A sensitive method for the quantification of the proteolysis targeting chimera (PROTAC), TL 13-12, in rat plasma using accurate mass spectrometry

Accurate and reproducible quantitative performance for PROTAC analysis using the ZenoTOF 7600 system

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This technical note demonstrates an accurate mass spectrometry method for quantifying a selective Anaplastic Lymphoma Kinase (ALK) degrader and PROTAC, TL 13-12, and its inactive control, TL 13-22 in rat plasma. A minimal sample preparation method combined with a 10-minute LC-MS analysis achieved a lower limit of quantification (LLOQ) of 0.3 ng/mL for TL 13-12 and TL 13-22 (Figure 1).

PROTACs have evolved into a small molecule-based drug modality 20 years after its potential for targeted degradation was shown in 2001.[1] New therapeutic candidates are now moving to clinical trial stages against cancer.[2] PROTACs have a heterobifunctional structure with 2 functional moieties that enables them to target both a protein of interest (POI) and the ubiquitin E3 ligase (Figure 2A). This targeting brings the POI and E3 ligase close together to facilitate the ubiquitination of the POI. Ubiquitination of the POI then signals endogenous protein degradation machinery to initiate the process of POI removal.[1]

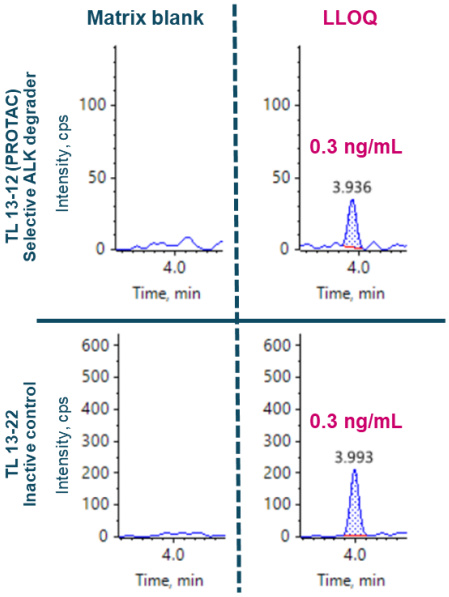

Figure 1. Representative extracted ion chromatograms (XICs) of the selective ALK degrader (PROTAC) and its inactive control at the LLOQ level. Sub-ng/mL quantification without matrix interference was achieved using the ZenoTOF 7600 system.

PROTACs have gained significant interest for drug development pipelines due to their high potency in nanomolar drug concentrations[3] and selectivity in protein targeting. However, low circulating drug levels in complex matrices with limited sample volumes present analytical challenges. Therefore, sensitive and selective assays for the high-confidence detection and quantification of PROTACs in complex matrices using minimal sample extraction methods are needed to ensure the safety and efficacy in the drug development pipeline.

A sensitive assay for the quantification of PROTACs in a complex matrix was demonstrated using the commercially available standards, TL 13-12 (PROTAC) and TL 13-22 (inactive control). Sub-ng/mL level quantification was achieved for both analytes in 100 µL of rat plasma using the ZenoTOF 7600 system.

Key features of the quantification of PROTACs using the ZenoTOF 7600 system

- **Enhanced sensitivity:** Achieve an LLOQ of 0.3 ng/mL for quantification of TL 13-12 and TL 13-22 in rat plasma on the ZenoTOF 7600 system with improved MS/MS sampling efficiency using the Zeno trap
- **Minimize sample volume and sample preparation time:** Perform selective and sensitive quantification with 100 µL of rat plasma and simple sample preparation method by leveraging high mass accuracy and MS/MS sensititvity of the ZenoTOF 7600 system
- **Robust analytical performance:** Achieve accurate quantitative performance with %CV <4% at all concentration levels across a linear dynamic range (LDR) of 3 orders of magnitude
- **Streamlined data management:** Employ fast, intuitive and integrated data acquisition and processing using SCIEX OS software

Methods

Sample preparation: The commercially available individual PROTAC degrader (TL 13-12) and its inactive control (TL 13-22) were reconstituted in DMSO according to the manufacturer's manual. Both analytes were spiked into 100 µL of rat plasma at concentrations ranging from 0.3 ng/mL to 200 ng/mL. Samples were extracted using a simple protein precipitation method by adding 600 µL of 1:1 (v/v), acetonitrile/methanol. Samples were vortexed for 30 seconds, then centrifuged at 13000 rpm for 12 minutes at room temperature. The supernatant was dried under a nitrogen stream at 40°C. Samples were reconstituted using 200 µL of 1:1 (v/v), methanol/acetonitrile for analysis.

Chromatography: Sample separation was performed using an ExionLC system at a flow rate of 0.3 mL/min using a Phenomenex Kinetex XB-C18 (2.1 x 50 mm, 1.7 µm, 100 Å) column. Chromatographic separation was performed with a 10-minute gradient (Table 1) using 0.1% (v/v) formic acid in water as mobile phase A and 0.1% (v/v) formic acid in acetonitrile as mobile phase B. The column temperature was kept at 40°C. A 5 µL injection volume was used for analysis. A solution containing equal parts acetonitrile, methanol and water by volume was used as the needle wash solvent.

Table 1. Chromatography gradient for the PROTAC and its inactive control.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	85	15
0.2	85	15
5	50	60
5.5	5	95
8.5	5	95
8.6	85	15
10	85	15

Mass spectrometry: Samples were analyzed using the ZenoTOF 7600 system. The optimized MS and source parameters used are listed in Table 2. The optimized analyte-dependent MRM^{HR} parameters used are listed in Table 3.

Table 2. MS and source parameters.

Parameter	MS	MS/MS
Scan mode	TOF MS	MRM^{HR}
Polarity	Positive	
Gas 1	40 psi	
Gas 2	60 psi	
Curtain gas	40 psi	
Source temperature	500°C	
Ion spray voltage	5000 V	
CAD gas	7	
Declustering potential	80 V	See Table 3
Start mass	400 m/z	400 m/z
Stop mass	1,000 m/z	970 m/z
Q1 resolution	NA	Unit
Accumulation time	0.25 s	0.04 s
Collision energy	6 V	See Table 3
CE spread	0 V	0 V
Zeno trap	NA	ON
ZOD threshold (CID)	NA	20,000 cps
Time bins to sum	6	8

Table 3. MRM parameters.

ID	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Collision energy (V)	Declustering potential (V)
TL 13-12	961.3403	543.1906	65	40
TL 13-22	947.3598	543.1912	65	80

Data processing: Data collection, analysis and quantification were performed using SCIEX OS software, version 3.1. Peaks were automatically integrated using the MQ4 algorithm. The XIC peak width was set to 0.05 Da for TL 13-12 and to 0.02 Da for TL 13-22. A weighting of $1/x^2$ was used for quantification.

Quantitative performance

This technical note demonstrates a sub-ng/mL level quantification assay of a PROTAC and its inactive control in rat plasma using the ZenoTOF 7600 system.

The calibration curve ranged from 0.3 ng/mL to 200 ng/mL and was prepared as described in the sample preparation section. Individual concentrations were run in triplicate. A LOD of 0.15 ng/mL and a LLOQ of 0.3 ng/mL were achieved for TL 13-12 and TL 13-22 (Figure 2B). No interferences were observed in the matrix blank (rat plasma) for either analyte (Figure 2B). Both analyte calibration curves yielded a correlation coefficient (r^2) of 0.99 and had a linear dynamic range (LDR) that spanned 3 orders of magnitude (Figure 3).

Analytical performance was evaluated for accuracy and precision. The accuracy of the calculated mean was expected to be between 80% and 120% at the LLOQ and between 85% and 115% for the higher concentrations. The %CV of the calculated mean for each concentration was expected to be <20% at the LLOQ and <15% for all higher concentrations. As expected, the accuracy was within 20% of the nominal concentration for both analytes and the %CV was <15% for both analytes (Table 4).

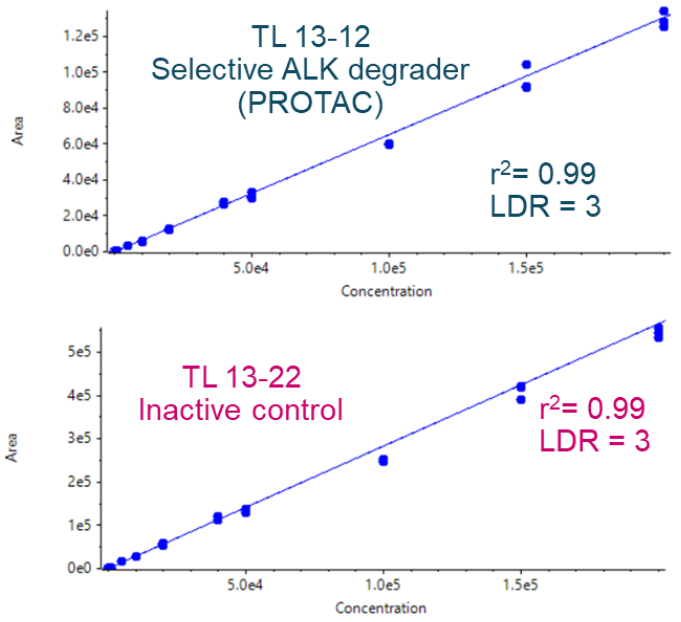

Figure 3. Calibration curves TL 13-12 and TL 13-22 in rat plasma. Strong linearity was achieved for TL 13-12 (top) and TL 13-22 (bottom) in rat plasma, with a correlation coefficient (r^2) of 0.99 for both targets**.** Each concentration level was run in triplicate.

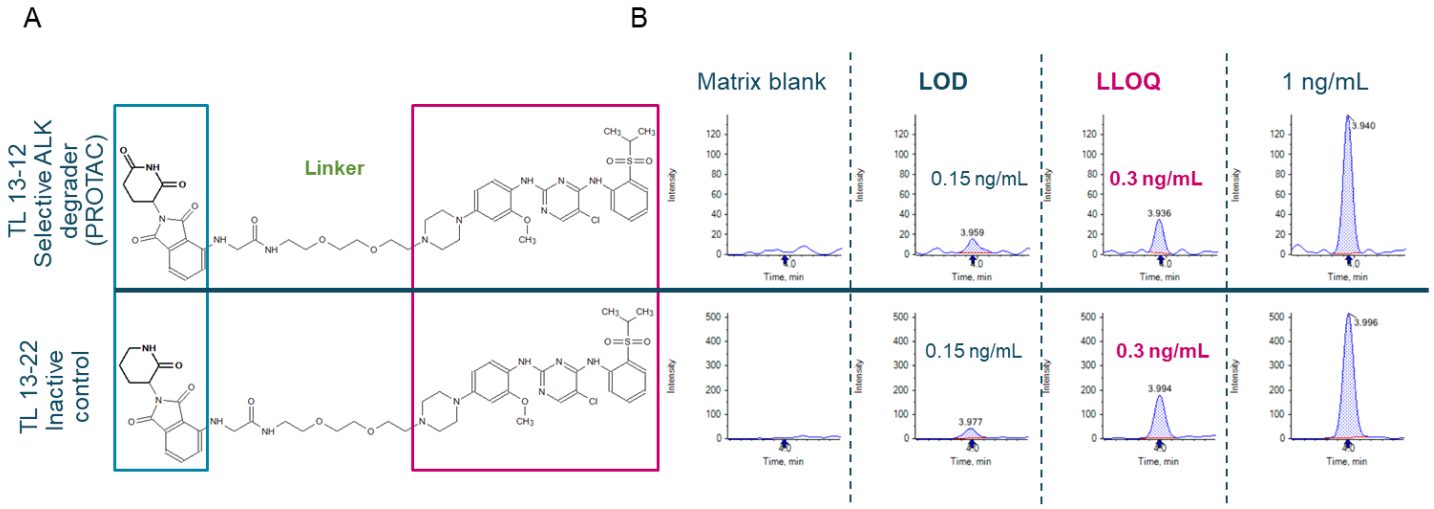

Figure 2. Sub-ng/mL level quantification was achieved for the PROTAC and its inactive control. The PROTAC used for this assay was TL 13-12 and the inactive control was TL 13-22. A) TL 13-12 contains additional carbonyl oxygen in the pomalidomide group compared to TL 13-12 (panel A, bolded structure). The blue rectangle highlights POI binding moiety and the magenta rectangle highlights the E3 ligase binding moiety. B) Representative XICs of the PROTAC and its inactive control are shown. An LLOQ of 0.3 ng/mL was achieved for both the PROTAC and its inactive control with a LOD of 0.15 ng/mL for both analytes. No matrix interferences were observed in the rat plasma blank for either analyte.

Table 4. Accuracy and %CV at each concentration level was measured for TL 13-12 and TL 13-22.

	TL 13-12		TL 13-22	
Concentration (ng/mL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.3	92.8	13.8	92.3	14
1	118	12.3	107	4.57
5	105	8.47	115	1.52
10	93.5	5.31	102.	3.43
20	95.9	4.18	103	5.48
40	104	2.61	109	4.21
50	95.3	5.36	99	3.07
100	91.7	0.67	92.4	1.38
150	98.1	7.38	101	4.15
200	98.8	3.55	101	2.03

Compliance-ready SCIEX OS software

To meet the regulations outlined in 21 CFR Part 11, SCIEX OS software is a closed system and requires records and signatures to be stored electronically. SCIEX OS software can open raw data files from any visible storage location within a closed network by using designated processing workstations. Figure 4 illustrates 3 types of controls that are required for 21 CFR Part 11 compliance. The workflow presented here is fully compliant with these guidelines, as SCIEX provides 1) technical controls over hardware and software configuration, 2) network security and secure operating systems and policies and 3) procedures and user training (Figure 4).

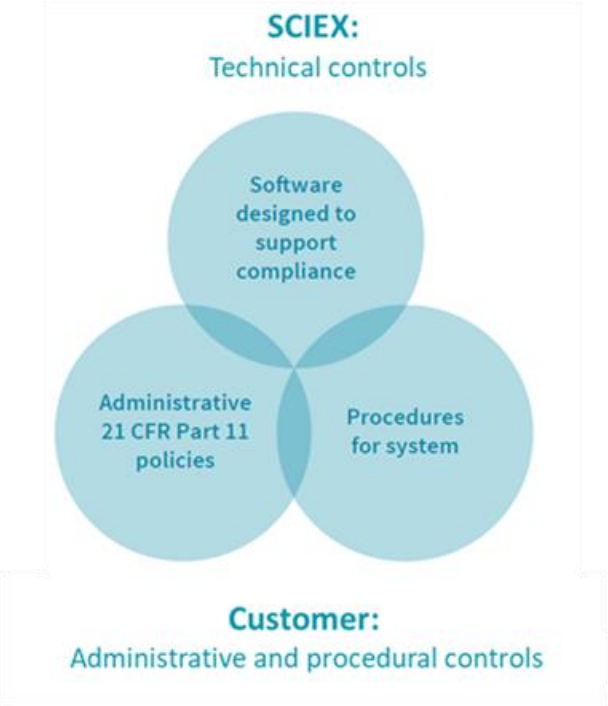

Figure 4. Controls required for 21 CFR Part 11 compliance.

Conclusions

- Low-level quantification levels were achieved at 0.3 ng/mL for TL 13-12 and TL 13-22 in rat plasma using a simple sample preparation method
- A sensitive and robust quantification using the MRM^{HR} workflow was demonstrated on the ZenoTOF 7600 system with improved MS/MS sampling efficiency using the Zeno trap
- The method demonstrated accurate quantitative performance with %CV <14% at all concentration levels across an LDR of 3 orders of magnitude
- Streamlined data acquisition, processing and management were achieved using SCIEX OS software

References

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3. Ming H. et al. (2021). Opportunities and Challenges of Small Molecule Induced Targeted Protein Degradation. Frontiers in Cell and Developmental Biology, 9.

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