Universal Solution for Monoclonal Antibody Quantification in Biological Fluids Using Trap-Elute MicroLC-MS Method

Featuring the SCIEX QTRAP® 6500+ LC-MS/MS System with OptiFlow™ Turbo V source and M5 MicroLC system

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LC-MS has been routinely adopted for biologics quantitation serving as the orthogonal technology to the traditional ligand binding assays (LBAs). As the amount of biological sample that can be collected from a small animal is limited and/or quantitative, studies requiring ultra-low-level detection demonstrate the importance of microflow LC-MS methodologies. The implementation of microflow chromatographic technique and immunoaffinity based sample preparation method provides significant improvement on assay sensitivity. Microflow LC provides multiple fold boost on signal intensity, while immunoaffinity based sample preparation dramatically improves the sample cleanness, thereby reducing baseline interference[1]. Herein a hybrid LBA/microflow LC-MS/MS workflow for ultra-sensitive quantification of SILuLite SigmaMAb universal antibody (SILuLite) in mouse plasma is presented. This method can be simply transferred to quantitation assays for any other human mAbs in an animal matrix with minimum modification.

Key Feature of the SCIEX Immunoaffinity-MicroLC-MS/MS Solution

- M5 MicroLC system provides:
  - Microfluidic flow control for accurate flow rate down to 1 µL/min
  - Trap-elute option for fast and large volume sample loading
  - Flexibility to couple with any microflow LC column
- OptiFlow™ Turbo V Source on the QTRAP® 6500+ LC-MS/MS system provides (Figure 1):
  - Easy setup with no probe or electrode position optimization required
  - Robust performance and long electrode lifetime
- Optimized immunoaffinity sample preparation provides:
  - Decreased sample complexity and matrix interference
  - Desired assay linear dynamic range
  - Shortened sample preparation time

Methods

Immunocapture of Target Analyte: (Figure 2) A streptavidin coated immunoaffinity magnetic bead slurry was aliquoted and washed with PBS Buffer (1x) three times. Biotinylated Goat Anti-Human IgG Antibody (0.5 mg/mL) was added to the beads and incubated at room temperature for 1 hour with shaking. The conjugated beads were washed three times and re-suspended in PBS Buffer (1x). Calibration standard samples were prepared as 25 µL mouse plasma spiked with the SILuLite standard. The concentrations of SILuLite in plasma are 2, 10, 50, 100, 500, 1000, 5000, 10000 and 20000 ng/mL. SILuMab was used as the internal standard. To each calibration standard sample, 100 µL of PBS Buffer (1x), 50 µL conjugated bead slurry and internal standard were added, and the mixtures were incubated at room temperature for 1 hour with shaking. The beads were accumulated by magnetic stand and washed sequentially with PBS Buffer (1x) and 10 mM ammonium bicarbonate. The target proteins were eluted by incubating the beads with 0.1% TFA in water with vortexing for 10 min.

Protease Digestion of Immuno-Enriched Eluents: The eluents were transferred to 96 well plate wells and neutralized with 1 mM calcium chloride in 500 mM ammonium bicarbonate in water.

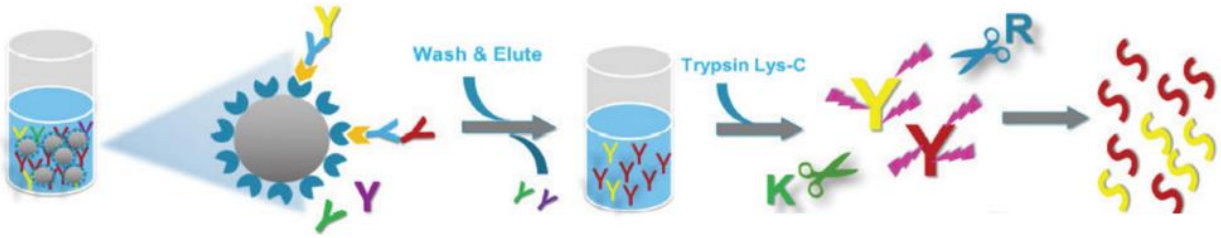

Figure 2. Sample Preparation Workflow.

The sample plate was placed into a deep well thermo-shaker and incubated at 95 °C for 10 mins with shaking. The plate was cooled to room temperature, and 1 µg of trypsin/lysineC was added to each sample. The trypsin digestion was conducted by incubating the samples for 1 hour at 50 °C with gentle shaking and aborted by adding formic acid. The supernatants from the samples were subjected to LC-MS/MS analysis.

LC-MS Conditions for Microflow Analysis: Each sample was analyzed in triplicate by a QTRAP 6500+ LC-MS/MS system coupled with M5 MicroLC system at the trap-elute mode. Table 1 describes the chromatographic conditions for analyte trapping. Table 2 describes the chromatographic conditions for analyte separation. During sample loading, the analytes were trapped and desalted on the trap column. During analyte separation, the auxiliary valve was at "inject" position for the first 5 min to connect the trap column with analytical column.

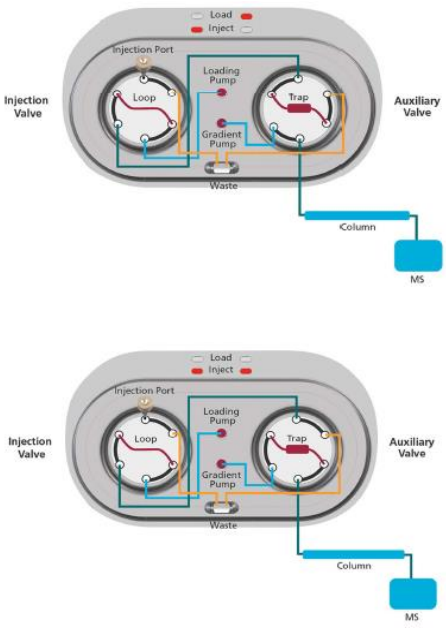

Figure 3. Valve Configurations. Diagrams for plumbing of valve for "Load" position (top) and "Inject" Position (bottom).

At 5 min, the auxiliary valve was switched to "load" position to connect the trap column with the loading pump for column washing (Figure 3).

MS analysis was performed on a QTRAP 6500+ LC-MS/MS system with OptiFlow Turbo V Source with a 25 µm SteadySpray™ probe and electrode. The OptiFlow Turbo V Source requires no physical adjustment of the probe or electrode positions. The optimized MS parameters are listed in Table 3 and 5. The data were processed using MultiQuant™ 3.0 software.

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% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Flow rate	*50 µL/min*
Column temperature	*Room Temperature*
Injection volume	*30 µL*

Time	Flow Rate (µL/min)	%A	%B
0	50	100	*0*
7	50	*100*	*0*
8	50	*10*	*90*
9	50	*10*	*90*
9.1	50	*100*	*0*
12	50	*100*	*0*

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% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Flow rate	*5 µL/min*
Column temperature	*40 ºC*
Injection volume	*NA*

Time	Flow Rate (µL/min)	%A	%B	Comment
0	5	*97*	*3*	
5	5	*65*	*35*	*Valve Load*
5.2	5	*10*	*90*	
9.8	5	*10*	*90*	
10	5	*97*	*3*	
12	5	*97*	*3*	

LC-MS Conditions for Analytical Flow Analysis: To identify the sensitivity difference between analytical flow and microflow analysis, each sample was analyzed by a QTRAP 6500+ LC-MS/MS system coupled with a a Shimadzu Prominence HPLC system. Table 4 describes the liquid chromatography conditions for analytical flow analysis. The MRM parameters are identical as the microflow analysis (Table 3). The source/gas parameters were optimized at 0.7 mL/min flow rate, as summarized in Table 5. The data were processed using MultiQuant Software 3.0.

Table 3. MS Conditions for Microflow Analysis.

Name	Q1	Q3	DP	CE	CXP
GPSVFPLAPSSK1	593.8	699.4	78	28	15
GPSVFPLAPSSK2[1]	593.8	846.5	78	28	15
FNWYVDGVEVHNAK1	560.0	708.8	60	22	15
FNWYVDGVEVHNAK2	560.0	615.7	60	23	15
GPSVFPLAPSSK[H][2]	597.8	854.5	78	28	15
FNWYVDGVEVHNAK[H][2]	562.9	713.3	60	23	15

1Most suitable transition for quantification
2Internal standard transitions

Table 4: Chromatographic Conditions for Analytical Flow Analysis.

Parameter	Value
Stationary phase	*Phenomenex Kinetex C18 column, 50 x 3.0 mm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Flow rate	*0.7 mL/min*
Column temperature	*40 ºC*
Injection volume	*30 µL*

Time	Flow Rate (ml/min)	%A	%B
0.0	0.7	95	*5*
0.7	0.7	*95*	*5*
0.8	*0.7*	*90*	*10*
3.5	*0.7*	*75*	*25*
4.0	*0.7*	*60*	*40*
4.5	*0.7*	*10*	*90*
6.0	*0.7*	*10*	*90*
6.1	*0.7*	*95*	*5*
7.5	*0.7*	*95*	*5*

Table 5: MRM Source / Gas Parameters for Microflow and Analytical Flow Analysis.

Source/Gas Parameter	Microflow Value	Analytical Flow Value
Curtain Gas:	25	30
Ion Source Gas 1:	20	65
Ion Source Gas 2:	*15*	*65*
CAD gGs:	*High*	*High*
Ion Spray Voltage:	*5000*	*5500*
Source Temperature:	*150*	*600*

Results and Discussion

The signature peptide selection follows the criteria considering ionization and fragmentation efficiency, baseline cleanness in matrix, and post-translational modifications. These peptides have conserved amino acid sequences that are identical in immunoglobulin G (IgG). Therefore, the MRM method can be applied to other IgG based biotherapeutics without modification. For each peptide, the MRM transition with the highest S/N was selected for quantitation purpose. To achieve the desired assay sensitivity with limited sample volume (25 µL plasma per sample), the microflow chromatographic technique and immunoaffinity based sample preparation method were implemented. A 5 µL/min HPLC flow rate was applied for improved ionization efficiency; a trap-elute LC profile was used to increase sample injection volume and shorten sample loading time. The MRM parameters for signature peptides were optimized for both microflow and analytical flow analysis. An immunocapture based sample preparation was performed to minimize the matrix interference.

With the optimized method condition, the presented microflow assay achieved an LLOQ of 2 ng/mL for SILuLite quantification in mouse plasma (Figure 4). As summarized in Table 6, the assay accuracy is 87-109%, and CV%s are below 15% for all tested samples. The calibration curve covered 4.5 orders of magnitude (1-20000 ng/mL) (Figure 5) and displayed a regression coefficient (r) of 0.996 using a weighting of $1/x^2$.

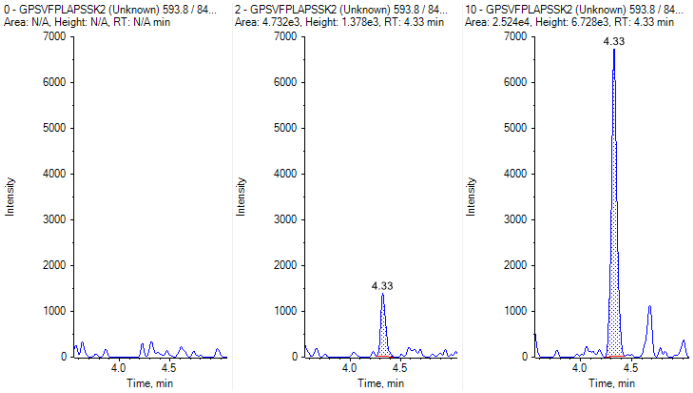

Figure 4. MRM Data for SILuLite. Extracted ion chromatograms (XICs) of selected MRM for SILuLite. From left to right are Blank, 2 ng/mL, 10 ng/mL.

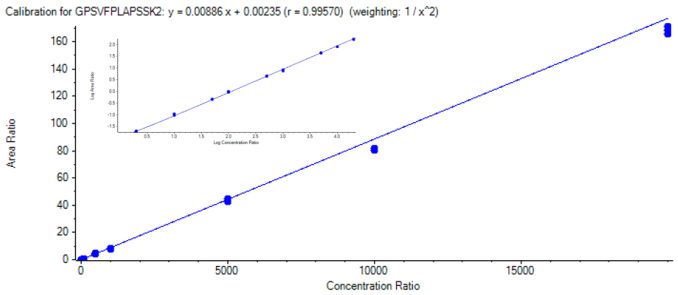

Figure 5. Calibration Curve for SILuLite. Concentration Ratio-Area Ratio and log Concertation Ratio-log Area Ratio for quantitation of SILuLite in mouse plasma (2 ng/mL to 20000 ng/mL).

To identify the sensitivity difference between the microflow and analytical flow analysis, the same set of samples were analyzed on both microflow and analytical flow LC-MS systems with the same injection volume. As shown in Figure 6, a >3 fold increase on peak area and >2 fold increase on S/N were observed on the low concentration standards.

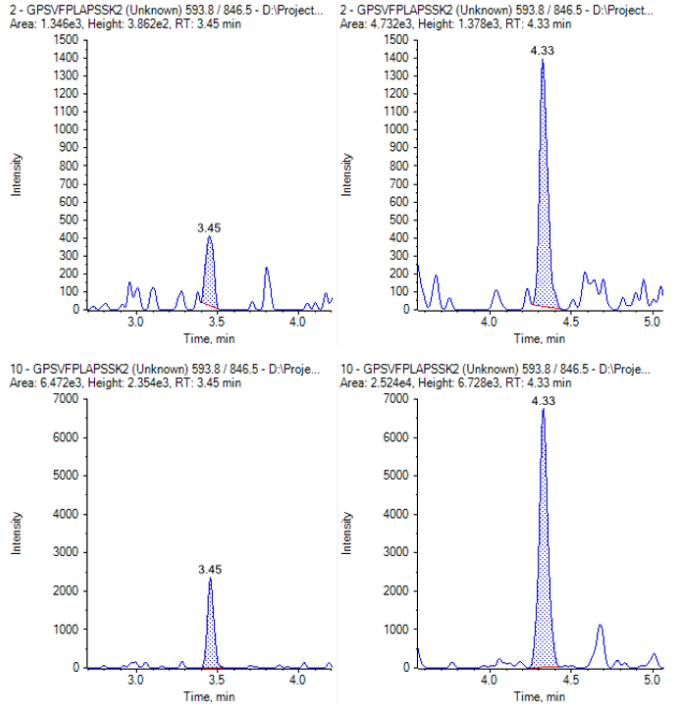

Figure 6. Microflow vs Analytical Flow Signal Comparison. Extracted ion chromatograms (XICs) of selected MRM for SILuLite at 2 ng/mL (top) and 10 ng/mL (bottom). The left are XICs generated with analytical LC flow rate, the right are XICs generated with microLC flow rate.

Conclusion

A universal immunoaffinity-microLC-MS/MS method for quantifying SILuLite in mouse plasma was developed. By utilizing the QTRAP 6500+ LC-MS/MS system with OptiFlow Turbo V Source coupled with M5 MicroLC system, SILuLite was quantified at 2 ng/mL level with high reproducibility, 4 orders of dynamic range and minimum source optimization requirement. This method can be applied to preclinical quantitation assays of other IgG based biotherapeutics with minimum modification.

References

1. Zhang F., Li Y., etc, Quantification of Trastuzumab in Rat Plasma using an Improved Immunoaffinity-LC-MS/MS Method, SCIEX Technical Note

Table 6: Quantitation Summary.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
2	1.94	96.8	6.1
10	11.5	114.5	7.5
50	52.3	104.7	2.3
100	108.6	108.6	3.5
500	508.2	101.6	1.0
1000	887.5	88.8	4.6
5000	4895.9	97.9	3.3
10000	9200.6	92.0	0.9
20000	19022.9	95.1	1.6

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