High-resolution LC-MS/MS workflow for quantification of disulfide-bridged cyclic peptides in rat plasma

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ABSTRACT

The use of cyclic peptides as therapeutic agents has substantially increased due to their stability, selectivity and high potency. Most cyclic peptides are linked together by disulfide bonds, making them frequently resistant to the collision induced dissociation (CID) required for MS/MS quantification. The resulting CID spectra do not form the prominent selective fragments needed for high-quality quantitative analysis. The limitations of CID MS/MS for such species have driven the need for more efficient fragmentation techniques for MS/MS quantification of cyclic peptides. In this study, quantification of disulfide-bridged cyclic peptides in plasma was performed using a TOF MS/MS system with Zeno trap enabled EAD, CID and MS modes.

INTRODUCTION

Cyclic peptides are polypeptides in the configuration of a ring formed by chemically stable bonds, such as disulfide bonds between 2 cysteine residues. Cyclic peptides have been identified as critical therapeutic candidates given their structural stability and conformational rigidity.

With emerging interest in the advancement of cyclic peptide therapeutics, there is an equivalent drive toward developing highly robust and sensitive quantitative methods. However, current bioanalytical methods for the quantification of cyclic peptides in biological matrices still present challenges. For LC-MS-based methods, high baseline interference and resistance to CID, given the complex tertiary structure of cyclic peptides, impact overall sensitivity.

This study evaluated the quantitative performance of cyclic peptides on the ZenoTOF 7600 system. The Zeno trap enabled MS, CID and EAD modes were evaluated for quantification. The Zeno MS approach enabled low-level quantification of cyclic peptides compared to Zeno CID and Zeno EAD. In addition, the Zeno MS workflow facilitated an overall reduction in method development time with minimal ion path tuning, offering a simple quantitative workflow for cyclic peptides.

MATERIALS AND METHODS

Sample preparation:
Rat plasma was protein precipitated and the supernatant was diluted 1:1 (v/v) by water to serve as the processed biological matrix. Eptifibatide and a labeled cyclic peptide serving as an internal standard (IS) were spiked into the processed rat plasma. The concentration of the IS in solution was 10 ng/mL. Serial dilution with processed plasma was performed to create the calibration curves for analysis.

LC conditions:
The separation was performed at a flow rate of 0.3 mL/min using an ExionLC system. A HALO BioClass Peptide ES-C18 column (2.1 x 50 mm, 2.7 µm, 160 Å) was used for separation. The column oven temperature was set to 40ºC. A volume of 20 µL was injected for analysis.

MS conditions:
The data were acquired in positive mode using Zeno CID, Zeno MS and Zeno EAD on a ZenoTOF 7600 system. An MRM^{HR} workflow was applied for all MS modes examined. The source was operated in positive ion mode. Collision energy (CE) and other source and MS parameters were optimized for eptifibatide. A summary of the source and MS parameters and the Zeno trap settings is displayed in Table 1.

For Zeno CID and Zeno EAD, a suitable *m/z* range of fragment ions was monitored. EAD parameters such as ETC, electron KE and reaction time were optimized for eptifibatide as shown in Table 2.

Data processing:
Zeno CID, Zeno MS and Zeno EAD data were processed using the Analytics function in SCIEX OS software 2.0 with the MQ4 integration algorithm. A 1/x^2 weighting was used for quantification.

Table 1. Chromatographic conditions.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	95	5
1.0	95	5
3.0	75	25
4.0	40	60
4.2	10	90
5.2	10	90
5.5	95	5
6.5	95	5

- The mobile phase A consisted of 0.1% formic acid in water, while the organic phase B was composed of 0.1% formic acid in acetonitrile.
- Eptifibatide eluted between 3 min and 4 min (Table 1).

- All source and MS conditions were optimized for the best sensitivity for the quantification of eptifibatide (Table 2).

Table 2. Source and MS conditions and Zeno trap settings.

Parameter	Value	Parameter	Value
Curtain gas	35 psi	Source temperature	650°C
Ion source gas 1	30 psi	Ion source gas 2	80 psi
CAD gas	11	Ion spray voltage	4000 V
Q1 resolution	low	ZOD threshold	20,000 cps

Table 3. EAD conditions.

Parameter	Value	Parameter	Value
Electron beam current	4750 nA	ETC	100
Electron KE	15	Reaction time	5 ms

- EAD parameters such as electron beam current, electron KE, ETC and reaction time were optimized (Table 3).

RESULTS

Figure 1. Structure of eptifibatide.

- Eptifibatide was used as a model analyte to evaluate the quantification of cyclic peptides on the ZenoTOF 7600 system.
- Eptifibatide was spiked into processed rat plasma at concentrations ranging from 0.4 ng/mL to 423 ng/mL.

- The precursor ion for eptifibatide, *m/z* 832.3215, was applied for quantitative workflows involving Zeno CID, Zeno EAD and Zeno MS.
- For Zeno CID, fragment ions were monitored in the *m/z* 400 to *m/z* 900 range. The Zeno EAD workflow captured fragment ions in the *m/z* 100 to *m/z* 900 range.
- For quantification using Zeno CID and Zeno EAD, the most intense fragment ions were summed for optimal assay sensitivity.

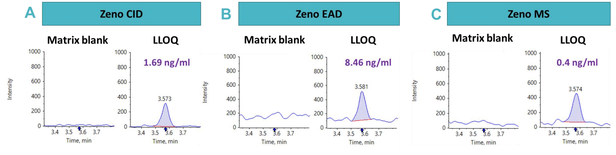

Figure 2. Extracted ion chromatograms (XICs) of matrix blank and the lower limit of quantification (LLOQ) using Zeno CID (A), Zeno EAD (B) and Zeno MS (C).

- No matrix interferences were observed for the MS modes examined.
- Lower limits of quantification (LLOQs) of 1.69 ng/mL, 8.46 ng/mL and 0.4 ng/mL were achieved using the Zeno CID, Zeno EAD and Zeno MS modes, respectively (Figure 2).
- In this case, the best sensitivity was reached using Zeno MS, with up to a 4-fold improvement in LLOQ compared with Zeno CID and a 21-fold improvement in LLOQ compared to Zeno EAD.
- This suggests that for complex peptides, such as those with disulfide-bridged modifications, resistance to fragmentation may cause a loss in sensitivity for quantitative assays, unless they are analyzed with a high duty cycle triple quadrupole system such as the SCIEX 7500 system.[1]
- With Zeno MS mode, the precursor ion was used for quantification with the application of the Zeno trap, which enhances overall sensitivity. In addition to the benefits of the Zeno trap in Zeno MS mode, the workflow offers reduced method development time with less ion path tuning.
- As a result, the Zeno MS approach presents the most sensitive and simple workflow for the quantification of cyclic peptides.

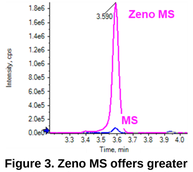

Figure 3. Zeno MS offers greater sensitivity for the quantification of cyclic peptides.

- The application of the Zeno trap in MS mode enhances overall sampling efficiency, which results in an increase in sensitivity.
- A 5-fold signal-to-noise (S/N) improvement was observed with the application of Zeno MS compared to traditional MS (Figure 3).

- The linear range covered 0.42 ng/mL to 423 ng/mL.
- An overall linear dynamic range (LDR) of 3 orders of magnitude was achieved.

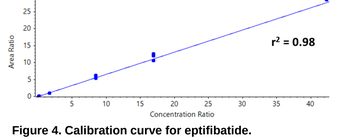

Figure 4. Calibration curve for eptifibatide.

Table 4. Calculated concentration, precision and accuracy for eptifibatide.

Concentration (ng/mL)	Accuracy (%)	Percent CV (%)
423.29	109.90	5.52
169.31	105.76	8.61
84.66	104.08	7.77
16.93	90.81	2.95
1.69	85.69	2.10
0.42	103.76	12.75

- Calculated concentrations for each calibration point were within ±15% of the nominal value, even at the LLOQ level (Table 4).

- The accuracy at the LLOQ level was ±5% of the nominal concentration with precision less than 13%, demonstrating high accuracy and reproducibility (Table 4).
- The quantitative results including—accuracy, precision and linearity—for the data acquired using Zeno CID and Zeno EAD were comparable to Zeno MS (data not shown).

CONCLUSIONS

- A 5-fold S/N improvement was achieved using Zeno MS compared to traditional MS for sensitive quantification of hard-to-fragment cyclic peptides.
- Development of a simple workflow for cyclic peptide quantification was accomplished with less method development time using the Zeno MS approach.
- Low-level quantification of cyclic peptides in rat plasma was reached with exceptional linearity, precision and accuracy.
- An LLOQ of 0.4 ng/mL was reached for eptifibatide, demonstrating an over 3-fold improvement in LLOQ compared to previous methods.[2-4]

REFERENCES

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TRADEMARKS/LICENSING