MicroLC-MRM^{HR} Workflow for Cyclic Peptide Quantitation in Human Plasma

Featuring the SCIEX TripleTOF® 6600+ System with OptiFlow™ Turbo V Source and M5 MicroLC system

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Introduction

In biotherapeutics discovery and development, there have been increased demands identified in developing LC-MS based large molecule bioanalysis assays with better sensitivity and selectivity. Decreased drug doses and limited sample volumes in preclinical and clinical models require higher quantification sensitivity, while the complexity of biological matrices calls for the need of assay selectivity improvment.

To fulfill these requirements, low flow HPLC techniques, e.g. microLC, have been successfully applied to large molecule bioanalysis to improve sensitivity by enhancing MS ionization efficiency. Meanwhile, scientists have started to evaluate different LC-MS workflows beside the traditional unit resolution MS analysis (e.g. MRM) to pursue improved selectivity. During this process, the high-resolution accurate mass spectrometry (HRAMS) has received specific attention. Its high resolving power and mass accuracy for both MS1 and MS/MS analysis can efficiently differentiate the target analyte from the interference molecules with similar m/z, therefore providing better selectivity than unit resolution quantitation methods. In this study, a novel HRAMS workflow, microLC-MRM^{HR} is demonstrated to quantify cyclic peptide desmopressin in human plasma, by using a SCIEX TripleTOF® 6600+ system with OptiFlow Turbo V Source and a M5 microLC system.

Key Features of the MicroLC-MRM^{HR} Workflow

- The M5 MicroLC system provides:
  - Microfluidic flow control for accurate flow rates down to 1 µL/min
  - Trap-elute option for fast and large volume sample loading
  - Flexibility to couple with any microLC column
- OptiFlow Turbo V Source provides:
  - Easy setup with no probe or electrode position optimization
  - Robust performance and long electrode lifetime
- MRM^{HR} analysis offered by SCIEX TripleTOF 6600+ system provides
  - High sensitivity and speed to perform the MRM-like quantitation
  - High resolution and mass accuracy that offer enhanced selectivity for large molecule bioanalysis in complex biological matrices
  - Full scan MS/MS that enables post-acquisition optimization of quantitative performance by selecting the best fragment ions and the best resolution for generating extracted ion chromatograms (XICs)

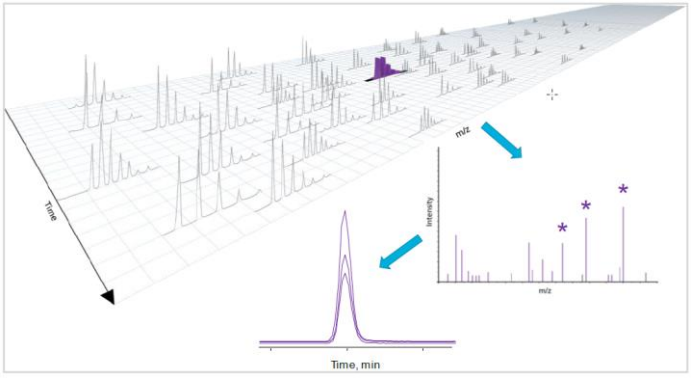

Figure 1. MRM^{HR} workflow using the TripleTOF Systems. In the MRM^{HR} workflow, a fixed number of analytes are targeted and high-resolution MS/MS spectra are collected across an LC run. Precursor masses are selected at narrow resolution such that mainly the target compound is passed into the collision cell (top). This produces a full scan MS/MS spectrum enriched for the analyte of interest (right). Then, any number of fragment ions can be extracted at high-resolution post-acquisition to generate MRM-like data (bottom).

Methods

Sample Preparation: The sample preparation procedure was modified from the previously published technical note[1] to improve extraction efficiency. Desmopressin standard was spiked in 0.3 mL of human plasma with serial dilution to reach the final concentrations as 1-500 ng/mL. Desmopressin D5 was spiked into the samples at 100 ng/mL as the internal standard. The human plasma samples were diluted with equal volume of 5% acetic acid in water, and subsequently loaded on weak cation exchange cartridges (WCX, Waters, Milford, MA, USA) which were preconditioned with 1 mL of methanol followed by 1 mL of 100 mM ammonium acetate in water. The loaded cartridges were washed with 1 mL of 5% ammonium hydroxide in water and 2 mL of methanol. Analytes were then eluted with 5% acetic acid in methanol and dried in a speedvac vacuum concentrator at 40 °C. Samples were reconstituted in 0.1 mL of 0.1% acetic acid in water and injected for LC-MS/MS analysis.

LC-MS conditions for microflow analysis: Samples were analyzed in triplicates using a SCIEX TripleTOF 6600+ system with an OptiFlow Turbo V Source containing a 25µm SteadySpray™ electrode, coupled with an M5 MicroLC system in trap-elute mode. The data was generated using Analyst® TF software 1.8 and processed using MultiQuant™ software 3.0. Table 1 and Table 2 describe the chromatographic conditions for analyte trapping and analyte separation, respectively. The optimized MS parameters are listed in Table 3 and Table 4.

Table 1: Chromatographic conditions for microflow analysis: analyte trapping.

Parameter	Value
Stationary phase	*Phenomenex Luna 5 µm, C18 Trap Column, 20 x 0.3 mm*
Mobile phase A	*0.1% acetic acid in water*
Mobile phase B	*0.1% acetic acid in acetonitrile*
Flow rate	*40 µL/min*
Column temperature	*Room Temperature*
Injection volume	*30 µL*

Time	Flow Rate (µL/min)	%A	%B
0	40	90	*10*
2	40	*90*	*10*
3	40	*90*	*10*

Table 2: Chromatographic conditions for microflow analysis: analyte separation.

Parameter	Value
Stationary phase	*Phenomenex Kinetex 2.6 µm, XB-C18 Column, 50 x 0.3 mm*
Mobile phase A	*0.1% acetic acid in water*
Mobile phase B	*0.1% acetic acid in acetonitrile*
Flow rate	*5 µL/min*
Column temperature	*40 ºC*

Time	Flow Rate (µL/min)	%A	%B
0	5	90	*10*
1	5	*90*	*10*
2.5	5	*60*	*40*
4	5	*60*	*40*
4.5	5	*5*	*95*
10	5	*5*	*95*
10.1	5	*90*	*10*
12	*5*	*90*	10

Table 3: MS conditions for microflow analysis.

TOF MS scan

Scan range (Da)	Accumulation Time (s)	DP	CE
100-1000	0.1	50	*10*

TOF MS/MS scan (MRM^{HR})

Name	Q1 (Da)	Enhanced MS (Da)	DP	CE	CXP
Desmopressin	535.222	328.209	50	26	10
Desmopressin D5	537.737	328.209	50	22	10

Table 4: Source/gas parameters for micro flow analysis.

Parameter	Value	Parameter	Value
Curtain gas:	25	CAD gas:	*High*
Ion source gas 1:	20	*Ion spray voltage:*	*4500*
Ion source gas 2:	*20*	*Source temperature:*	*100*

LC-MS conditions for analytical flow analysis: To compare the sensitivity difference between analytical flow and microflow analysis, the samples were also analyzed by the same TripleTOF 6600+ system coupled with an ExionLC™ AD HPLC system. The data was generated using Analyst TF 1.8 software and processed using MultiQuant software 3.0. Table 5 describes the LC conditions for analytical flow analysis. The source/gas parameters were reoptimized at 0.5 mL/min flow rate and summarized in Table 6.

Table 5: Chromatographic conditions for analytical flow analysis.

Parameter	Value
Stationary phase	*Phenomenex Kinetex C18 column, 50 x 2.1 mm*
Mobile phase A	*0.1% acetic acid in water*
Mobile phase B	*0.1% acetic acid in acetonitrile*
Flow rate	*0.5 mL/min*
Column temperature	*40 ºC*
Injection volume	*30 µL*

Time	Flow Rate (ml/min)	%A	%B
0.0	0.5	95	*5*
1.0	0.5	*95*	*5*
2.5	*0.5*	*60*	*40*
3.0	*0.5*	*60*	*40*
3.5	*0.5*	*5*	*95*
8.0	*0.5*	*5*	*95*
8.1	*0.5*	*95*	*5*
10.0	*0.5*	*95*	*5*

Table 6. Source/gas parameters for analytical flow analysis.

Parameter	Value	Parameter	Value
Curtain gas:	35	CAD gas:	*High*
Ion source gas 1:	60	*Ion spray voltage:*	*5500*
Ion source gas 2:	*60*	*Source temperature:*	*650*

MRM^{HR} Method Development

The mechanism of the MRM^{HR} workflow offered by the TripleTOF 6600+ system is to acquire full scan TOF MS/MS spectra for species of interest. The high resolution extracted ion chromatograms (XICs) of specific product ions or a sum of ions can then be used post-acquisition for quantitation to achieve optimum sensitivity and selectivity (Figure 1).

MRM^{HR} workflow is ideal for quantifying various biotherapeutics in the biological matrix, for two core experimental attributes. Firstly, MS/MS-based high-resolution quantitation offers significantly better selectivity than TOF MS1 based quantitation. As shown in Figure 2, when comparing TOF-MS and MRM^{HR} data for the plasma sample with 2.5 pg/mL desmopressin, the XIC peak of fragment ion at m/z 328.21 from the MS/MS scan (right) shows significantly higher s/n over the XIC peak of desmopressin precursor ion at m/z 535.22 from the TOF-MS scan (left). Secondly, the selection of signature fragment ions and the definition of XIC extraction window width can be performed after data acquisition. To identify the most appropriate extraction window width, a series of XIC extractions with window widths 0.2, 0.1, 0.05, 0.02 and 0.01 Da was performed for the plasma sample with 2.5 pg/mL desmopressin. The XIC with 0.05 Da extraction window shows no significant decrease of signal intensity and better resolution comparing with XICs with 0.2 and 0.1 Da extraction windows (Figure 3), therefore 0.05 Da window was used for the following quantitation data processing.

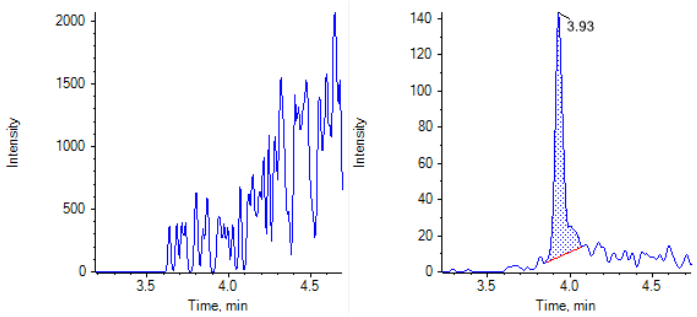

Figure 2. XIC comparison between TOF MS and MS/MS analysis of the plasma sample with 2.5 pg/mL desmopressin. Left: the XIC of desmopressin precursor ion at m/z 535.22 from the TOF MS scan; Right: the XIC of the fragment ion at m/z 328.21 from the MS/MS scan.

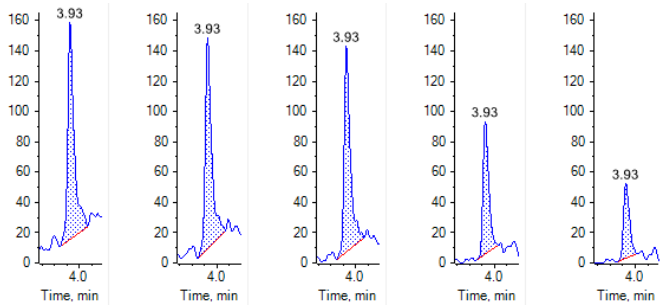

Figure 3. XIC comparison of different XIC extraction window widths in the MS/MS analysis of the plasma sample with 2.5 pg/mL desmopressin. From left to right are with window widths 0.2, 0.1, 0.05, 0.02, 0.01 Da.

Sensitivity Enhancement by Using MicroLC

To determine the sensitivity difference between the microflow and analytical flow analysis, the same set of samples was analyzed with both microflow and analytical flow LC-MS systems with the same injection volume. A ~3 folds improvement in sensitivity was observed among all plasma samples with different desmopressin levels (Figure 4).

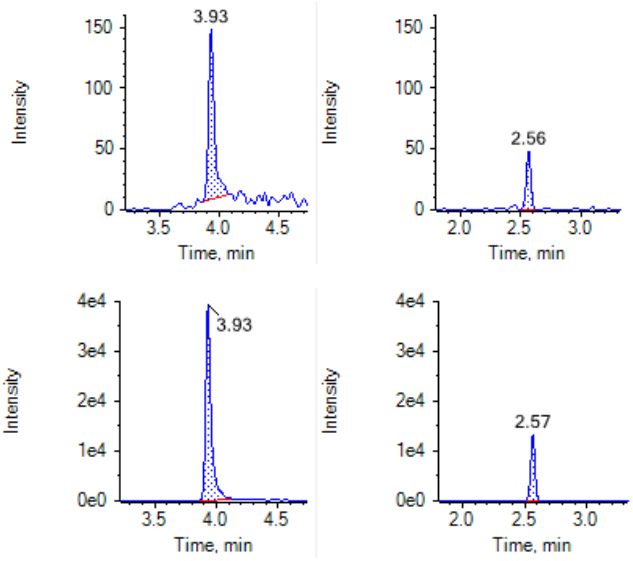

Figure 4. XIC comparison between micro flow (left) and analytic flow (right) analysis for plasma samples with desmopressin at 2.5 pg/mL (top) and 500 pg/mL (bottom).

Quantitation Results

With the optimized LC-MS conditions, the presented microLC-MRM^{HR} assay provided solid quantification of cyclic peptide in human plasma at 2.5 pg/mL as the LLOQ and 1 pg/mL as the LOD (Figure 5). As summarized in Table 7, the quantitation reproducibility and accuracy were evaluated. The assay accuracy is 85-111%, and CV%s are below 7% for all tested samples. The calibration curve cover the linear dynamic range (LDR) from 2.5 to 500 pg/mL and displayed a regression coefficient (r) of 0.99 using a weighting of $1/x^2$. Please note that plasma samples with concentrations higher than 500 pg/mL were not evaluated in this work, because they are out of the concentration range for desmopressin in clinical or preclinical models.

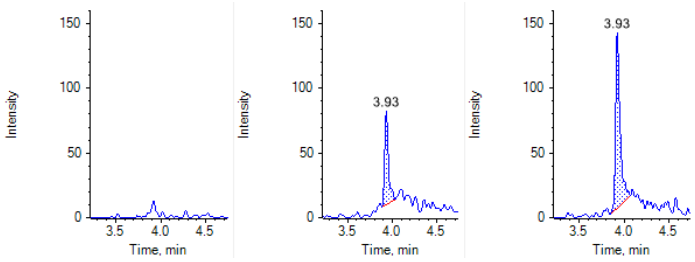

Figure 5. Selected XICs of MRM^{HR} analysis for desmopressin in human plasma. From left to right: matrix blank, 1 pg/mL (LOD), 2.5 pg/mL (LLOQ).

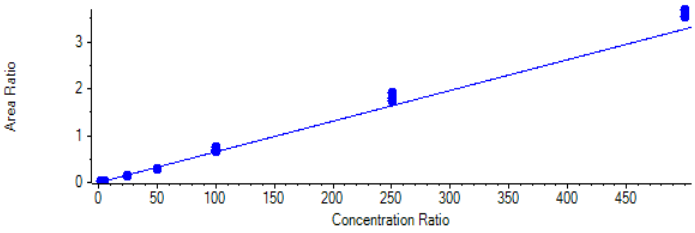

Figure 6. The calibration curve for desmopressin quantification in plasma (2.5-500 pg/mL).

Table 7: Quantitation summary.

Actual Conc. (pg/mL)	Calculated Conc. (pg/mL)	Accuracy (%)	CV (%)
2.5	2.7	107.8	3.6
5	4.4	87.6	8.4
25	21	85.4	2.6
50	45	89.9	4.8
100	109	108.7	6.9
250	278	111.2	4.7
500	547	109.4	2.4

Conclusion

A microLC-MRM^{HR} workflow for cyclic peptide quantification in human plasmawas presented by using SCIEX TripleTOF 6600+ system with OptiFlow Turbo V Source and M5 microLC system. The utilization of microLC significantly improved the quantitation sensitivity, while the MRM^{HR} analysis dramatically reduced the biolgical interferences in human plasma, therefore, improved the assay selectivity. Desmopressin was detected at 1 pg/mL level and quantified at 2.5 pg/mL level with high accuracy and reproducibility and was in human plasma. This study demonstrates a successful integration of microLC with HRAMS analysis (MRM^{HR}) for large molecule bioanalysis for improved sensitivity and selectivity.

References

1. Baghla R., Guttikar S., et al. A Sub-picogram quantification method for desmopressin in plasma using the AB SCIEX Triple Quad^{TM} 6500 System, SCIEX Technical Note

Document number: RUO-MKT-02-9440-A

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