A novel high-throughput microflow UHPLC method for LC-MS/MS quantitation

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ABSTRACT

Microflow liquid chromatography is becoming a compelling alternative to conventional UHPLC for many analyses due to its potential for higher throughput, low sample consumption and solvent savings. These benefits are realized while maintaining or enhancing sensitivity in comparison to conventional UHPLC
In high-throughput laboratories, where sensitivity is the primary goal of an LC/MS method, a larger injection volume (comparable to that used in conventional LC) is often desired. Injecting larger sample volumes with microLC can often present challenges such as sample loading ability and limiting higher throughput.
This study investigates the feasibility of on-line pre-concentration as a method to load larger volumes of sample onto a microLC column while maintaining the LC/MS system throughput and column lifetime.

INTRODUCTION

The complexity of the biopharmaceutical molecular entity brings challenges to the biopharmaceutical industry to manufacture safe and effective biosimilar products. For a successful biopharmaceutical drug development, reliable bioanalytical techniques, that enable quantitation of drugs in biological fluids (plasma, urine, tissue, etc.), are required to generate toxicokinetic, pharmacokinetics, and bioavailability data. Analytical techniques, such as ELISA, are frequently used to provide this information. Liquid chromatography coupled with mass spectrometry (LC/MS) is a compelling technique that has great advantages in biopharmaceutical applications. Advantages of LC/MS methods are fast to develop, potential to overcome interference from anti-antibody response, improved assay precision, and using isotope labeled internal standard making absolute quantification possible.
This study investigates the feasibility of on-line pre-concentration using microLC configured in trap elute as a method to load large volumes of sample onto a microLC column. This configuration maintains the LC/MS system throughput and column lifetime. Infliximab in serum samples were analyzed using the trap elute microLC-MS/MS method and the results were compared with those obtained using a direct inject microLC and a conventional flow LC-MS/MS methods.

MATERIALS AND METHODS

Sample Preparation:
Sample: Infliximab spiked in buffer (Phosphate-buffered saline, PBS ,with 0.01% BSA) to prepare concentrations 5 to 1000 ng/mL.
Sample matrix: Rat serum aliquots (100 µL each) were spiked with 10 µL of appropriate sample solutions to obtain the desired concentration.
Internal standard: SILu™MAB (Stable-Isotope Labeled antibody standard, Sigma) as the IS at a constant level.
Processing: Immunoenriched with magnetic beads (Dynabeads® Streptavidin, Life Technologies) and processed as outlined below and demonstrated in Figure 1.
Capture: Sample is loaded onto the magnetic beads to bind to the magnetic beads.
Elute: Sample is eluted from the magnetic beads using 0.1% TFA.
Digest: Sample is digested using trypsin.

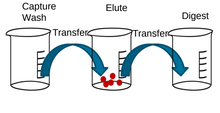

Figure 1. Representation of the workflow of sample processing

Figure 2. SCIEX M3 MicroLC-TE System

HPLC Conditions:

MicroLC System:
The liquid chromatography system is a SCIEX M3 MicroLC-TE System (Figure 2). It is configured with a 5-50 µL/min binary gradient pump, and a 20-200 µL/min binary pump as the loading pump. The system has dual valves for injection and trap loading and a column oven. A fast dynamic load and wash (DLW) autosampler is integrated with the system.

LC Conditions:
Mobile Phase A: Water with 0.1 % Formic acid
Mobile Phase B: Acetonitrile with 0.1 % Formic acid
Flow rate: 10 µL /min
Analytical Column: Eksigent Halo Peptide C18 50x0.3 mm 2.7 µm
Trap column: Eksigent C18 10x0.3 mm, 5 µm
Sample loop: 50 µL
Injection volume: 30 µL
Column temperature: 40 ºC
Autosampler wash solvents: Wash 1: Mobile phase B. Wash 2: Mobile phase A
Gradient run is outlined in table 1.
Trap loading: Isocratic at 60 µL /min with 2% mobile phase B in mobile phase A
Loading time: 1.5 minute

Table 1. Gradient program for microLC

Time	%B Concentration
0.00	5
0.25	5
0.85	20
2.5	60
2.90	95
3.30	95
3.40	5
4.20	5

Conventional LC System:
Shimadzu Prominence LC is a low pressure gradient system configured with binary pumps, autosampler and a column oven.

LC Conditions:
Mobile Phase s: as in microLC.
Flow rate: 700 µL /min.
Analytical Column: Phenomenex Kinetex C18 50x3 mm 2.65 µm.
Sample loop: 50 µL .
Injection volume: 30 µL.
Column temperature: 40 ºC.
Autosampler rinse solvent: 60% Isopropanol, 20% acetonitrile and 20%. methanol
Gradient run is outlined in table 2.

Table 2. Gradient conditions for conventional method

Time	B Concentration
1.00	5
1.10	15
3.50	25
4.00	40
4.10	95
5.00	95
5.10	5
6.50	5

MS/MS Conditions:
SCIEX QTRAP 5500® LC-MS/MS system with Turbo V™ source was used to acquire the data. Electrospray Ionization (ESI) 25 µm ID probe was used with the microLC system while the standard probe (100 µm ID) was used with the conventional LC system. MS data were collected in Multiple Reaction Monitoring (MRM) mode. 3 signature peptides and internal standard were detected using 2 MRM transitions per peptide to allow quantitation and identification based on the ratio of quantifier and qualifier transitions, MRM transitions for the signature peptides are listed in table 3 . Each concentration was analyzed in triplicate.

Table 3. Peptide ID and MRM transitions

Peptide ID	Q1 mass (Da)	Q3 mass (Da)	
ASQFVGSSIHWYQQR.+3y10 LC	598.6	917.3	
ASQFVGSSIHWYQQR.+3y11 LC	598.6	780.4	
GLEWVAEIR.+2y4 HC	536.8	773.4	
GLEWVAEIR.+2y5 HC	536.8	587.4	
YASESMSGIPSR.+2y3 LC	642.8	1050.4	
YASESMSGIPSR.+2y10 LC	642.8	359.1	
DTLMIS[R].heavy 1	423.2	629.4	IS
DTLMIS[R].heavy 2	423.2	516.3	IS

RESULTS:

LC performance comparison:
LC-MS/MS novel technique based on microLC was developed for quantitation of therapeutic monoclonal antibody in serum. The workflow uses on-line pre-concentration in a trap column using a loading pump then eluting the trapped analytes onto an analytical column. Figures 3-5 show the XIC chromatogram of the peptides using conventional LC, microLC direct injection and microLC trap elute respectively.

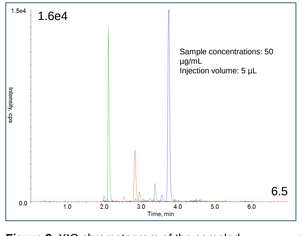

Figure 3. XIC chromatogram of the sampled peptides using conventional LC system

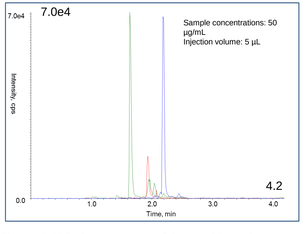

Figure 4. XIC chromatogram of the peptides using microLC direct inject system

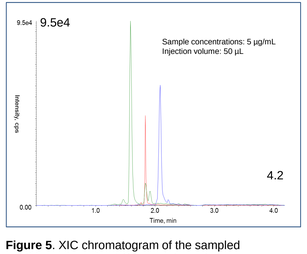

Figure 5. XIC chromatogram of the sampled peptides using microLC trap elute system

Quantitation:
The improved performance and the ability to load larger sample volume allow improved quantitation for peptides. Figure 6 shows an example of a low intensity peak with excellent S/N ratio using microLC trap elute. The concentration of the sample is 10 ng/mL in serum.

When injecting the same amount on the column from same concentration, the direct inject microLC based separation showed a gain in signal intensity for all peptides over that of conventional LC based separation by at least 3X. Using on-line pre-concentration microLC, it was possible to load larger sample volume than that of direct inject microLC and in a shorter time. Figure 5 shows a gain in signal intensity 1.5X over microLC direct injection (or 4.5X over conventional LC). The microLC provides higher throughput than that of conventional LC and the direct inject microLC. The run time using microLC was 35% shorter than the run by conventional LC. The results also show no loss of chromatographic performance due to the use of trap column.
The results of this work demonstrate the ability to inject larger sample volumes and improve the quantitation limit without changing the chromatographic performance and sacrificing throughput.

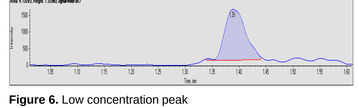

Figure 6. Low concentration peak

The results obtained with the calibration samples prepared once and analyzed in triplicate are shown in figures 7 and 8 and table 4. Figures 7- 8 show the calibration curve for the signature peptide YASESMSGIPSR.+2y10 LC using the peak area ratio to the internal standard.

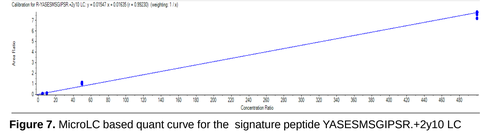

Figure 7. MicroLC based quant curve for the signature peptide YASESMSGIPSR.+2y10 LC

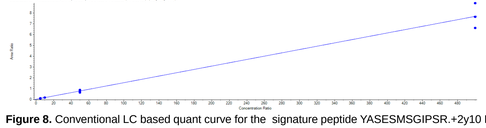

Figure 8. Conventional LC based quant curve for the signature peptide YASESMSGIPSR.+2y10 LC

MicroLC				
C (ng/mL)	Num. Values	Measured C (ng/mL)	% CV	Accuracy
5	3 of 3	5.65	6.35	112.97
10	3 of 3	8.52	15.49	85.19
50	3 of 3	51.00	6.68	101.91
500	3 of 3	502.00	7.52	100.34
Conventional LC				
5	3 of 3	5.38	20.14	106.15
10	3 of 3	9.77	1.96	97.74
50	3 of 3	48.75	14.88	95.96
500	3 of 3	502.1	14.98	100.41

Table 4. Assay results and statistics for microLC and conventional LC

Table 4 shows the quant results, %CV and accuracy of the assay for both microLC and conventional LC. Figure 7 and table 4 show that the microLC trap elute results show better %CV for the lower point of measurement compared to that of the conventional LC, this improved %CV allows better quantitation.

CONCLUSIONS

A fast novel LC-MS/MS workflow for peptide quant was developed using therapeutic monoclonal antibody. The sample was prepared using immunoenriched magnetic beads binding, followed by elution and digestion. The workflow using microLC trap elute demonstrated the ability to inject large sample volume in short time. The results also demonstrated higher signal intensity with microLC trap elute when compared to microLC direct inject and conventional LC. The lower %CV on peak area for the lowest concentration observed with microLC allows for improved quantitation. In this workflow, the lower limit of quantitation (LLOQ) of signature peptide YASESMSGIPSR.+2y10 LC 642.8/359.1 transition was found to be 5ng/mL with excellent accuracy.
The results of this work demonstrate excellent performance of the microLC trap elute system without compromising the chromatographic performance and sacrificing throughput. These benefits were realized in addition to extending the life of the analytical column, and protecting the MS source from contamination. Future work is focusing on investigating LLOQ at a lower concentration.

TRADEMARKS/LICENSING