TN-1319

Low-pg/mL Quantification of Cyclic Peptides in Rat plasma Using Microflow LC

Eshani Nandita, PhD[1], Remco van Soest[1], Zoe Zhang, PhD[1], Elliot Jones[1], and Bryan Tackett, PhD[2]
[1]AB SCIEX LLC, 500 Old Connecticut Path, Framingham, MA 01701, USA
[2]Phenomenex Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Introduction

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. 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.

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.

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.

LC Conditions – Trap Column

Column: Luna™ 5 µm C18(2)
Dimensions: 20 x 0.3 mm
Part No.: 05M-4252-AC
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:	Time (min)	%B
	0	0
	0.1	0
	5	0
	5.2	90
	6.8	90
	7	0
	8.5	0

Flow Rate: 50 µL/min
Injection Volume: 20 µL
Temperature: Ambient
LC System: SCIEX M5 MicroLC

LC Conditions – Analytical Column

Column: Kinetex™ 2.6 µm XB-C18
Dimensions: 50 x 0.3 mm
Part No.: 00B-4496-AC
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:	Time (min)	%B
	0	40
	5	60
	5.2	90
	6.8	90
	7	40
	8.5	40

Flow Rate: 5 µL/min
Injection Volume: 20 µL
Temperature: 40 °C
LC System: SCIEX M5 MicroLC
Detection: MRM
Detector: SCIEX Triple Quad™ 7500

MRM Conditions

Polarity: Positive
Source Temperature: 300 °C
GS1: 30
GS2: 80
CUR: 35
CAD: 11
IS: 4000 V

MRM Parameters

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

Revision: 0
PHEN-RUO-00183

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Results and Discussion

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.

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.

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**).

Calculated concentrations for all calibration points were within ±15 % of the nominal value (**Table 1**). As shown in **Table 1**, 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, 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.

Figure 1. Amino Acid Sequence of Human ANP.

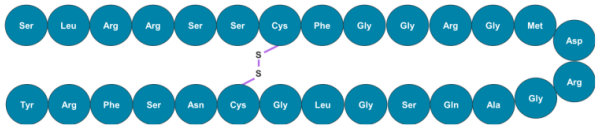

Figure 2. XICs of Matrix Blank and LLOQ of Human ANP.

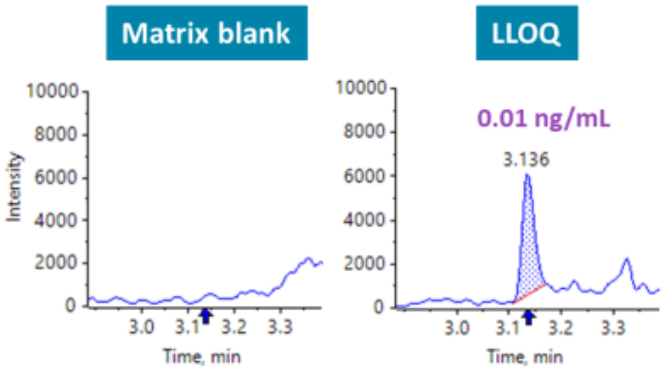

Figure 3. Calibration Curve for Human ANP.

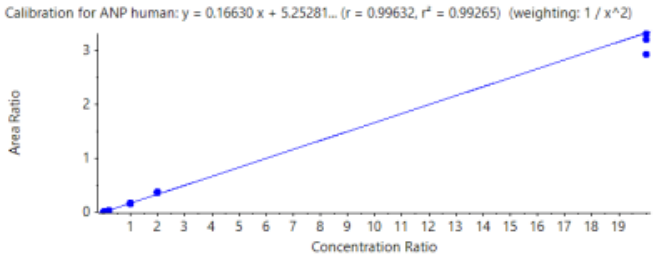

Table 1. Concentration, Accuracy, and Precision for Human ANP.

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

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Luna™ Ordering Information

3 µm and 5 µm Micro LC Columns (mm)								Trap Column	Trap Column
Phases	**50 x 0.30**	**100 x 0.30**	**150 x 0.30**	**50 x 0.50**	**100 x 0.50**	**150 x 0.50**	**250 x 0.50**	**20 x 0.30**	**20 x 0.50**
3 µm C8(2)	00B-4248-AC	—	—	00B-4248-AF	—	—	—	—	—
3 µm C18(2)	00B-4251-AC	00D-4251-AC	00F-4251-AC	00B-4251-AF	00D-4251-AF	00F-4251-AF	—	—	—
3 µm Phenyl-Hexyl	—	00D-4256-AC	—	—	00D-4256-AF	—	—	—	—
3 µm NH2	—	—	00F-4377-AC	—	—	—	—	—	—
3 µm HILIC	—	—	—	00B-4449-AF	—	—	—	—	—
5 µm C8(2)	—	—	00F-4249-AC	—	—	—	—	05M-4249-AC	05M-4249-AF
5 µm C18(2)	—	—	00F-4252-AC	—	—	00F-4252-AF	00G-4252-AF	05M-4252-AC	05M-4252-AF
5 µm Phenyl-Hexyl	00B-4257-AC	—	—	00B-4257-AF	—	—	—	—	—

Kinetex™ Ordering Information

2.6 µm Micro LC Columns (mm)						
Phases	**30 x 0.3**	**50 x 0.3**	**100 x 0.3**	**150 x 0.3**	**50 x 0.5**	**150 x 0.5**
XB-C18	00A-4496-AC	00B-4496-AC	00D-4496-AC	00F-4496-AC	00B-4496-AF	00F-4496-AF
Biphenyl	—	00B-4622-AC	—	00F-4622-AC	00B-4622-AF	—
C18	00A-4462-AC	00B-4462-AC	—	00F-4462-AC	00B-4462-AF	—
EVO C18	—	00B-4725-AC	—	00F-4725-AC	00B-4725-AF	—
F5	—	00B-4723-AC	00D-4723-AC	00F-4723-AC	00B-4723-AF	—

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Australia
t: +61 (0)2-9428-6444
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
info@phenomenex.com

China
t: +86 400-606-8099
cninfo@phenomenex.com

Czech Republic
t: +420 272 017 077
cz-info@phenomenex.com

Denmark
t: +45 4824 8048
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
anfrage@phenomenex.com

Hong Kong
t: +852 6012 8162
hkinfo@phenomenex.com

India
t: +91 (0)40-3012 2400
indiainfo@phenomenex.com

Indonesia
t: +62 21 5019 9707
indoinfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
italiainfo@phenomenex.com

Japan
t: +81 (0) 120-149-262
jpinfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
nordicinfo@phenomenex.com

Poland
t: +48 22 104 21 72
pl-info@phenomenex.com

Portugal
t: +351 221 450 488
ptinfo@phenomenex.com

Singapore
t: +65 6559 4364
sginfo@phenomenex.com

Slovakia
t: +420 272 017 077
sk-info@phenomenex.com

Spain
t: +34 91-413-8613
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
nordicinfo@phenomenex.com

Switzerland
t: +41 (0)61 692 20 20
swissinfo@phenomenex.com

Taiwan
t: +886 (0) 0801-49-1246
twinfo@phenomenex.com

Thailand
t: +66 (0) 2 566 0287
thaiinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
www.phenomenex.com/chat

All other countries/regions
Corporate Office USA
t: +1 (310) 212-0555
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