Improving Sensitivity for an Immunocapture LC-MS Assay of Infliximab in Rat Plasma 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 sample using different approaches, e.g. solid phase extraction or immunocapture, and 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 then 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 rates, can improve sensitivity in LC-MS by a factor of up to 10, compared to using the more commonly adopted 2.1 - 3 mm ID columns at flow rates of 0.5-1 mL/min.[1,2] In a previously published technical note[3] we described using Trap-Elute MicroLC-MS with 0.3 mm ID columns to achieve lower LLOQ's for the quantitation of a digest of neat infliximab, a mAb used for the treatment of Crohn's disease. The digest was 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.

Separately, SCIEX has developed a generic anti-human IgG immunocapture workflow for the selective extraction of human IgG antibodies from rat plasma[4]. In this technical note we show the results of samples prepared using this immunocapture workflow using both High Flow LC-MS and the Trap-Elute MicroLC-MS method.

Key Benefits of using the M3 MicroLC system for Antibody Quantitation

- Antibody quantitation 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 that were removed during the Trap-Elute workflow

Materials and Methods

Sample preparation: Infliximab was acquired from Myoderm (Norristown, PA, USA). An internal standard SILuMab, a recombinant stable isotope labeled human mAb, was used (Sigma-Aldrich, St. Louis, MO, USA). Rat Plasma (Sprague Dawley; K2 EDTA) was acquired from BioreclamationIVT, (Westbury, NY, USA) and spiked with various concentrations

Infliximab and 500 ng/mL SILuMab. Samples were prepared from this using a magnetic bead based immunocapture assay developed by SCIEX[4]. Streptavidin coated beads (Dynabeads M-280) were acquired from Thermo Fisher Scientific (Waltham, MA, USA), and goat anti-human IgG antibody was acquired from SouthernBiotech (Birmingham, AL, USA). In order to prepare a sufficient amount of sample allowing for 5 replicate injections with both high flow LC and microLC, the amount of plasma used for each sample was increased to 200 uL, with a final dilution volume of 400 uL, versus the standard protocol. For tryptic digestion, N-octylglucoside (OGS) was used as denaturant, TCEP for reduction and MMTS for alkylation (all included in the SCIEX Protein Preparation kit) and TPCK treated trypsin (SCIEX) was used for digestion.

Table 1. 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

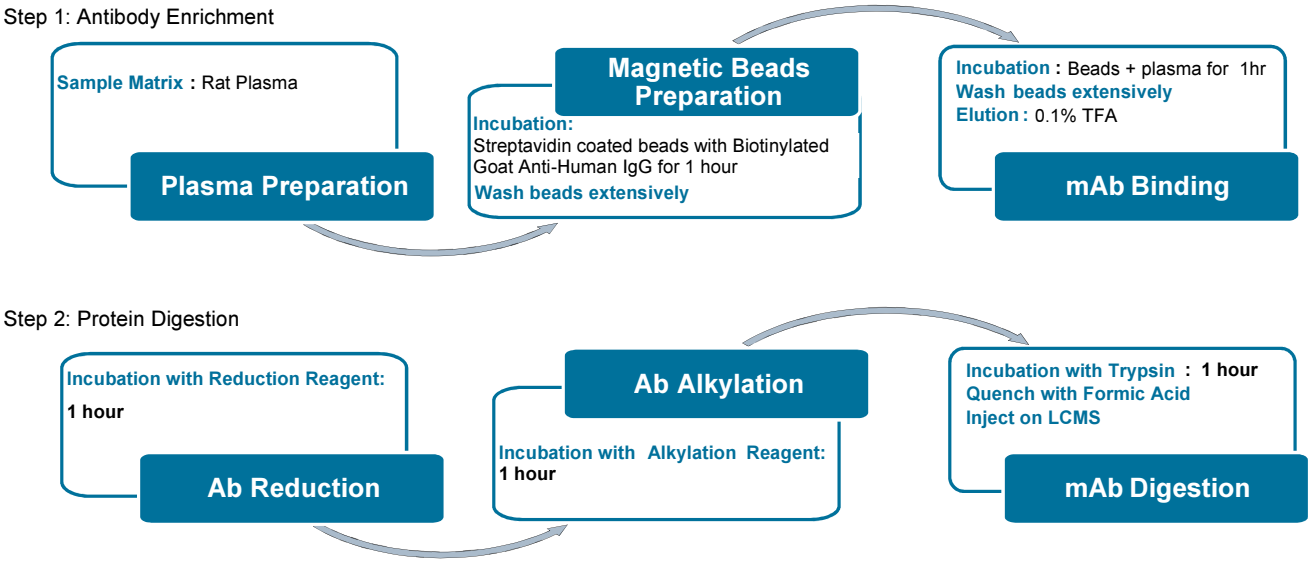
Table 2. Signature peptides used for Infliximab and SILuMab quantitation

Antibody	Signature
Infliximab	ASQFVGSSIHWYQQR
Infliximab	GLEWVAEIR
Infliximab	YASESMSGIPSR
SILuMab	DTLMIS[R]

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.

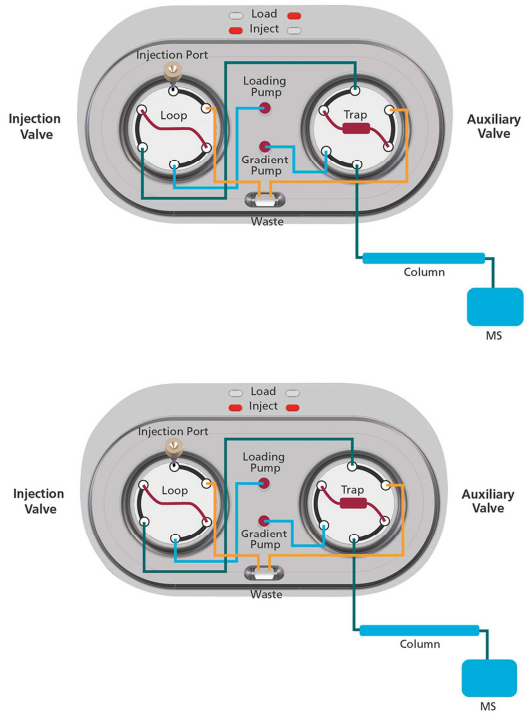
HPLC conditions – MicroLC: A SCIEX M3 MicroLC-TE system, consisting of two MicroLC gradients and an integrated autosampler, was used in combination with a source mounted column oven (SCIEX). As the trap, a 10 x 0.3 mm 5 µm 120 Å ChromXP™ C18 CL column (SCIEX) was used, and the analytical column was a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

Figure 1. Infliximab quantitation sample preparation workflow



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.

Figure 2. Valve positions for the trap loading part of the method (top) and the analysis part of the method (bottom).



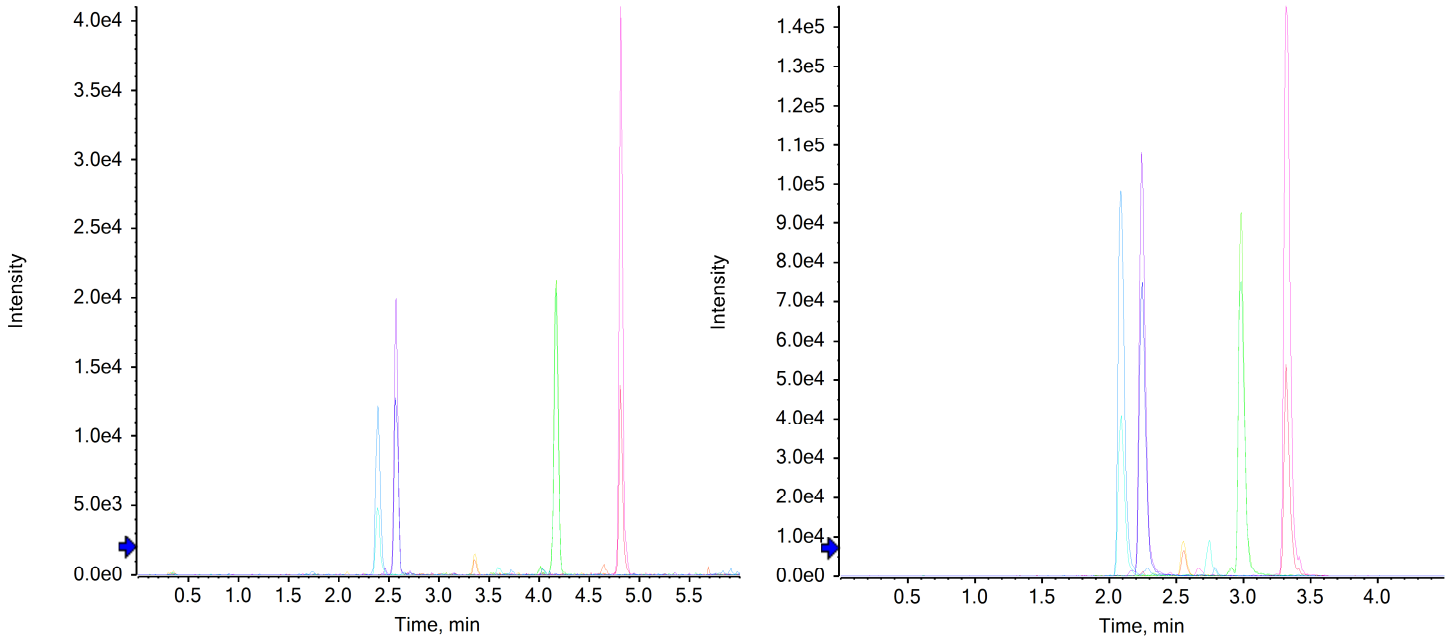
Separation conditions – Mobile phase A in the analytical gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. The gradient was 3 - 40% B in 3 min, with a 1.5 min 90% B wash step. Flow rate was 8 µL/min. The column temperature was 40°C, injection volume was 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.

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.

Table 3. 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

Figure 3. XIC of all signature peptides for the High Flow LC-MS method (left) and MicroLC method (right)



Sensitivity Improvement

Rat plasma samples were prepared by spiking varying concentrations of Infliximab and 500 ng/mL of the internal standard SiluMab. The samples were processed using immunocapture followed by enzymatic digestion. Five replicate injections with both the High Flow and Trap-Elute MicroLC methods were made using the same sample to exclude variations in response due to sample preparation. Figure 4 shows the XIC's for the Infliximab signature peptide used for quantitation (YASESMSGIPSR) in both methods at the 50 ng/mL level. S/N improved from 16 for High Flow LC-MS to 100 for the MicroLC method.

Table 4 lists the calculated concentrations for the calibration curves with the CV % and accuracies. The LLOQ's for both methods were determined using the requirements of precision < 20% and accuracy between 80 and 120% at LLOQ, and at any higher concentration a precision <15% and accuracy between 85% and 115%. LLOQ improved by a factor of 4 using the MicroLC Trap-Elute method.

Both the High Flow and MicroLC methods showed good linearity with r >0.99.

Carryover

Carryover was determined by injecting the digest of the immunocaptured extract from a blank plasma sample after an injection of the ULOQ of 5,000 ng/mL. Using High Flow LC-MS, no carry over was observed. The Trap-Elute microLC method showed slight carryover, with an area of 0.12 %. The carryover was 35% of the response at the LLOQ (12.5 ng/mL.), which is slightly higher than the required max. of 20% . See Figure 5. With additional washing of the trap column the carryover can be further reduced. Alternatively a lower ULOQ could be declared.

Conclusions

We have shown that quantitation of infliximab using its signature tryptic peptide YASESMSGIPSR in samples prepared using a magnetic bead based immunocapture method can be performed with a 4x lower LLOQ 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 ensures similar throughput while injecting the same 20 µL of sample, and protecting the MicroLC column and MS from contamination.

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

Figure 4. Sensitivity comparison between the High Flow LC-MS (left) and MicroLC-MS (right) methods at the 50 ng/mL level. S/N improved by a factor of 6.

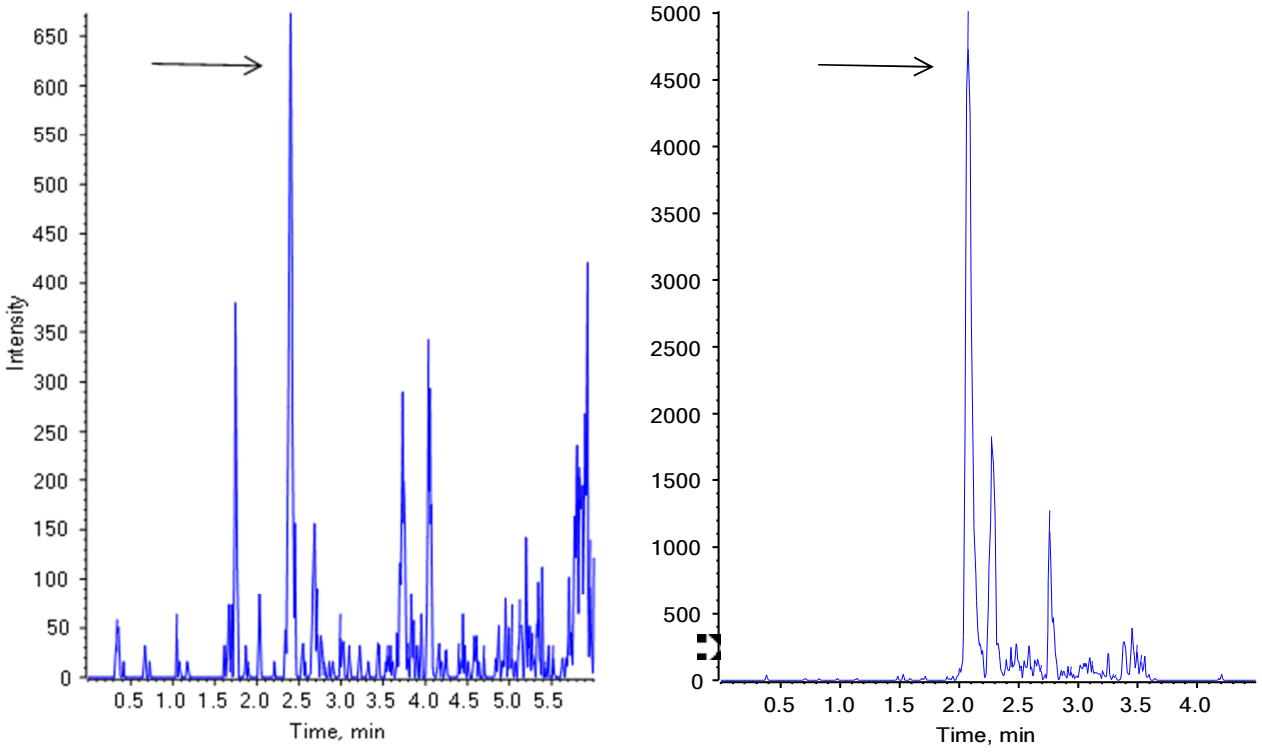
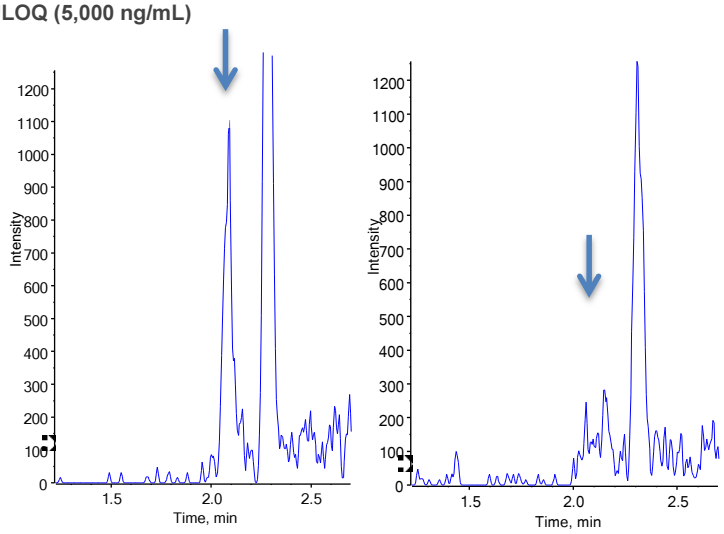


Table 4 Standard curve data for the high flow and MicroLC-MS methods

	High Flow LC-MS			Micro LC-MS		
Actual Concentration (ng/mL)	Mean calculated concentration (ng/mL)	Accuracy (%)	CV (%)	Mean calculated concentration (ng/mL)	Accuracy (%)	CV (%)
12.5				14.27	114.12	4.18
15				14.79	98.63	10.12
20				18.76	93.78	8.13
25				22.24	88.97	7.2
35				30.57	87.34	5.41
50	48.52	97.05	17.46	53.31	106.62	3.8
75	83.35	111.13	11.68	75.39	100.52	3.5
100	92.46	92.46	7.58	91.86	91.86	4.85
200	196.5	98.26	9.33	201.9	100.96	5.26
500	485	97	6.13	511	102.2	3.87
1000	1013	101.3	2.67	1089	108.93	4.74
2000	1920	96	3.72	1884	94.18	4.37
5000	5340	106.79	3.98	5594	111.88	6.26

Figure 5. XIC for the signature peptide used for quantitation at the LLOQ for the MicroLC method (12.5 ng/mL), and for a blank after injecting the ULOQ (5,000 ng/mL)



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