Improved sensitivity for LC-MS quantitation of biologics in plasma using Trap-and-Elute MicroLC-MS

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INTRODUCTION

Quantitation of peptide/protein biotherapeutics in plasma is important during all stages of drug development. While traditionally immunoassays have been used for the quantitation of such analytes, more recently LC-MS has been adopted because of its high selectivity, accuracy, and precision. The antibodies or peptides can be enriched from the sample using different approaches, e.g. solid phase extraction or immunoaffinity enrichment. As the volume of blood drawn from a small animal during DMPK studies is limited, sensitivity of an LC-MS based method has become critically important. In this presentation we describe how sensitivity can be improved by using microflow LC for the analysis of the peptide drugs glucagon and insulin glargine, the monoclonal antibody infliximab, and the Antibody Drug Conjugate (ADC) ado-trastuzumab emtansine.

MATERIALS AND METHODS

Sample Preparation: Solid phase extraction using a mixed-mode sorbent was used for glucagon [1]. Insulin glargine, infliximab and ado-trastuzumab emtansine were enriched using immunoaffinity with high capacity streptavidin coated magnetic beads (SCIEX) and biotin labeled antibodies against the targeted analytes. Immuno-enriched proteins were eluted and digested using Trypsin or Trypsin/Lys-C. Signature peptides of the proteins of interest were selected based on criteria such as digestion efficiency, stability after digestion, chromatographic behavior and MS/MS sensitivity. Isotopically labeled analogs to the targeted analytes were used as internal standards.

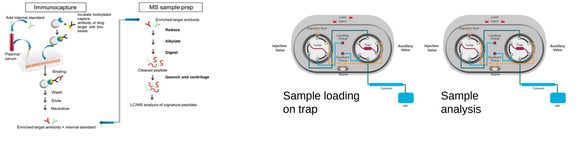

Figure 1. BioBA immuno-capture enrichment workflow.

Figure 2. Valve positions for the Trap-and-Elute microflow LC.

Analytical Liquid Chromatography: A SCIEX ExionLC™ AD HPLC system was used for the high flow LC-MS analysis. The columns used were a 50 x 3 mm Kinetex C18 2.6 µm 100 Å column or a 100 x 2.1 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. A reversed phase gradient method was used for separation of the signature peptides[1-4]. The column temperature was kept at 40˚ C. Injection volume was 20-35 µL.

Microflow Liquid Chromatography: A SCIEX M3 MicroLC-TE system, with two microLC gradient pumps and an integrated autosampler was used in combination with a source mounted column oven (SCIEX). A 10 x 0.3 mm trap column packed with 5 µm 120 Å ChromXP C18 CL (SCIEX) and either a 50 x 0.3 mm HALO Peptide ES-C18 2.7 µm 160 Å (SCIEX) or 0.2 x 100 mm MonoCap C18 Monolith (GL SCIENCES) analytical column was used. Mobile phase A for the analytical gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. Flow rate was 8-10 µL/min. The column temperature was set to 40˚ C. Injection volume was 20-35 µL. For sample trapping, mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. Sample was loaded from the injection loop onto the trap column using 100% A at 25 or 50 µL/min flow rate. After analysis, the trap was washed with 95% B at 50 µL/min for 5 minutes.

Mass Spectrometry: A SCIEX QTRAP® 6500+ with IonDrive™ Turbo V source was used. For the microflow LC experiments, the standard electrode was replaced with a 25 µm ID electrode (SCIEX). Source and gas parameters were optimized for both analytical and microflow LC-MS/MS [1-4]. MultiQuant™ 3.0.2 software (SCIEX) was used for 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. Sample for both microflow and Analytical flow LC-MS analysis was prepared on the same day to exclude variations in response due to sample preparation. Three to five replicate LC-MS injection were acquired for both the analytical flow and trap-and-elute microflow LC analysis.

THROUGHPUT AND ROBUSTNESS

Similar chromatography of the peptides was seen between analytical and microflow LC. As an example Figure 3 shows a comparison of the analytical flow direct injection LC-MS method and the microflow LC-MS method for infliximab. Taking into account the additional 1 min loading time for the Trap-And-Elute Microflow LC-MS method, throughput is maintained, while loading the same 20 uL of sample as was injected in the analytical flow method.

In order to determine the robustness of the Trap-And-Elute Microflow LC-MS 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 analyzed over a period of 5 consecutive days (figure 4). 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%.

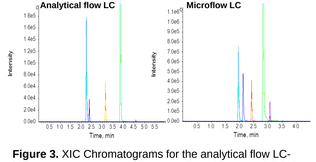

Figure 3. XIC Chromatograms for the analytical flow LC-MS method (left) and the Trap and-Elute Microflow LC-MS method (right) for the infliximab and IS signature peptides.

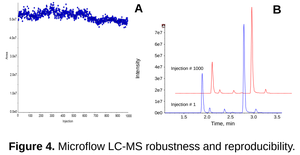

Figure 4. Microflow LC-MS robustness and reproducibility. Peak area reproducibility for the infliximab peptide YASESMSGIPSR over 1,000 injections was 4.35 % (A) . Total Ion Chromatograms for injection 1 and 1000 show identical separation and peak shapes. (B)

IMPROVED SIGNAL TO NOISE RATIO (S/N)

S/N ratio of the (signature) peptides for the assays studied increased from 3.5 – 6 x using microflow LC instead of analytiol flow LC (See table 3). As an example, figure 5 shows the XIC's for glucagon with both methods at the 0.5 ng/mL level. Signal improved by a factor 10, and S/N improved by a factor of approximately 5.

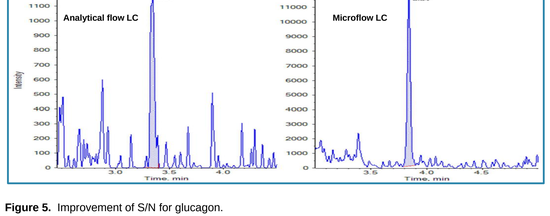

Figure 5. Improvement of S/N for glucagon.

IMPROVED SENSITIVITY

In order to determine how much the LLOQ's for the studied assays were improved by the increased S/N ratio when using MicroLC, standard curves for each assay were measured with both analytical flow LC-MS/MS and microflow LC-MS/MS. Tables 1 and 2 show the comparisons for insulin glargine and ado-trastuzumab emtansine . 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 at any higher concentration a precision <15% and accuracy between 85% and 115%. The LLOQ's for all assays were 4-5x improved with microflow LC (see table 3).

Table 1. Microflow LC–MS/MS analysis of insulin glargine in human plasma. Calibration curve data for analytical flow and microflow LC shows 5x lower LLOQ with microflow LC.

	Traditional LC-MS/MS			MicroLC-MS/MS		
Actual Concentration (pg/mL)	Mean calculated concentration (pg/mL)	Accuracy (%)	CV (%)	Mean calculated concentration (pg/mL)	Accuracy (%)	CV (%)
10	N/A	N/A	N/A	10.2	102	10
25	N/A	N/A	N/A	23.0	92	14
50	49.2	98	13	50.9	102	3.4
100	95.7	96	9.6	104	104	5.8
500	482	96	1.4	486	97	10
1,000	990	99	0.61	1,056	106	3.4
5,000	5,276	105	1.3	4,888	98	1.3
10,000	10,300	103	1.3	9,922	99	0.50

Table 2. Microflow LC–MS/MS analysis of ado-trastuzumab emtansine in rat plasma. The calibration curve data for the signature peptide IYPTNGYTR from the total antibody assay of ado-trastuzumab emtansine showed a 5X increase in sensitivity with a wider linear dynamic range with microflow LC-MS compared to analytical LC-MS.

	Traditional LC-MS/MS			Microflow LC-MS/MS		
Actual Concentration (ng/mL)	Mean Calculated Concentration (ng/mL)	Accuracy (%)	CV (%)	Mean Calculated Concentration (ng/mL)	Accuracy (%)	CV (%)
1	N/A	N/A	N/A	1.00	100.42	3.24
5	4.63	92.63	7.48	4.87	96.92	7.61
10	11.50	115.00	7.30	10.43	104.29	9.37
50	49.65	99.29	8.05	46.94	93.89	6.35
100	99.52	99.52	1.62	89.38	89.38	2.41
1,000	921.6	92.16	0.71	921.6	92.16	4.38
10,000	10,120	101.24	4.00	10,930	109.27	0.56
25,000	26,270	105.08	2.04	27,720	110.86	3.04
50,000	52,720	105.44	1.41	54,780	109.57	3.04
100,000	89,650	89.65	2.93	93,250	93.25	4.35

CARRYOVER

In order to reduce carryover when using microflow LC to the same level as what is seen with analytical flow LC, the trap column is washed at a high flow rate off-line from the analytical column utilizing the M3 MicroLC gradient loading pump. As an example, using this wash method, we observed carryover of <0.01% for insulin glargine (Figure 6). For all analytes the carryover after injecting the highest concentration of the linear range (ULOQ) was <20% of the response at the LLOQ.

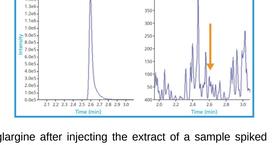

Figure 6. Carryover for insulin glargine after injecting the extract of a sample spiked at the 10,000 pg/mL level. Carryover based on peak intensity was less than 0.01%, and low enough to declare 10,000 pg/mL as the ULOQ.

Table 3. Sensitivity improvements using M3 MicroLC. Improvement in S/N resulted in improved LLOQ's for the quantitation of the peptide drugs glucagon and insulin glargine, the monoclonal antibody infliximab, and the antibody drug conjugate ado-trastuzumab emtansine.

Analyte	Matrix	Sample Used (µL)	S/N Improvement	LLOQ Improvement	Linear Dynamic Range (ng/mL)
Infliximab	Rat plasma	50	6	4	12.5-5,000
Ado-trastuzumab emtansine	Rat plasma	50	4	5	1-100,000
Insulin glargine	Human plasma	250	3.5	5	0.01-10
Glucagon	Rat plasma	25	5	5	0.1-100

CONCLUSIONS

- MicroLC-MS/MS improves the LLOQ's for the studied peptide and mAb drugs with a factor of 4-5x compared to using analytical LC-MS/MS
- Linear dynamic range and carryover are equal or better when using microLC versus using analytical flow LC
- The Trap-And-Elute workflow allows for similar throughput and robustness as analytical flow LC-MS/MS, while injecting the same injection volume
- Immunocapture-Based Target Enrichment is an effective sample preparation method for insulin glargine and mAb's quantitation by LC-MS/MS

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