Ultra-Sensitive Quantification of Trastuzumab Emtansine in Mouse Plasma using Trap-Elute MicroLC MS Method

Featuring SCIEX QTRAP® 6500+ System with OptiFlow™ Turbo V source and M5 MicroLC system

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Quantification of monocolonal antibodies (mAbs) and antibody-drug conjugates (ADCs) in biological fluids play a critical role across multiple stages of the biotherapeutic development. 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, sensitivity improvement for biologic quantitation experiments has driven the need for sensitivity improvement for LC-MS assays. The implementation of microflow chromatography and immunoaffinity based sample preparation methodologies provide significant improvement on assay sensitivity. Micro-LC provides a multiple fold increase in signal/noise, while immunoaffinity based sample preparation dramatically improves the sample cleanness, thereby reducing baseline interference[1]. Herein we introduce a hybrid LBA/MicroLC-MS/MS workflow for ultra-sensitive quantification of Trastuzumab Emtansine in mouse plasma.

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

- 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 on the QTRAP® 6500+ LC-MS/MS system provides:
  - 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 1) 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 trastuzumab emtansine standard. The concentrations of trastuzumab emtansine in plasma are 1, 5, 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. 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 was added to

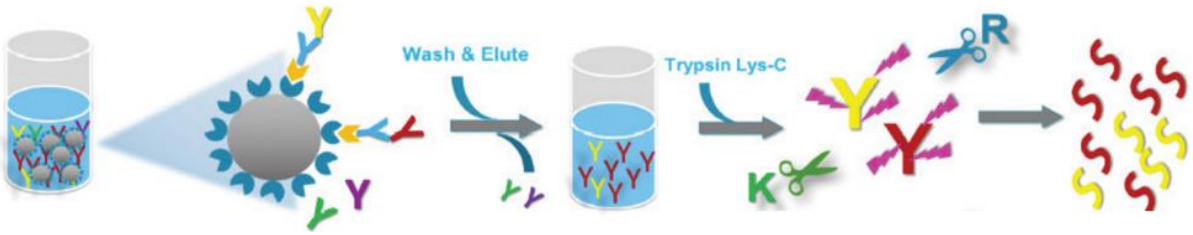

Figure 2. Sample Preparation Workflow.

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 QTRAP 6500+ mass spectrometer 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 analyte 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.

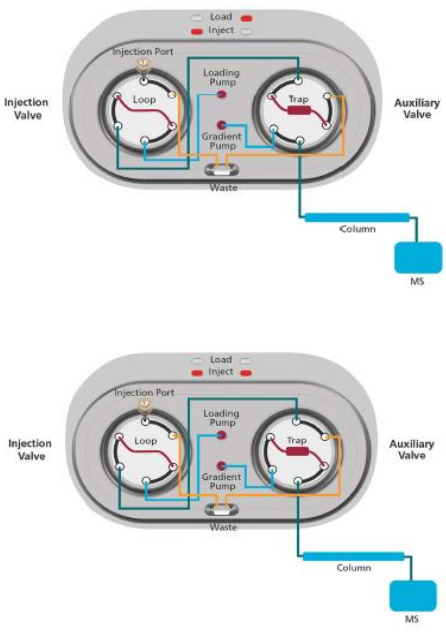

Figure 2. Valve Configurations. Diagrams for plumbing of valve for “Load” position (top) and “Inject” Position (bottom).

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 SCIEX QTRAP 6500+ 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. The data were processed using MultiQuant™ Software 3.0.

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 QTRAP 6500+ LC-MS/MS system coupled with 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, 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
IYPTNGYTR1[1]	542.8	808.4	50	24	12
IYPTNGYTR2	542.8	405.1	50	23	6
FTISADTSK1	485.2	721.3	60	21	15
FTISADTSK2	485.2	608.2	60	22	25
GPSVFPLAPSSK1	593.8	699.4	78	28	15
GPSVFPLAPSSK2	593.8	846.5	78	28	15
FNWYVDGVEVHNAK[H][2]	562.9	713.3	60	23	15
GPSVFPLAPSSK[H][2]	597.8	707.4	78	28	15

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

In order 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 method allowed to increase sample injection volume while keeping sample loading time low. The MRM parameters for signature peptides were optimized for both microflow and analytical flow analysis. A immunocapture based sample preparation was performed to minimize the matrix interference.

With the optimized method condition, the presented microflow assay achieved a LLOQ of 1 ng/mL for trastuzumab emtansine quantification in mouse plasma (Figure 3). 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 4) and displayed a regression coefficient (r) of 0.996 using a weighting of $1/x^2$.

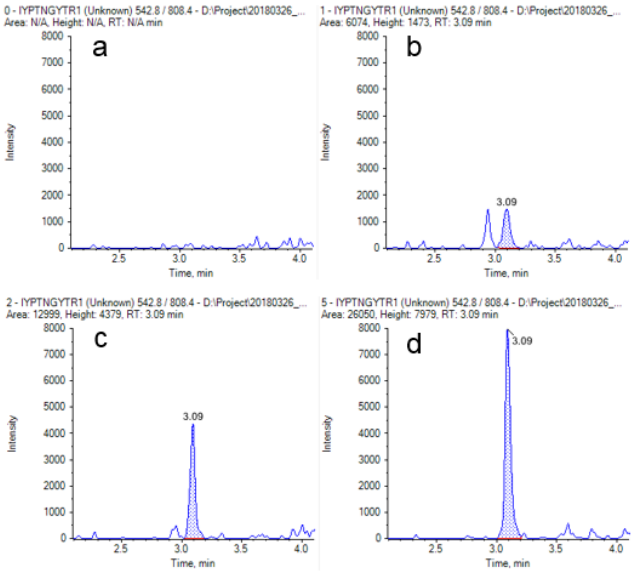

Figure 3. MRM Data for Trastuzumab. Extracted ion chromatograms (XICs) of selected MRM for trastuzumab emtansine. a) blank; b) 1 ng/mL; c) 2 ng/mL; d) 5 ng/mL.

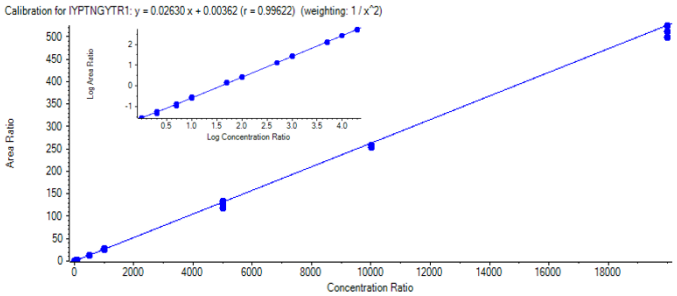

Figure 4. Calibration Curve for Trastuzumab. Quantitation of trastuzumab emtansine in mouse plasma (1 ng/mL to 20000 ng/mL).

To determine the sensitivity difference between the microflow and analytical flow analysis, the same set of samples were analyzed with both microflow and analytical flow LC-MS systems with the same injection volume. As shown in Figure 5, a >5 fold increase on peak area and 3 fold increase on S/N were observed on the low concentration standards.

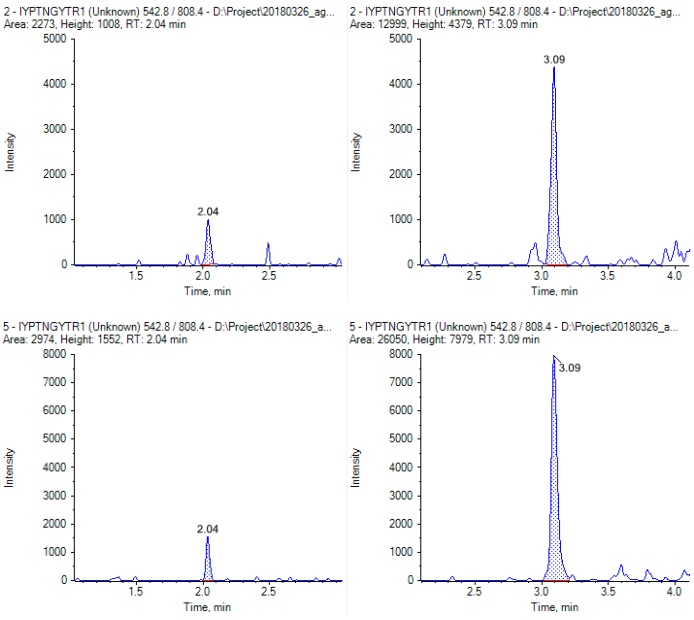

Figure 5. Microflow vs Analytical Flow Signal Comparison. Extracted ion chromatograms (XICs) of selected MRM for trastuzumab emtansine at 2 ng/mL (top) and 5 ng/mL (bottom). The left are XICs generated with analytical LC flow rate, the right are XICs generated with microLC flow rate.

Conclusion

A hybrid immunoaffinity-MicroLC-MS/MS method for quantifying transtuzumab emtansine in mouse plasma was developed. The QTRAP 6500+ LC MS/MS system with OptiFlow Source coupled with M5 MicroLC system provides reliable quantitation of ADCs at 1 ng/mL level with high reproducibility, 4.5 orders of dynamic range, high throughput and minimum source optimization requirement.

References

1. Zhang F., Li Y., etc, Quantification of Trastuzumab in Rat Plasma using an Improved Immunoaffinity-LC-MS/MS Method, SCIEX Technical Note
2. Universal Solution for Monoclonal Antibody Quantification in Biological Fluids Using Trap-Elute MicroLC-MS Method, SCIEX technical note RUO-MKT-02-8279-A.

Table 6: Quantitation Summary.

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
1	1.02	102.0	1.9
2	1.99	99.7	13.2
5	4.35	87.2	13.9
10	10.5	104.9	6.2
50	54.7	109.3	4.7
100	104.0	104.0	2.2
500	500.9	100.2	1.6
1000	1023.6	102.4	4.7
5000	4772.9	95.5	5.8
10000	9757.1	97.6	0.8
20000	19472.3	97.4	2.5

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