Improving Sensitivity in Bioanalysis using Trap-and-Elute MicroLC-MS

Using the SCIEX M3 MicroLC system for Increased Sensitivity in Antibody Quantitation

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Quantitation of monoclonal antibodies (mAbs) in biological fluids is important during all stages of antibody drug development. While traditionally immunoassays are used, more recently LC-MS has been adopted because of its high selectivity, accuracy, and precision. The antibodies can be enriched from the sample using different approaches, e.g. solid phase extraction or immunocapture, and are then digested using trypsin. Unique signature peptides are selected based on criteria such as digestion efficiency, stability after digestion, chromatographic behavior and MS-MS sensitivity, and measured using LC-MS in MRM mode. As the amount of sample that can be drawn from a small animal during DMPK studies is limited, sensitivity of an LC-MS based method becomes very important. MicroLC, using 0.3 mm ID columns at 5-20 µL/min flow rate, can improve sensitivity in LC-MS by a factor of up to 10 compared to using the more commonly used 2.1 - 3 mm ID columns at flow rates of 0.5-1 mL/min.[1,2] In this technical note we explore the use of MicroLC-MS using 0.3 mm ID columns at 8 µL/min flow rate to achieve lower LLOQ's for the quantitation of infliximab, a mAb used for the treatment of Crohn's disease. A Trap-Elute MicroLC method was used to maintain the same throughput as with High Flow LC-MS, while injecting the same sample volume. Sample is loaded at 35 µL/min onto a short trap column, followed by switching the trap column in-line with the separation column for analysis at 8 µL/min.

Key Benefits of using the M3 MicroLC system for Antibody Quantitation

- Quantitate antibodies at levels up to 10 x lower than what can be measured with High Flow LC-MS
- High throughput by using a Trap-Elute workflow
- Increased column lifetime and reduced need for cleaning of the MS by protecting the analytical column and MS from salts and other impurities with the Trap-Elute workflow

Materials and Methods

Sample preparation: Infliximab was acquired from Myoderm (Norristown, PA, USA). As internal standard SILuMab, a recombinant stable isotope labeled human mAb, was used (Sigma-Aldrich, St. Louis, MO, USA). Stock tryptic digests of both mAb's were prepared using N-octylglucoside (OGS) as denaturant, TCEP for reduction and MMTS for alkylation, all included in the SCIEX Protein Preparation kit, and TPCK treated trypsin (SCIEX) for digestion. Standards were diluted from stock with 98/2 water/acetonitrile 0.1% formic acid.

HPLC conditions – High Flow LC: A Shimadzu Prominence HPLC system was used, consisting of two LC-20AD pumps, a SIL-20AC autosampler and CTO-20A column oven. The column used was a 50 x 3 mm Kinetex C18 2.6 µm 100 Å column from Phenomenex (Torrance, CA, USA). Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with

0.1% formic acid. Wash solvent for the autosampler was 20/20/60 methanol/acetonitrile/IPA. The gradient method used is listed in Table 1. Flow rate was 0.7 mL/min. Injection volume was 20 µL, and the column was kept at 40°C.

Table1. Gradient used with the High Flow LC-MS workflow

Time (min)	%B
0	5
0.8	10
3.5	25
5	40
5.1	95
5.9	95
6	5
7	5

HPLC conditions – MicroLC: A SCIEX M3 MicroLC-TE system, consisting of two MicroLC gradients and an integrated autosampler, was used in combination with a source mounted column oven (SCIEX). As the trap, a 10 x 0.3 mm 5 µm 120 Å ChromXP™ C18 CL column (SCIEX) was used, and the analytical column was a 50 x 0.3 mm HALO Peptide ES-C18 2.7 µm 160 Å column (SCIEX).

Trapping conditions – Mobile phase A in the loading gradient was water with 0.1% formic acid, Mobile phase B was acetonitrile with 0.1% formic acid. Sample was loaded from the injection loop onto the trap column using 100% A for one minute at 35 µL/min. The trap was washed with 90% B at 70 µL/min for 2 minutes after every injection.

Separation conditions – Mobile phase A in the analytical gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. The gradient used was 3 - 40% B in 3 min, with a 1.5 min 90% B wash step. Flow rate was 8 µL/min. The column temperature was 40°C. Injection volume was 20 µL, and the autosampler needle and valve wash consisted of two cycles using mobile phase B, followed by one cycle using mobile phase A.

Table 2. Signature peptides used for Infliximab and SILuMab quantitation

Antibody	Signature Peptide
SILuMab	DTLMIS[R]
SILuMab	VVSVLTVLHQDWLNG[K]
Infliximab	ASQFVGSSIHWYQQR
Infliximab	GLEWVAEIR
Infliximab	YASESMSGIPSR

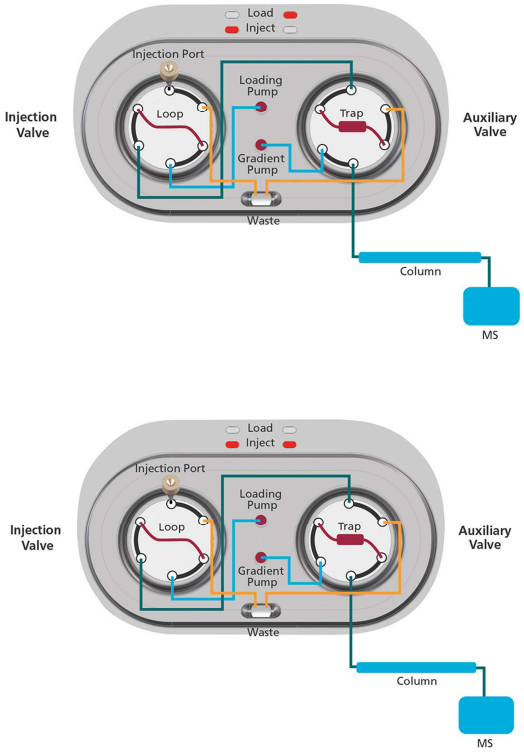

Figure 1. Valve positions for the trap loading part of the method (top) and the analysis part of the method (bottom).

Mass Spectrometry – A SCIEX QTRAP® 6500 LC-MS/MS system was used. For the MicroLC experiments the standard electrode was replaced with a 25 µm ID electrode (SCIEX). MRM transitions were developed for the peptides listed in table 2, and the source and gas parameters are listed in table 3. MultiQuant 3.0.2 software was used for data analysis.

Table3. Source and gas parameters

	High Flow LC	MicroLC
Electrode ID	100 µm	25 µm
Curtain Gas	30	20
Collision GAS	High	High
IonSpray Voltage	5500	5000
Temperature (°C)	650	300
Ion Source Gas 1	60	40
Ion Source Gas 2	60	10

Separation and Analysis time

Analysis time and separation resolution are similar between the direct inject High Flow LC-MS method and the trap-elute microLC-MS method, when taking into account the 1 minute trap loading time for the microLC method (see Figure 2).

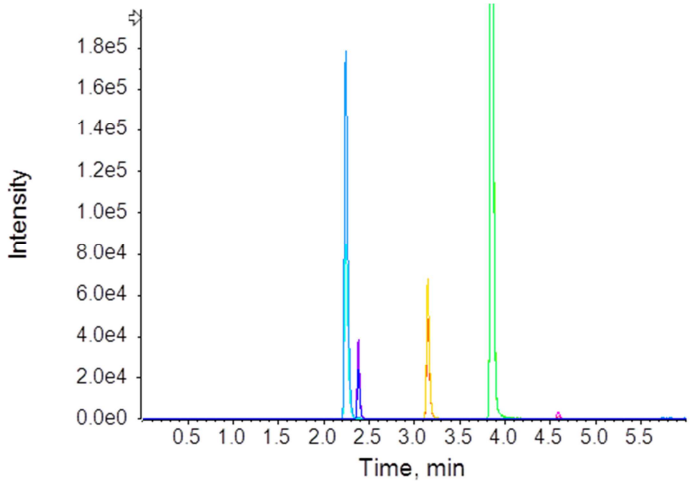

Figure 2A, XIC chromatogram for the High Flow LC-MS method.

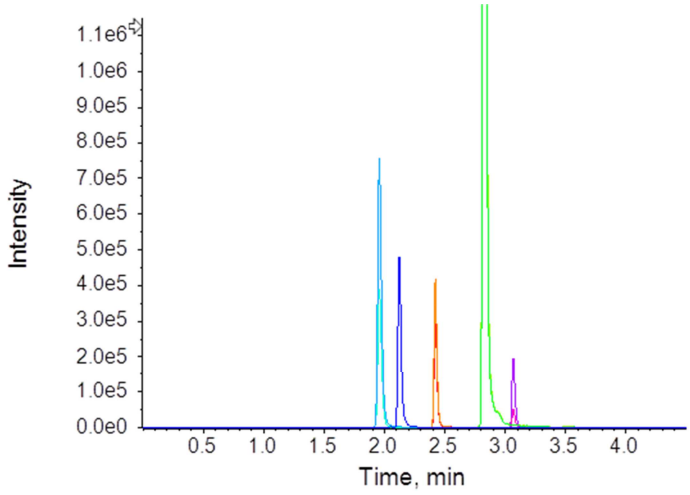

Figure 2B, XIC chromatogram for the Trap-elute MicroLC-MS method.

Sensitivity Improvement

In order to determine the improvement in sensitivity that can be achieved using MicroLC Trap-Elute versus High Flow LC, two standard quantitation curves were obtained with each concentration injected five times. The peptide used to quantitate infliximab was YASESMSGIPSR, while DTLMIS[R] was used as an internal standard. For the High Flow experiment 5 – 5000

ng/mL infliximab was measured, while 1-1000 ng/mL was measured with MicroLC. The internal standard was maintained at 100 ng/mL. An improvement in S/N of 5x was seen for the infliximab signature peptide with the MicroLC-MS method at the 5 ng/mL level. S/N at the 1 ng/mL level using MicroLC was approximately the same as S/N with the High Flow method at 5 ng/mL (see figure 3).

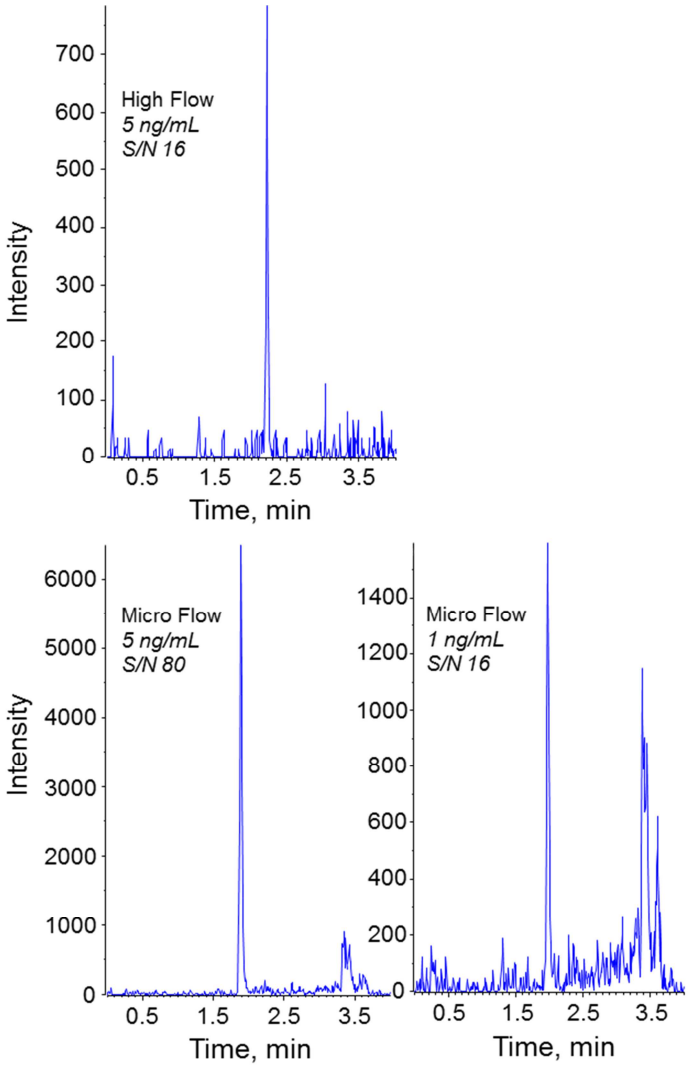

Figure 3. Sensitivity comparison between the High Flow LC-MS and MicroLC trap-elute methods.

Accuracy and precision data from both curves are summarized in Tables 4 and 5. Linear regression and a weighting of 1/x was used. R^2 for both the High Flow and MicroLC curves were 0.993. The LLOQ for both the High Flow and MicroLC-MS methods was determined using the criteria that accuracy at the LLOQ should be between 80 and 120 %, and precision should be < 20% CV. Using the MicroLC-MS method the LLOQ was improved from 10 ng/mL to 2 ng/mL, a 5-fold improvement. In order not to exceed 20% response in the first blank after an injection at the ULOQ level, the ULOQ for the MicroLC Trap-Elute method was reduced from 1000 ng/mL to 200 ng/mL. Extra washing steps may be required to reduce carry-over if a larger linear range is required.

Table 4. Standard curve date for High Flow LC-MS

Actual Concentration (ng/mL)	Mean calculated concentration (ng/mL)	Accuracy (%)	CV (%)
5.0	6.1	121.32	13.49
10.0	9.8	98.43	6.23
20.0	16.3	81.62	11.63
50.0	45.5	90.97	6.72
100.0	94.7	94.73	5.31
500.0	547.3	109.46	3.23
1000.0	1052.2	105.22	4.77
5000.0	4913.1	98.26	9.30

Table 5. Standard curve date for MicroLC-MS

Actual Concentration (ng/mL)	Mean calculated concentration (ng/mL)	Accuracy (%)	CV (%)
2.0	1.6	80.25	18.70
3.0	3.1	101.96	9.54
5.0	6.2	123.14	11.07
10.0	11.3	113.14	11.01
20.0	22.9	114.73	5.81
50.0	58.8	117.57	6.92
100.0	110.3	110.34	7.04
500.0	515.3	103.07	7.35
1000.0	961.1	96.11	2.65

Robustness

In order to determine the robustness of the MicroLC trap-elute method, a high concentration of infliximab (10 µg/mL) and SILuMab (1 µg/mL) was digested. A total of 1000 20 µL injections were made over a period of 5 consecutive days. No clogging of tubing, electrode or columns was observed. All 1000 injections were completed using the same trap and analytical column. CV % on peak area for the signature peptide used for quantitation was 4.35%, while the CV % for the SILuMab standard peptide was 6.13%.

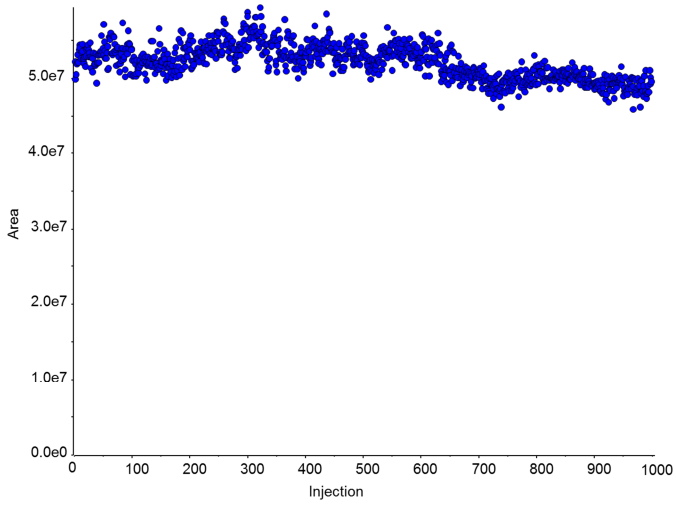

Figure 4. Area reproducibility for the peptide YASESMSGIPSR over 1,000 injections was 4.35 %.

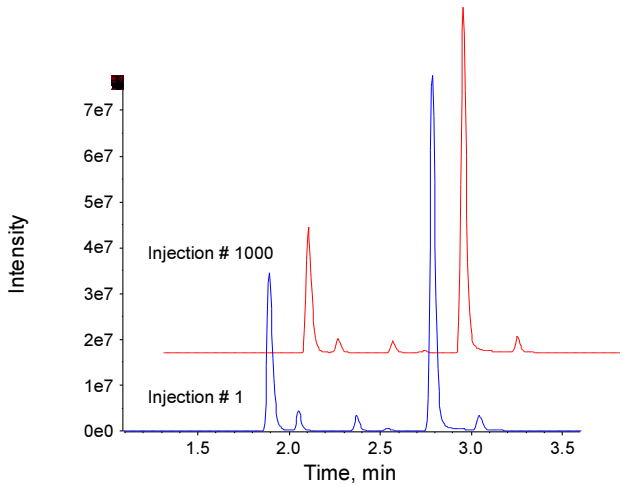

Figure 5 . Total Ion Chromatograms for injection 1 and 1000 of the robustness test. Separation and peak shapes are identical.

Conclusions

We have shown that quantitation of infliximab using its signature peptide YASESMSGIPSR can be performed with an approximately 5x higher sensitivity using a trap-elute MicroLC-MS method at 8 µL/min, compared to using a direct inject high-flow LC-MS method at 700 µL/min. The trap-elute method used ensures similar throughput while injecting the same 20 µL of sample, and protecting the MicroLC column and MS from contamination. The trap-elute MicroLC-MS workflow proved to be robust over a 1000 injections.

This workflow offers a solution for applications where Mab's need to be quantitated in small volume samples and/or low concentrations.

References

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2. Covey T.R., Schneider B.B., Kovarik, Corr, Javahari et al.;The Central Analytical Figures of Merit of ESI, MALDI, and APCI.; *Cole RB, ed. Electrospray and MALDI mass spectrometry*. 2010, Chapter 13. Hoboken: John Wiley & Sons, Inc.

Document number: RUO-MKT-02-2849-B

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