# Improved Sensitivity for LC-MS Quantitation of Trastuzumab Emtansine in Rat Plasma with Trap-and-Elute MicroLC Using a New Microflow Source

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## ABSTRACT

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. Due to Antibody-drug conjugates (ADCs) heterogeneous nature, they require multiple bioanalytical assays to quantify their various forms. One important assay is the total antibody measurement which is the sum of all the conjugated antibody species plus the unconjugated antibody. Different protein sample preparation techniques can be combined with LC-MS/MS analysis to make a total antibody measurement. As the volume of blood drawn from a small animal during Drug Metabolism and Pharmacokinetics (DMPK) studies is limited sensitivity becomes critically important and combining immunoenrichment sample preparation with microflow LC-MS/MS detection more selective assays with lower limit of quantitation (LLOQ) are possible. Here we describe how sensitivity can be improved with MicroLC on the new SCIEX OptiFlow™ Quant Solution for the analysis of ADC, ado-trastuzumab emtansine.

## MATERIALS AND METHODS

**Sample Preparation:** Ado- trastuzumab emtansine was enriched from the sample using immunoaffinity with high capacity streptavidin coated magnetic beads and biotin labeled antibodies against the targeted analytes. Immuno-enriched proteins were eluted and digested using Trypsin/Lys-C, and 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.

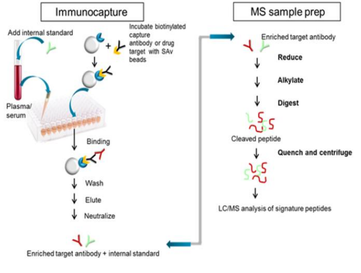


**Figure 1. Immuno-Capture Enrichment Workflow.**

**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 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 and injection needle rinsing solution was Isopropanol:Methanol:Acetonitrile in 60:20:20 ratio. A reversed phase gradient method was used for separation of the signature peptides[1]. The column temperature was kept at 40 ºC. Injection volume was 25 µL.

**Microflow Liquid Chromatography**: A SCIEX M5 MicroLC-TE system, with two microLC gradient pumps and an integrated autosampler was used. A 10 x 0.3 mm trap column packed with 5 µm 120 Å ChromXP C18 CL (SCIEX) and a 50 x 0.3 mm Kinetex C18 XB 2.6 µm 100 Å column from Phenomenex 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 10 µL/min. The analytical column temperature was set to 40 ºC. Injection volume was 25 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 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 analytical flow experiments. For the microflow LC experiments, the IonDrive™ Turbo V source was replaced with OptiFlow™ Turbo V Source Ion Source (SCIEX) with a 25 µm SteadySpray™ probe and electrode. Probe position, electrode protrusion, temperature and gas parameters were optimized for the IonDrive™ Turbo V source using analytical flow LC-MS/MS. The OptiFlow Turbo V Source requires no physical adjustment of the probe or electrode positions and only temperature and gas parameters were optimized for microflow LC-MS/MS at 10 µL/min .

**Data Processing:** MultiQuant™ 3.0.2 software (SCIEX) was used for data analysis. Data was subjected to a 1 point Gaussian smoothing, and a 1/x 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. Four replicate LC-MS injections were acquired for both the analytical flow and trap-and-elute microflow LC analysis.

## SEPARATION AND THROUGHPUT

Similar chromatography of the peptides was seen between analytical and microflow LC. As an example Figure 2 shows a comparison of the analytical flow direct injection LC-MS method and the microflow LC-MS method for ado-trastuzumab emtansine. 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 25 µL of sample as was injected in the analytical flow method.

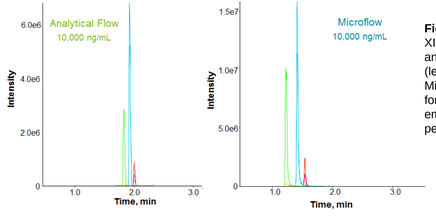


**Figure 2. XIC Chromatograms.** XIC Chromatograms for the analytical flow LC-MS method (left) and the Trap and-Elute Microflow LC-MS method (right) for the ado-trastuzumab emtansine and IS signature peptides.

## ROBUSTNESS

In order to determine the robustness of the Trap-And-Elute Microflow LC-MS method, a high concentration of ado-trastuzumab emtansine was digested. A total of 1150 injections were analyzed over a period of ~10 consecutive days. No clogging of tubing, electrode or columns was observed. All 1150 injections were completed using the same trap and analytical column.

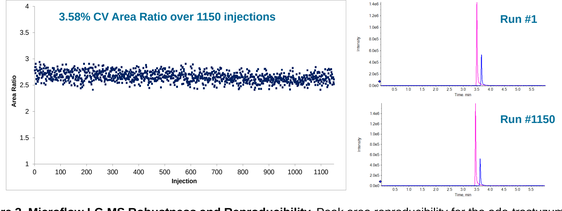


**Figure 3. Microflow LC-MS Robustness and Reproducibility.** Peak area reproducibility for the ado-trastuzumab emtansine peptide FTISADTSK over 1150 injections was 3.58%. Extracted Ion Chromatograms (XICs) for injection 1 and 1150 show identical separation and peak shapes.

## IMPROVED SIGNAL TO NOISE RATIO (S/N)

S/N ratio of the (signature) peptides for the ado-trastuzumab emtansine assay increased 3x using microflow LC instead of analytical flow LC (See table 2). Figure 4 shows the XIC's for FTISADTSK peptide with both methods at the 5 ng/mL level. Response (peak area) improved by a factor 3x.

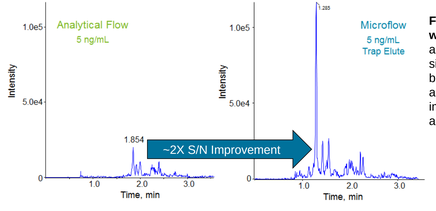


**Figure 4. Improvement of S/N with Microflow.** The S/N for ado-trastuzumab emtansine signature peptide was compared between microflow and analytical flow and was 3X improved at microflow LC, at the analytical LC LLOQ of 5 ng/mL.

## IMPROVED SENSITIVITY

In order to determine how much the LLOQ's for the ado-trastuzumab emtansine assay was 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. Table 1 shows the comparisons for ado-trastuzumab emtansine. The LLOQ was determined using the requirements of a precision < 20% and accuracy between 80 and 120% at the LLOQ. The LLOQ was 5X improved with microflow LC (Table 1).

**Table 1. Microflow LC–MS/MS Analysis of Ado-trastuzumab emtansine in Rat Plasma.** The calibration curve data for the signature peptide FTISADTSK (485.2 → 721.3) from the total antibody assay of ado-trastuzumab emtansine showed a 5X increase in sensitivity with 4 order of linear dynamic range with microflow LC-MS compared to analytical LC-MS.

| | Microflow LC | | | Analytical Flow LC | | |
|---|---|---|---|---|---|---|
| Actual Concentration | Mean Measured Concentration (ng/ml) | Accuracy (%) | CV (%) | Mean Measured Concentration | Accuracy (%) | CV (%) |
| 1 | 0.85 | 85.45 | 5.72 | - | - | - |
| 2.5 | 2.41 | 96.17 | 9.06 | - | - | - |
| 5 | 5.29 | 105.86 | 7.93 | 5.32 | 106.83 | 7.56 |
| 10 | 9.73 | 97.32 | 7.28 | 9.54 | 95.37 | 7.67 |
| 50 | 51.86 | 103.73 | 8.85 | 47.28 | 94.56 | 8.93 |
| 100 | 107.3 | 107.30 | 4.26 | 103.9 | 103.93 | 8.68 |
| 1000 | 1047.0 | 104.73 | 5.08 | 992.4 | 99.24 | 9.49 |
| 10,000 | 9944 | 99.44 | 4.42 | 10010 | 100.07 | 9.68 |

**Table 2. Sensitivity Improvements using OptiFlow Source with Microflow LC.** Improvement in S/N as well as improved CVs at the lowest concentrations resulted in improved LLOQ's for the quantitation of the antibody drug conjugate ado-trastuzumab emtansine of about 5x versus the analytical flow experiment.

| Analyte | Matrix | Sample Used (µL) | S/N Improvement | LLOQ Improvement |
|---|---|---|---|---|
| Ado-trastuzumab emtansine | Rat Plasma | 25 | 1.8 | 5 |

## LINEAR DYNAMIC RANGE

Calibration curves for both LC methods showed good linearity with r >0.99. Dynamic range for the microflow LC and analytical flow LC methods are summarized in Table 2. As an example, the calibration curve of ado-trastuzumab emtansine in rat plasma using microflow LC is shown in Figure 5.

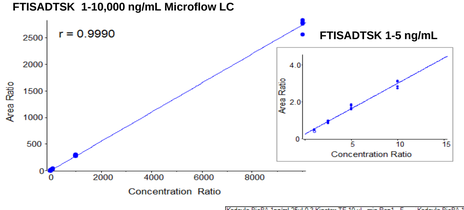


**Figure 5. Linear Dynamic Range.** Quantitation over 4 orders of linear dynamic range was achieved for ado- trastuzumab emtansine, with an r value of 0.999, from 1 to 10 000 ng/mL.

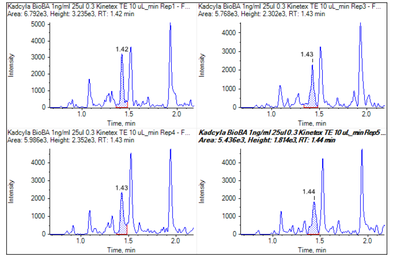


**Figure 6. Peptide Signal at Microflow LLOQ**. Four replicates of the 1 ng/mL sample are shown to highlight the reproducibility obtained at the LLOQ of the microflow experiment.

## CONCLUSIONS

- Microflow LC-MS/MS improves the LLOQ's by a factor of 5x compared to using analytical LC-MS/MS for this immuno-capture experiment on this ADC
- Linear dynamic range is equal or better when using microflow LC 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

## TRADEMARKS/LICENSING

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