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Low-pg/mL quantification of cyclic peptides in rat plasma using microflow LC

Featuring the SCIEX 7500 system, powered by SCIEX OS software

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This technical note describes the enhancement of lower limits of quantification (LLOQs) for cyclic peptides by using a microflow trap-and-elute method. Low-pg/mL quantification was achieved for human atrial natriuretic peptide (ANP) with outstanding reproducibility, precision, accuracy and linearity. The microflow LC method achieved a 5-fold improvement in LLOQ, compared to previously published data acquired using analytical flow LC on a SCIEX 7500 system.[1]

Cyclic peptides are polypeptides held in a ring configuration by chemically stable bonds, such as disulfide linkages. For example, the natriuretic peptide (NP) family is a group of genetically distinct cyclic peptides that contains an amino acid ring formed by disulfide bonds (Figure 1). The unique structure of these peptides confers structural stability and conformational rigidity. As a result, cyclic peptides can exhibit enhanced biological activity compared to traditional peptides. These features have helped identify cyclic peptides as important therapeutic candidates and successful therapeutic agents in cardiovascular diseases.[2]

With emerging interest in the advancement of cyclic peptide therapeutics, there is an equivalent drive towards the development of highly robust and sensitive quantitative methods. Current bioanalytical methods lack the sensitivity necessary to reliably quantify cyclic peptides. For LC-MS based methods, high baseline interference in single MS mode and resistance to CID in MS/MS mode, given the tertiary structure, have an impact on overall sensitivity.

In this study, human ANP was selected as a model analyte to evaluate improvement in sensitivity with the application of microflow LC. Low-level quantification was achieved for human ANP at an LLOQ of 0.01 ng/mL. The application of microflow LC yielded excellent accuracy, precision and linearity, while providing outstanding quantitative performance in parallel with high sensitivity.

Key features of using microflow LC on the SCIEX 7500 system to quantify cyclic peptides

- Achieve low-pg/mL quantification of cyclic peptides in rat plasma with exceptional reproducibility, accuracy and linearity.
- Enable large sample volume analysis without increased run time resulting in high sample throughput with a microflow LC setting.
- Achieve improved sensitivity through hardware improvements including:
    - D Jet ion guide—increased capture and transmission of analyte ions[3]
    - OptiFlow Pro ion source—latest generation ion source with maximum flexibility and robustness[3]
    - E Lens probe—increased field strength improves desolvation and ion generation[3]
- Employ a single platform for streamlined data acquisition, processing, and management with SCIEX OS software.

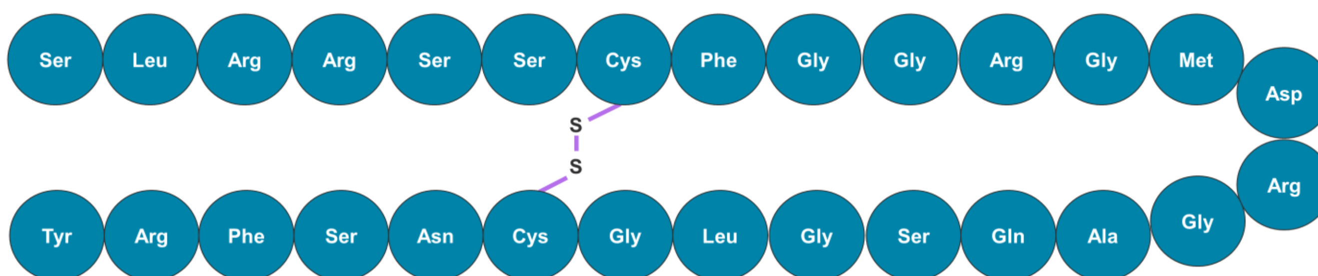

Figure 1. Amino acid sequence of human ANP. The cyclic peptide is composed of 28 amino acids and 1 disulfide-bridge between cysteine residues.

Methods

Sample preparation: Rat plasma was protein precipitated and the supernatant was diluted 1:1 (v/v) with water which served as the processed biological matrix. Human ANP and a labeled cyclic peptide, internal standard (IS), were spiked into the processed rat plasma. The IS concentration was 10 ng/mL. Serial dilution with processed plasma was performed to create the calibration curves for analysis.

Chromatography: A SCIEX M5 MicroLC system was used for separation in trap-and-elute mode, run in contact closure mode. A volume of 20 µL of sample was loaded onto the trap column for analysis. The mobile phase A consisted of 0.1% formic acid in water and the organic phase B was composed of 0.1% formic acid in acetonitrile.

Chromatographic conditions for analyte trapping and separation are summarized in Tables 1 and 2, respectively. For analyte trapping, the operating flow rate was set to 50 µL/min using a Phenomenex Luna C18(2) column (20 x 0.3 mm, 5 µm, 100 Å). The column was operated at room temperature.

Table 1. Chromatographic conditions for analyte trapping.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	*100*	*0*
*0.1**	*100*	*0*
5.0	*100*	*0*
5.2	*10*	*90*
6.8	*10*	*90*
7.0	*100*	*0*
8.5	*100*	*0*

** Analytical gradient was initiated*

For analyte separation, the operating flow rate was 5 µL/min using a Phenomenex Kinetex XB-C18 column (50 x 0.3 mm, 2.6 µm, 100 Å). The column oven temperature was 40ºC.

Table 2. Chromatographic conditions for analyte separation.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	*60*	*40*
5.0	*40*	*60*
*5.2**	*10*	*90*
6.8	*10*	*90*
7.0	*60*	*40*
8.5	*60*	*40*

** Valve load was initiated*

Mass spectrometry: Sample analysis was performed using a SCIEX 7500 system in MRM mode. Source was operated in positive ion mode. Collision energy (CE) and other source and MS parameters were optimized to achieve the best sensitivity for MS/MS quantification. A summary of the source and MS parameters is displayed in Table 3.

Table 3. MS parameters on SCIEX 7500 system.

Parameter	Value	Parameter	Value
Curtain gas	*35 psi*	*Source temperature*	300 °C
Ion source gas 1	*30 psi*	*Ion source gas 2*	*80 psi*
CAD gas	*11*	*Ion spray voltage*	4000 V

The MRM transition and method parameters for human ANP is outlined in Table 4.

Table 4. MRM method parameters.

Compound	Q1 mass (*m/z*)	Q3 mass (*m/z*)	CE (V)	CXP (V)
Human ANP	*617.1*	*584.1*	*34*	*15*

Data processing: MRM data was processed using the Analytics function in SCIEX OS software 2.0 using the MQ4 integration algorithm. A weighting of $1/x^2$ was used for quantification.

Cyclic peptide quantification results

In this workflow, a sensitive LC-MRM method was developed for the quantification of cyclic peptides in rat plasma. Human ANP was spiked into processed rat plasma at concentrations ranging from 0.01 ng/mL to 200 ng/mL. Calibration curves were measured in triplicates.

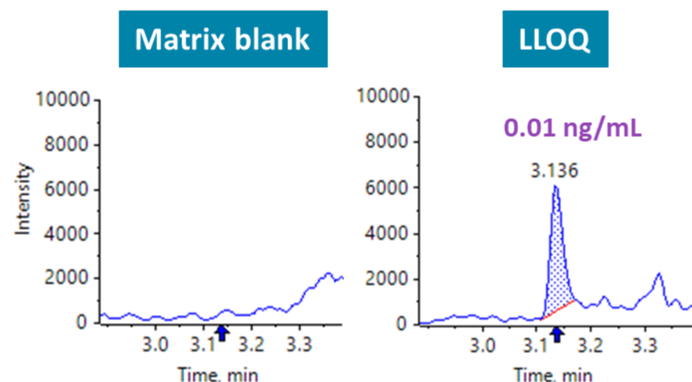

Figure 2. XICs of matrix blank and LLOQ of human ANP. No matrix interferences were observed at the retention time of the analyte.

The LLOQ was determined based on the requirements that the %CV is below 20% and accuracy is between 80% and 120%. For concentrations above the LLOQ, the %CV was required to be below 15%, with accuracy between 85% and 115%.

An LLOQ of 0.01 ng/mL was achieved, as shown in Figure 2. No significant matrix interferences were observed at the retention time of the analyte. The implementation of microflow LC resulted in a 5-fold improvement in sensitivity, compared to prior implementations of analytical flow LC on a SCIEX 7500 system.[1]

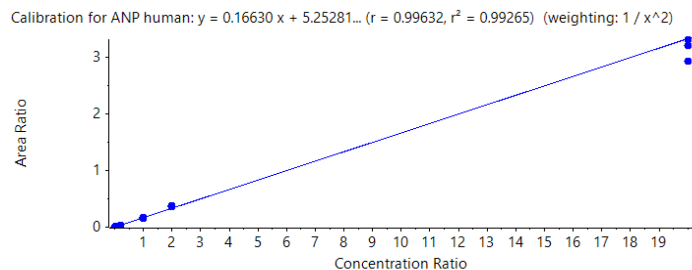

Figure 3. Calibration curve for human ANP. The linear range covered 0.01 ng/mL to 200 ng/mL with an overall linear dynamic range (LDR) of 4.3 orders of magnitude.

The linear range was between 0.01 ng/mL to 200 ng/mL for human ANP, providing 4.3 orders of magnitude in linear dynamic range (LDR) (Figure 3).

Table 5. Concentration, accuracy and precision for human ANP.

Concentration (ng/mL)	Accuracy (%)	CV (%)
200	*94.60*	*6.24*
20	*108.94*	*3.36*
10	*100.94*	*8.60*
2	*107.67*	*3.91*
1	*101.18*	*3.24*
0.5	*93.99*	*9.26*
0.2	*99.51*	*12.30*
0.1	*93.08*	*3.20*
0.02	*98.62*	*8.44*
0.01	*101.47*	*4.10*

Calculated concentrations for all calibration points were within ±15% of the nominal value (Table 5). As shown in Table 5, the precision was less than 12.5%, demonstrating high reproducibility.

Overall, a highly sensitive method for the quantification of cyclic peptides was demonstrated. For human ANP, quantification at low-pg/mL levels was achieved.

Conclusions

- An ultra-sensitive microflow LC-MRM based cyclic peptide quantification workflow using SCIEX 7500 system has been demonstrated in this work
- Compared to previously published data acquired using analytical flow LC on a SCIEX 7500 system[1], a 5-fold improvement in LLOQ was achieved with the implementation of a microflow LC workflow
- Low-level quantification was achieved for human ANP at an LLOQ of 0.01 ng/mL with exceptional reproducibility, accuracy, and linearity
- The combination of the D Jet ion guide, OptiFlow Pro ion source, and E Lens probe enabled a cumulative gain in sensitivity through improvement in ion generation, capture and transmission

References

1. Improved LC-MRM quantification sensitivity for cyclic peptides from the natriuretic peptide family. SCIEX technical note, RUO-MKT-02-11883-A.
2. Das BB, Solinger R (2009) *Cardiovasc. Hematol. Agents Med. Chem.* **7(1)**, 29-42.
3. Enabling new levels of quantification. SCIEX technical note, RUO-MKT-02-11886-A.

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