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

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INTRODUCTION

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 are quantified 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 is becoming a compelling alternative to conventional HPLC because of the sensitivity improvement it can offer when coupled with MS. The observed sensitivity improvement is analyte-dependent, and can be as high as 10x [1,2]. When injecting similar volumes as typically injected in conventional HPLC, throughput is sacrificed because of the time it takes to load the sample onto the column using flow rates as low as 5 µL/min. In this presentation we describe an on-line pre-concentration (Trap-and-Elute) MicroLC method that allows for sample loading at flow rates of 35 µL/min or higher on a short trap column, while performing the LC-MS analysis at much lower flow rates to achieve the highest sensitivity possible.

We studied the sensitivity improvement that can be achieved using the trap-and-elute microLC method for the quantitation of infliximab, a mAb used for the treatment of Crohn's disease, in rat plasma. Samples were prepared using a generic anti-human IgG immunocapture workflow.

MATERIALS AND METHODS

Sample Preparation:

Infliximab was acquired from Myoderm (Norristown, PA, USA). As an 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.

Rat Plasma (Sprague Dawley; K2 EDTA) was acquired from BioreclamationIVT, (Westbury, NY, USA) and spiked with Infliximab and SILuMab. Samples were prepared from this using a magnetic bead based immunocapture assay developed by SCIEX[3]. 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.

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.

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

Table 1. Gradient used with the High Flow LC-MS workflow

	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

Table 3. Source and gas parameters

MicroLC

A SCIEX M3 MicroLC-TE system, consisting of two MicroLC gradient pumps 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 1.5 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.

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

Table 2. Signature peptides used for Infliximab and SILuMab quantitation

MS/MS Conditions:

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 all data analysis. Data was subjected to a 1 point Gaussian smoothing, and a $1/x^2$ weighting was used for the linear regression of the calibration curves.

RESULTS

Separation and Throughput comparison

Figure 1 shows a comparison of the high flow direct injection LC-MS method and the MicroLC-MS trap-elute method. Separation between the two method is similar. Taking into account the additional 1 min. loading time for the MicroLC trap-elute method, throughput is maintained with the MicroLC method, while loading the same 20 uL of sample as was injected in the high flow method.

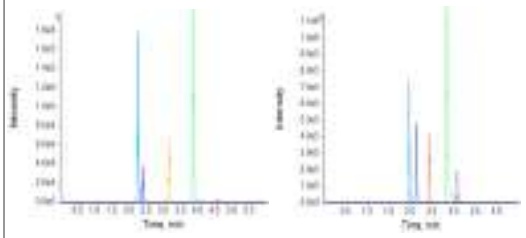

Figure 1. XIC chromatograms for the High Flow LC-MS method (left) and Trap-Elute Micro LC-MS method (right)

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 1,000 20 µL injections were made over a period of 5 consecutive days. No clogging of tubing, electrode or columns was observed. All 1,000 injections were completed using the same trap and analytical column. CV % for peak area for the signature peptide used for quantitation was 4.35%, while the CV % for the SILuMab standard peptide was 6.13%.

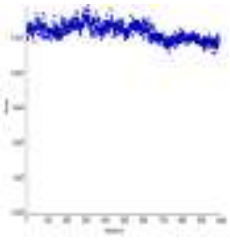

Figure 2. Area reproducibility for the peptide YASESMSGIPSR over 1,000 injections was 4.35 %.

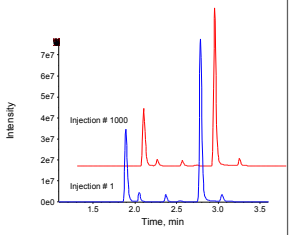

Figure 3. Total Ion Chromatograms for injection 1 and 1000 of the robustness test. Separation and peak shapes are identical.

Sensitivity Improvement

Rat plasma was spiked with both Infliximab and the internal standard SiluMab, and prepared using immunocapture and digestion. 5 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.

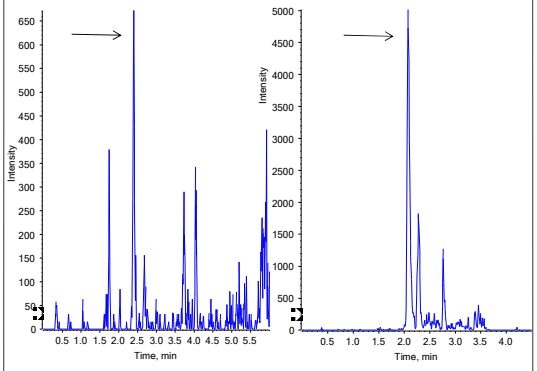

Figure 4. Sensitivity comparison between the High Flow LC-MS (left) and MicroLC-MS (right) methods at the 50 ng/mL level. S/N improved with a factor of 6.

Table 5 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 a precision < 20% and accuracy between 80 and 120% at the LLOQ, and a precision <15% and accuracy between 85% and 115% for any higher concentration. LLOQ improved with a factor 4 using the MicroLC Trap-Elute method.

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

	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

Table 5. Standard curve data for the high flow and MicroLC-MS methods

Carryover

Carryover was determined by injecting the 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, determined by 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% . With additional washing of the trap column the carryover can be reduced. Alternatively a lower ULOQ could be declared.

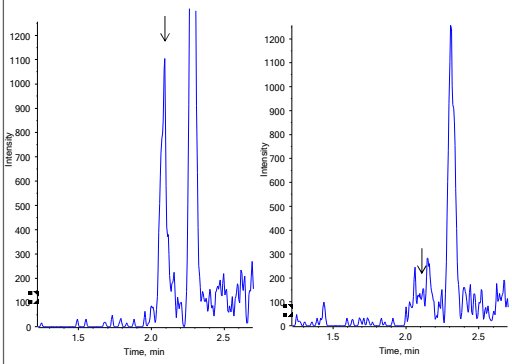

Figure 5. XIC for the signature peptide used for quantitation at the LLOQ for the MicroLC method (12.5 ng/mL; left), and for a blank after injecting the ULOQ (5,000 ng/mL, right)

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 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 quantified in small volume samples and/or low concentrations.

REFERENCES

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