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