Sensitive, Fast and Robust Quantification of Antibodies in Complex Matrices by Capillary Flow UHPLC and High-resolution Accurate-mass MS

Alexander Boychenko, Stephan Meding, Martin Samonig, Remco Swart
Thermo Fisher Scientific, Dornierstr. 4, Germering, Germany, 82110

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ABSTRACT

The detection of very low concentrations of antibodies in complex matrices poses a large challenge in bioanalysis. The applied detection methods require high sensitivity and selectivity. Additionally, they need to be robust and fast in order to provide consistent data quality and high sample throughput. We developed a capillary flow LC-MS method which overcomes the sensitivity limitation of analytical flow setups and the throughput limitation of nano flow separations.
Chromatographic separation was achieved using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system in capillary flow configuration and 300 µm inner diameter Acclaim™ PepMap column. The UHPLC system was set-up in pre-concentration mode exploiting its micro flow pump for fast sample loading.
Capillary flow LC together with high-resolution accurate-mass (HRAM) provided by the Thermo Scientific™ Q Exactive™ HF mass spectrometer allows robust, high throughput detection of antibody with amol sensitivity and is around 20 times more sensitive in comparison with analytical flow LC-MS analysis.

INTRODUCTION

There is a continuous demand for improved analytical depth and higher liquid chromatography mass-spectrometry (LC-MS) throughput capabilities in research and applied markets. However, sensitivity and throughput very often counteract each other. Currently two extreme cases of LC-MS analysis are widely used in practice: nano flow LC-MS with flow rates less than 1 µL/min and analytical flow LC-MS with flow rates above 400 µL/min. The acceptance of nano flow LC-MS in biopharma and academic research is mainly related with rapidly growing proteomics field where high sensitivity is an absolute requirement for analysis of small amounts of biological samples. However, nano flow separations are often time consuming due to relatively high delay volumes and operation of very long columns under low linear velocity that allows achieving high peak capacity in one-dimensional separations and identify around 6000 proteins with modern high-resolution mass-spectrometer [1]. On the other side analytical flow LC-MS analysis provides uncompromised throughput required for routine applications. Recently, new challenges in bioanalysis of biotherapeutics where high throughput is needed to analyze 1000s of samples in reasonable time push forward the development of capillary flow LC-MS platforms. Capillary flow LC-MS analysis unites a high sensitivity required for targeting of low abundant species with high throughput. Capillary flow LC is usually defined by the applied flow rate (1-10 µL/min) and the used column dimensions (100-500 µm inner diameter). Despite the fact that capillary flow LC separations on sub 500 µm column IDs are known for decades the coupling of capillary LC with MS detectors is not always straightforward. ESI interfaces that allow to couple capillary flow LC with MS can strongly affect ESI signal. The performance of capillary flow LC-MS analysis is dependent on emitter ID, flow rate, emitter position/source type that should be optimized prior to LC-MS method setup.
In this work we thoroughly investigated and optimized capillary flow LC-MS platform and compared the sensitivity of analytical, micro, capillary and nano flow LC-MS separations. The proposed combination of capillary separation column, flow rate and ESI source were used for targeted quantification of Infliximab with HR/AM MS detection.

MATERIALS AND METHODS

Sample preparation
Pierce™ HeLa protein digest standard and Thermo Scientific Dionex™ Cytochrome C digest were used as test samples to compare the sensitivity of analytical, micro, capillary and nano flow LC-MS analysis as well as to optimize gradient conditions for targeted analysis of Infliximab.
Remicade® that is supplied as a sterile, white, lyophilized powder and contained 100 mg of infliximab, 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate, monohydrate, 6.1 mg dibasic sodium phosphate, dehydrate was diluted with water and digested using the Thermo Scientific SMART Digest™ kit to obtain tryptic peptides in accordance with standard protocol (Fig. 1). No additional sample cleaning steps were implemented. The obtained solution was sequentially diluted and used for capillary flow LC-MS analysis. The sample was cleaned-up onto trap column prior to capillary flow separations using UltiMate 3000 RSLCnano. HeLa protein digest standard was dissolved in 0.1% formic acid (FA) solution and diluted till 100 ng/µL. 20 µL of obtained HeLa cell lysate was spiked with different amounts of infliximab tryptic digest.

Figure 1. The scheme of sample preparation using SMART digest kit prior to capillary flow LC-MS analysis

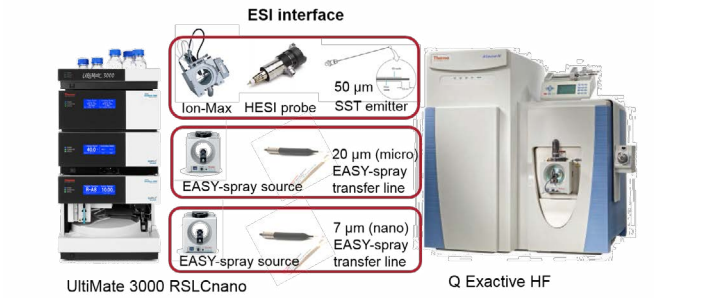

Figure 2. LC-MS setup for nano and capillary flow LC-MS analysis with corresponding ESI interfaces and emitters. Capillary column with 0.3 mm ID operated at flow rates from 1 to 10 µL/min and nano column with 75 µm ID was operated with flow rate from 0.1 to 1 µL/min were used with this LC-MS setup.

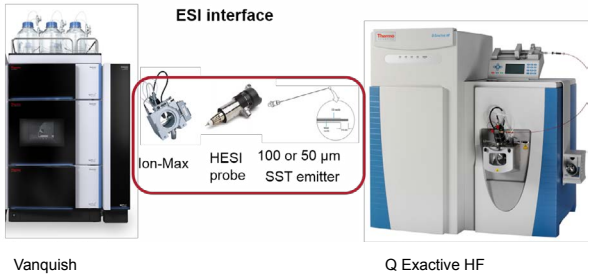

Figure 3. LC-MS setup for analytical and micro flow LC-MS analysis with corresponding ESI interface and emitters. Analytical column with 2.1 mm ID operated at 450 µL/min and micro column with 1.0 mm ID operated from 25 to 200 µL/min were used for all experiments with this LC-MS setup.

LC conditions

LC system used for analytical and micro flow separations: a Thermo Scientific Vanquish™ UHPLC system with a 2.1 x 100 mm Accucore™ AQ, C18, 2.6 µm column and gradients of water and acetonitrile (ACN) with 0.1% FA each were used to separate the peptide mixtures at flow rate 450 µL/min (analytical flow). The same UHPLC system with 1.0 x 150 mm ID. Acclaim PepMap, C18, 2 µm column was used to separate peptides at flow rates 200, 150, 100, 50, 25 µL/min (micro flow).
LC system used for capillary flow separations: the UltiMate 3000 RSLCnano system with Capillary flow meter was used to separate peptides on 0.3 mm x 150 mm, 2 µm Acclaim PepMap C18 column at flow rates 10, 7, 5, 3, 2, 1 µL/min. The UHPLC system was set-up in pre-concentration mode exploiting its micro flow pump for fast sample loading at 100 µL/min onto 0.3 x 5 mm Acclaim PepMap trap cartridge.
LC system used for nano flow separations: the UltiMate 3000 RSLCnano system with ProFlow flow meter was used to separate peptides on 75 µm x 150 mm, 2 µm EASY-Spray™ PepMap C18 column at flow rates 1200, 800, 500, 300, 200, 100 nL/min

ESI interfaces and emitters
HESI probe equipped with 100 µm stainless steel needle (SST) was used for analytical flow separations. HESI probe with 50 µm SST needle was used for micro flow LC-MS analysis. HESI probe with 50 µm SST needle, micro EASY-spray transfer line with 20 µm silica emitter and nano EASY-spray transfer lines were tested used for capillary flow LC-MS analysis. EASY-spray column (75 µm ID) with 7 µm silica emitter was used for nano flow LC-MS analysis. All ESI interfaces and emitters that were used in this work are commercially available.

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MS conditions

LC systems were coupled with the Q Exactive HF Orbitrap™ mass spectrometer. The instrument has been operated in Full scan and data-dependent acquisition (DDA) for comparison of sensitivity at different flow rates or Parallel Reaction Monitoring (PRM) for targeted analysis of Infliximab.

Data Acquisition and Analysis

Data were acquired with Thermo Scientific Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2. Thermo Scientific Proteome Discoverer™ 2.0 software was used for DDA analysis and data base search with the Sequest™ HT algorithm. FullMS data were processed with Chromeleon 7.2, PRM data were processed with Skyline 3.1 software.

RESULTS

Sensitivity gain for low flow rates and column inner diameters

The main factors that affect sensitivity gain in ESI MS are better desolvation of the analytes, a more efficient electrospray ionization process and a higher sampling of analytes into the mass spectrometer. The sensitivity increase is also strongly dependent on ESI source setup and emitter inner diameter. An improvement in sensitivity of approximately two orders of magnitude for nano compared to analytical flow LC-MS systems can be observed [3]. It is difficult to model the influence of all factors on ESI response. Thus, empirical evaluation is required to select optimal LC ESI setup. We have compared different combinations of column IDs, ESI sources and emitters at wide flow range to estimate the influence of different parameters on ESI signal. The results were normalized to sensitivity observed at analytical flow rate 450 µL/min and column with 2.1 mm ID (Fig. 4-5). The increase in peak area and peak height with decrease in flow rate can be approximated by power law (Fig. 4). The obtained relationships predict that to gain approximately one order of sensitivity the flow rate must be reduced for approximately two orders of magnitude (e.g. from 450 µL/min to 4.5 µL/min).

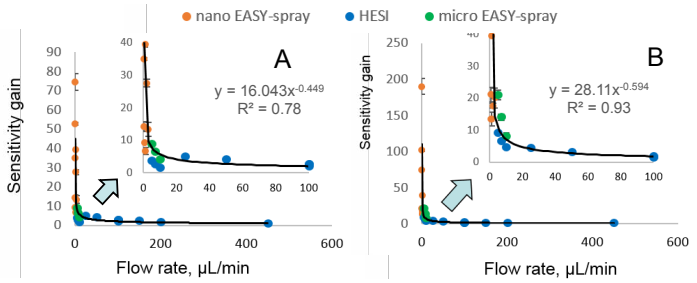

Figure 4. The relative sensitivity gain based on peak height (A) and peak area (B) EDLIAYLK (m/z 482.77, charge +2) for flow rates from 100 nL/min to 450 µL/min using different source types, emitter and column IDs. Each dot represents a set of replicate measurements under the same LC ESI MS conditions. The color highlights corresponding ESI sources and emitters

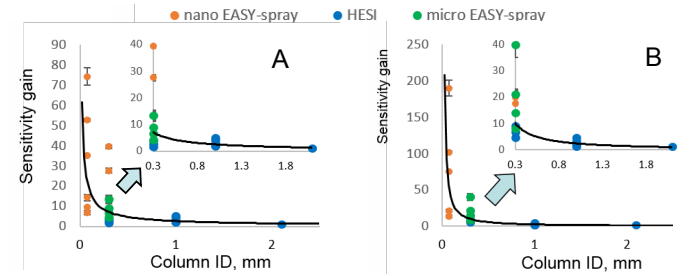

Figure 5. The relative sensitivity gain based on peak height (A) and peak area (B) for different column IDs, emitter IDs and flow rates. Each dot represents a set of replicate measurements under the same LC ESI MS conditions.

We found that HESI probe with 50 µm ID SST needle and micro EASY-spray transfer line with 20 µm ID silica emitter can be successfully used for capillary flow LC-MS analysis. However, the maximum sensitivity at these flow rates can be obtained with micro EASY-spray transfer line (Fig. 6). Thus, it was selected for all next capillary flow LC-MS experiments.

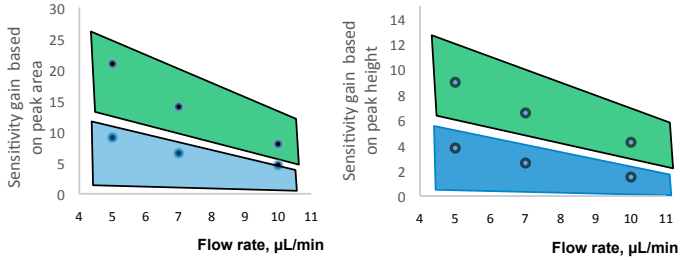

Figure 6. The relative sensitivity gain for 0.3 ID x 150 mm column and different flow rates using HESI probe with 50 µm ID SST emitter (blue) and micro EASY-spray transfer line with 20 µm silica emitter (green).

Optimal ESI interface for different flow rates

The sensitivity of LC ESI MS analysis (peak area as well as peak height) increases significantly with decreasing of column ID and flow rate (Figs. 4-6). Thus, the compromise should be found to maintain high sensitivity as well as acceptable throughput of LC-MS analysis for particular column ID. The reducing of linear velocity results in reduced throughput due to significant impact of delay volume on separation. Moreover, this may require to operate LC system at non-optimal flow rates or pressure that affects retention time precision. Additionally to flow rate and column ID, the emitter ID influences signal intensity by affecting peak shape and peak width. As a summary (Fig. 7), the optimal results for (i) analytical flow rates (> 100 µL/min) were obtained with HESI probe equipped with 100 µm SST needle; (ii) micro flow rates (from 10 to 100 µL/min) with HESI probe equipped with 50 µm SST needle; (iii) capillary flow rates (from 1 to 10 µL/min) with micro EASY-spray transfer line (20 µm silica emitter); (iv) nano flow rates (< 1 µL/min) with nano EASY-spray transfer line (7 µm silica emitter).

In general we observed from to 2 to 4 times increase in sensitivity for 1 mm ID column in comparison with 2.1 mm ID column depending on applied flow rate. Capillary flow allowed to gain from 5 to 30 times more peak area and peak height for flow rates ranging from 10 to 3 µL/min on 0.3 mm ID column. The higher sensitivity was observed for nano flow separations on 75 µm ID column. At standard in proteomics 300 nL/min flow rate the sensitivity was from 50 to 100 times higher in comparison with analytical flow separations. The sensitivity was improved even further (up to 300 times) for 100 nL/min flow rate.

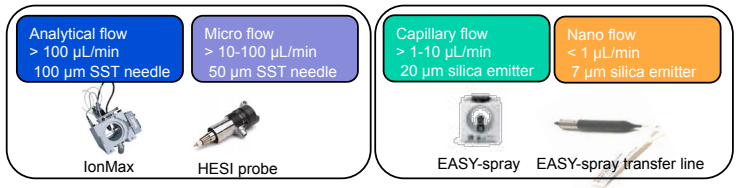

Figure 7. Optimal ESI interface for analytical, micro, capillary and micro flow rates and corresponding emitters inner diameters

Sensitivity gain for complex protein digest

In order to verify our findings we analyzed HeLa cell lysate digest with different flow rates and columns IDs (Fig. 8). The obtained TIC profiles confirmed that sensitivity for capillary flow LC-MS is from 10 to 20 times higher than for analytical flow LC-MS analysis. Additionally, nano flow LC-MS is around 5 times more sensitive in comparison with capillary flow LC-MS.

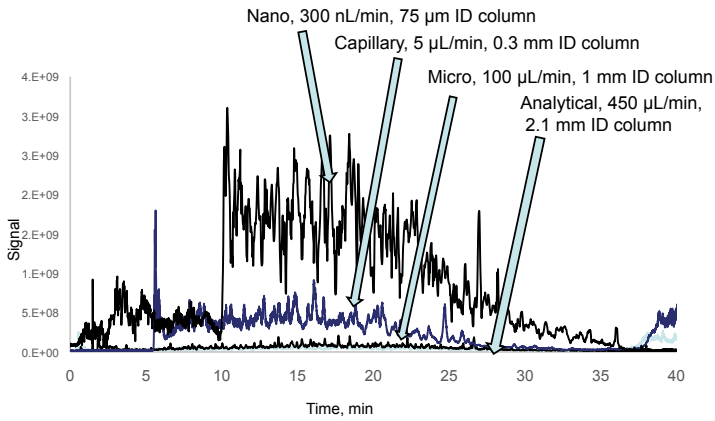

Figure 8. TIC profiles of 1 µg of HeLa cell lysate digest with analytical, micro, capillary and nano flow LC-ESI-MS setups

Nano flow LC-MS: EASY-spray 0.075 x 150 mm, C18, 2 µm column
Capillary flow LC-MS: 0.3 x 150 mm Acclaim PepMap, C18, 2 µm column, micro EASY-spray transfer line with 20 µm ID silica emitter
Micro flow LC-MS: 1.0 x 150 mm Acclaim PepMap, C18, 2 µm column, HESI probe with 50 µm ID SST emitter
Analytical flow LC-MS: 2.1 x 100 mm Accucore AQ, C18, 2.6 µm column, HESI probe with 100 µm IS SST emitter

Targeted analysis of Infliximab with capillary flow LC-MS

Capillary flow LC-MS has attracted most attention for bioanalysis applications in pharma and especially biopharma laboratories. Dedicated solutions have been developed in order to provide robust, easy-to-use applications. Thus, as a proof-of-principle study we developed a method for targeted quantification of Infliximab with HR/AM MS detection and capillary flow LC separations. The separation method consisted of very fast sample loading onto the trap column with using loading pump and peptides separation at 5 µL/min. After that the trap and analytical columns were thoroughly washed with increased flow rate 10 µL/min to avoid carryover onto the column (Fig. 9). Three uniquely attributed to Infliximab peptides DILLTQSPAILSVSPGER, SINSATHYAESVK, DSTYSLSSTLTLSK were selected for PRM quantification and showed good ESI MS response. The highest sensitivity was observed for DILLTQSPAILSVSPGER (m/z 632.68, charge +3). The quantification was done using y7 ion with m/z 731.38. The lowest calibration point was 16 amol and the linearity range covered 5 orders of magnitude in "pure" Infliximab digest (Fig. 10). In the spiked HeLa digest (500 ng) the lowest detectable amount of Infliximab was around 3 fmol.

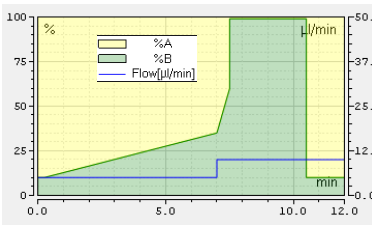

Figure 9. The gradient and flow rate profile for Infliximab quantification with capillary LC-MS
Column: 0.3 mm x 150 mm, 2 µm Acclaim PepMap C18 column. ESI: micro EASY-spray transfer line (20 µm ID silica emitter)

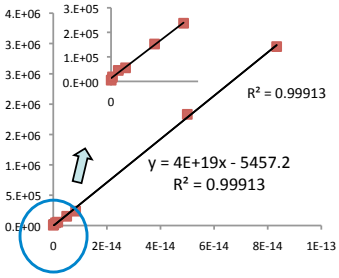

Figure 10. The calibration plot for PRM quantification of Infliximab based on DILLTQSPAILSVSPGER peptide
MS1 resolution: 60K, IT 150 ms; MS2 resolution 120K, IT 50 ms

CONCLUSIONS

We systematically investigated the effect of flow rate, column inner diameter and source type on the sensitivity in LC ESI MS analysis. A fast and sensitive method for targeted quantification of Infliximab was developed using capillary flow LC separations and high-resolution accurate-mass mass-spectrometric detection.

- The optimal setup for capillary LC-MS analysis comprised the UltiMate RSLCnano system with capillary flow selector, column with 0.3 mm inner diameter for peptides separation, trap cartridge with 0.3 mm inner diameter for fast sample loading and clean-up, EASY-spray source and micro EASY-spray transfer line with 20 µm silica emitter for efficient ESI ionization.
- The fast sample loading with micro flow loading pump integrated in UltiMate 3000 RSLCnano system and column washing at increased flow rate allowed to achieve high-throughput separations with 12 min total analysis time
- The absolute sensitivity of capillary flow LC-MS was around 20 times higher at 5 µL/min in comparison with analytical flow LC-MS analysis at 450 µL/min.
- Capillary flow LC separation with short gradient in combination with PRM quantification resulted in amol sensitivity for quantification of Infliximab after fast digestion with SMART digest.

REFERENCES

1. D. Lopez-Ferrer , M. Blank, S. Meding, A. Paulus, R. Huguet, R. Swart, A.Huhmer Pushing the Limits of Bottom-Up Proteomics with State-Of-The-Art Capillary UHPLC and Orbitrap Mass Spectrometry for Reproducible Quantitation of Proteomes, Application Note 639, 2015, P. 1-6.
2. S. Meding, A. Boychenko Capillary Flow LC-MS Unites Sensitivity and Throughput Chromatography Today, 2016, accepted for publication
3. M. Wilm, M. Mann Analytical Properties of the Nanoelectrospray Source. Anal Chem. (1996) Vol. 68(1), P. 1-8.