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Novel capillary-flow LC HRAM MS platform for fast targeted analysis and robust profiling of complex samples

Alexander Boychenko[1], Peter Bults[2], Martin Ruehl[1], Christopher Pynn[1], Mike Baynham[3], Wim Decrop[1], Alexander Harder[4], Nico van de Merbel[2], Rainer Bischoff[2], Remco Swart[1]

[1]Thermo Fisher Scientific, Germering. Germany, [2]University of Groningen, Groningen, The Netherlands, [3]Thermo Fisher Scientific, Runcorn, UK, [4]Thermo Fisher Scientific, Bremen, Germany

ABSTRACT

Purpose: Here we present a highly robust novel capillary-flow LC-MS platform that combines the Thermo Scientific™ capillary-flow UltiMate™ 3000 RSLCnano system (capLC), the new 150 µm ID Thermo Scientific™ EASY-Spray™ column and the new Thermo Scientific™ Q Exactive™ HF-X mass spectrometer.

Methods: We used typical shotgun and targeted proteomics experiments (Full-scan MS, Data-Dependent Acquisition (DDA), and Parallel-Reaction Monitoring (PRM)) to verify the performance and robustness of the novel capLC-MS platform.

Results: We show that the novel capLC-MS platform is a sensitive and reliable solution for shotgun and targeted high-resolution accurate-mass (HRAM) proteomics experiments that can be used for routine proteome profiling of complex samples including bio-fluids as well as targeted high-throughput quantification.

INTRODUCTION

Capillary flow LC-MS (capLC-MS) with 100-300 µm inner diameter (ID) columns and flow rates from 1 to 10 µL/min provide increased MS sensitivity, lower solvent consumption, and reduced MS contamination compared with typical analytical flow LC-MS applications which runs at flow rates of more than 100 µL/min. Additionally, capLC-MS provides higher throughput in comparison to nano-flow LC, whilst affording a similar sensitivity achievable by loading higher sample amounts. Here we describe a novel capLC-MS platform (Fig. 1) that can be used for high-throughput analysis, whilst providing higher MS sensitivity for routine shotgun proteomics due to the brighter ion-source of the Q Exactive HF-X mass-spectrometer.

Figure 1. Novel capLC-MS platform that combines: (i) the capillary-flow UltiMate 3000 RSLCnano system; (ii) the new 150 µm ID EASY-Spray column; (iii) the new Q Exactive HF-X mass-spectrometer

- Direct flow control
- Reliability and RT stability
- Application versatility
- High intra-column reproducibility
- Wide flow and pressure range
- High loading capacity
- Brighter ion source
- Up to 40 Hz acquisition speed
- More resolution options

MATERIALS AND METHODS

LC system setup

The capillary-flow UltiMate 3000 RSLCnano system (capLC) was used to separate peptides on an EASY-Spray 150 µm x 150 mm, 2 µm Acclaim PepMap C18 column at flow rates of 1.2 µL/min for highest sensitivity and 3 µL/min for highest throughput. The UHPLC system was set-up in pre-concentration mode with a 20 µL injection loop exploiting its micro flow pump for fast sample loading at 100 µL/min onto a 0.3 mm x 5 mm Acclaim PepMap trap cartridge or direct injection mode with a 5 µL injection loop. The loop was switched off-line after the sample was transferred onto the column and washed with 50% acetonitrile (ACN) in water with 0.1 % formic acid (0.1% FA) to minimize carryover. The mobile phase A was water with 0.1 % FA and mobile phase B was acetonitrile with 0.1 % FA.

MS instrumentation and ESI interface

The EASY-Spray source was used to connect the capLC system and EASY-Spray column to the Q Exactive HF-X mass spectrometer. The instrument was operated in Full-scan MS and DDA for comparison of sensitivity at different flow rates or Parallel Reaction Monitoring (PRM) for targeted analysis of Infliximab.

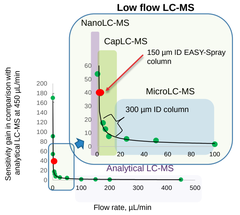
Data Acquisition and Analysis

Data were acquired with Thermo Scientific™ Xcalibur 4.0 software. The capLC system was controlled with Standard Instrument Integration (SII) software. PRM data were processed with Thermo Scientific™ TraceFinder™ 4.0 software and Skyline. DDA data were processed with Thermo Scientific™ Proteome Discoverer™ software.

CAPLC-MS SENSITIVITY GAINS

At ASMS2016 we presented a comprehensive study that compared sensitivity gains using columns with different internal diameters (IDs) at different flow rates while loading the same sample amount [1]. A clear experimental relationship between flow rate and sensitivity was observed that could be described by a power law function (Fig. 2). Thus, major improvements in sensitivity can be achieved at nano and capillary-flow rates compared to analytical flow rates.
The EASY-Spray capillary column with 150 µm ID can be operated at a wide flow range from 1 to 3 µL/min, corresponding to linear velocities from 2 to 4 mm/s. The results with capLC-MS are 40 times more sensitive than analytical flow LC-MS at 450 µL/min and only 2 to 4 times less sensitive than nanoLC-MS at 300 nL/min (Fig. 2) when loading the same amount of sample onto the column.

Figure 2. The increase in sensitivity experienced with low-flow LC-MS compared to analytical flow LC-MS.



CAPLC-MS CHROMATOGRAPHIC PERFORMANCE

During our preliminary experiments we determined a flow rate of 3 µL/min to be optimal for high-throughput, targeted analysis and a flow rate of 1.2 µL/min to be ideal for profiling of proteomics samples with our capLC-MS platform. Typical chromatograms for capLC-MS separations at 3 and 1.2 µL/min (Fig. 3 and 4) showed that the separation performance is not compromised at low flow rates and is comparable to analytical flow separations. The peak width at half maximum (PWHM) is about 3 s for fast capLC-MS separations at 3 µL/min and less than 15 s for longer gradients at 1.2 µL/min (Table 1 and 2) which results in peak capacities of circa 300 for both gradients.

Figure 3. Typical chromatogram for 1 pmol of CytC protein digest on column

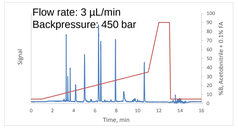


Figure 4. Typical chromatogram for 1 µg of HeLa cell lysate digest on column.

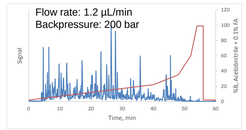


Table 1. Chromatographic characteristics for 3 selected CytC peptides averaged over 150 consecutive injections of CytC protein digest, separated at 3 µL/min. The total analysis time was 16 min.

Peptide	GRAVY	m/z	Ret. time	Ass.	PWHM, s	PW base, s
KYIPGTK	-1.04	403.74	3.0	1.8	0.3	1.0
MIFAGIK	1.6	779.45	5.9	1.0	3.0	5.3
EDLIAYLK	0.21	482.77	7.5	1.0	3.0	5.2

Table 2. Chromatographic characteristics for 5 selected proteotypic peptides averaged over 18 consecutive injections of 100 ng HeLa cell lysate digest, separated at 1.2 µL/min. The total analysis time was 60 min.

Peptide	Protein	Acc. No.	m/z	Ret. time	Ass.	PWHM, s	PW base, s
[K].VDNDENEHQLSLR.[T]	Nucleophosmin	P06748	523.584	12.0	1.3	11	20
[K].LTDcVVMR.[D]	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	497.248	14.3	1.2	11	20
[K].STELLIR.[K]	Histone H3.2	Q71DI3	416.252	17.4	1.3	14	26
[K].VNQIGSVTESIQAcK.[L]	Beta-enolase	P13929-1	817.417	22.8	1.2	9	17
[K].SLTNDWEDHLAVK.[H]	Heat shock protein HSP 90-beta,	P08238	509.921	22.9	1.3	12	23
[K].TVTAMDVVYALK.[R]	Histone H4	P62805	655.858	36.3	1.3	13	24

ROBUSTNESS OF CAPILLARY LC-MS

To investigate the robustness of the capLC-MS platform retention time and MS signal stability were monitored over an extended injection sequence. The capLC system was configured for direct injections and evaluated over 8 days of operation (Fig. 5). An excellent retention time stability (RSD < 1%) was observed for 350 injections. The peak area stability was evaluated on MS1 level for 150 consecutive injections conducted between day 5 and 8 (Fig. 6). The peak are RSD values of less than 10% were obtained even without internal standard correction during long term testing.

Figure 5. Retention time stability for capLC-MS analysis of CytC protein digest

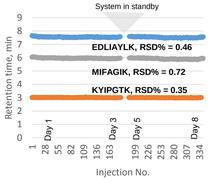
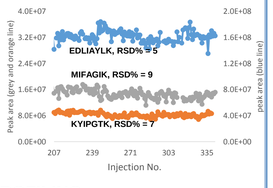


Figure 6. Peak area stability for capLC-MS analysis of CytC protein digest



CELL LYSATE PROTEIN PROFILING

CapLC-MS is an ideal technique for routine and robust proteome profiling of complex samples, especially for the analysis of large sample cohorts. To understand the performance of the novel capLC-MS platform for shotgun proteomics with data dependent acquisition we analyzed HeLa cell lysate digest with 60 and 90 min total analysis time. We loaded 1, 2 or 5 µg of protein digest onto the column (Fig. 6 and 7). The new Q Exactive HF-X MS instrument allows an increase of the acquisition speed of up to 40 Hz and gives more options for resolution settings. The following MS settings were applied to explore the speed of the Q Exactive HF-X: MS Resolution: 60K; MS1 IT: 100 ms; MS2 Resolution 7.5K, MS2 IT 35 ms, TopN: 40.

Figure 7. Protein groups identified at different gradient lengths and different loading amounts of HeLa cell lysate digest

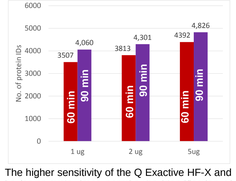
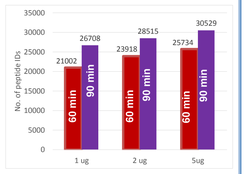
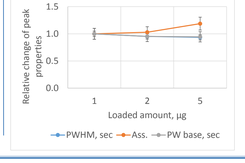


Figure 8. Peptide groups identified at different gradient lengths and different loading amounts of HeLa cell lysate digest



The higher sensitivity of the Q Exactive HF-X and its faster cycle times permits the identification of a similar number of protein and peptide groups with capLC-MS compared to nanoLC-MS using the previous generation Orbitrap MS [2]. CapLC-MS with a 150 mm long column allows shorter sample loading, column washing, and equilibration times by increasing the flow rate during these steps. Thereby, the elution window for peptides, number of MS/MS events and peptide to spectra matches (PSM) are increased, while having the same total analysis time as in nanoLC-MS. Typically, more than 3500 protein groups and 20000 unique peptides were identified from only 1 µg of HeLa cell lysate digest in 60 min (Fig. 7 and 8). The number of identifications can be boosted to more than 4300 with increased sample loading (Fig. 7) without compromising chromatographic performance (Fig. 9).

Figure 9. Dependence of the average PWHM, PW at base, and asymmetry on the loading amount of HeLa protein digest. The values are relative to results obtained with 1 µg HeLa cell lysate digest



CRUDE PLASMA PROTEOME

The abundance of proteins in the plasma proteome is above 10 orders of magnitude. Additionally, the 14 most abundant proteins cover more than 94% of the total protein mass. Thus, it is very challenging to perform deep proteome profiling of blood products without intensive pre-fractionation. However, alterations of the high-abundant proteome as well as changes in several inflammatory markers can be monitored by analysis of crude plasma or serum protein digests. The simple procedure described elsewhere [3] was used to digest plasma samples (Fig. 10) in this work.

Figure 10. Scheme of crude plasma digestion pipeline

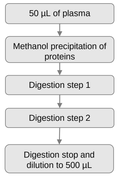
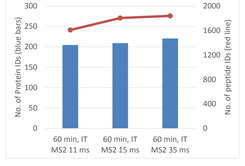


Figure 11. Peptide (red line) and protein (blue bars) groups identified with 1 % FDR in crude plasma digest at different MS2 injection times and 60 min total analysis time

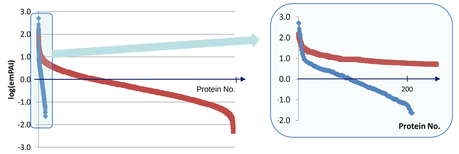


We were able to identify more than 200 protein groups and almost 2000 peptides with single shot DDA analysis of 1 µL of crude plasma protein digest solution loaded onto the capillary column (Fig. 11). The increase of MS2 injection time to 15 ms permitted some increase in proteome depth, however only minor improvements were obtained with 35 ms injection time (Fig. 11) due to the high sample loading amount.

DEPTH OF HUMAN PROTEOME PROFILING

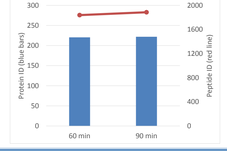
The depth of proteome coverage from a crude plasma digest and a HeLa cell lysate digest achieved by capLC-MS was estimated based on the logarithmically transformed emPAI index, which is proportional to the protein abundance in the sample. The proteins identified in cell lysate digest as well as in plasma covered 4 orders of magnitude (Fig. 12). The difference in protein abundance for those proteins identified in in plasma is significantly more pronounced compared to those identified from HeLa cells.

Figure 12. Sorted exponentially modified protein abundance index (emPAI) of proteins identified in HeLa cell lysate digest (red) and crude plasma protein digest (blue).



It is more difficult to increase the number of identified protein and peptide groups in plasma by increasing the gradient length due to the high difference in protein abundances (Fig. 13). We observed only minor improvements using longer separation gradients for capLC-MS analysis of crude plasma digest (Fig. 13). Thus, immunodepletion or orthogonal separation techniques are required in order to look deeper into the plasma proteome.

Figure 13. Peptide (red line) and protein (blue bars) groups identified with 1 % FDR in crude plasma digest at 60 min and 90 min total analysis time



TARGETED CAPLC-MS PRM QUANTIFICATION

The quantitation and monitoring of in-vivo degradation of protein therapeutics, e.g. monoclonal antibodies, are required to get information about protein inactivation after admission. LC-MS/MS analysis permits the measurement of multiple targets within one run and also to monitor original as well as degradation products [3]. We developed a capLC-MS method for fast targeted analysis of the trastuzumab signature peptide and its three degradation products (two deamidated isomers and succinimide intermediate) in plasma (Fig. 14). CapLC-MS allowed baseline resolution of deamidated isomers of YPTNGYTR within 12 minutes of total analysis time. A shallow 7 min gradient from 5 to 9 % solvent B was used to separate the peptide targets. The linearity range for the non-deamidated product in plasma was from 500 ng to 500 µg/mL of trastuzumab that covers the typical range of the drug in plasma during the medical treatment (Fig. 15). With an injection volume of only 0.5 µL of digested plasma solution, this corresponds to the extremely low detection limit of 2 fmol trastuzumab loaded onto the capillary column.

Figure 14. CapLC-MS chromatograms of trastuzumab signature peptide (YPTNGYTR) and three degradation products of trastuzumab PRM targeted in plasma sample

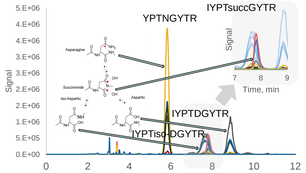
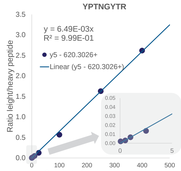


Figure 15. Calibration plot for YPTNGYTR peptide in plasma.



CONCLUSIONS

The novel capillary-flow LC-MS platform combines the advantages of high-throughput analytical flow LC separations and the high sensitivity of nano-flow LC-MS analysis. The high robustness and sensitivity it provides, permit routine profiling of proteomics samples and targeted analysis of large sample cohorts.

- Capillary-flow UltiMate 3000 RSLCnano system delivers high gradient reproducibility and retention time stability with RSDs less than 1 % for long term operation
- New 150 µm ID EASY-Spray columns provide excellent chromatographic performance, ease-of-use and robustness with PWHM as low as 3 s at 3 µL/min flow rate
- New Q Exactive HF-X MS with brighter ion source significantly improves sensitivity and permits the identification of more than 4000 proteins within a 60 min capLC-MS analysis cycle

REFERENCES

1. A. Boychenko, S. Meding, M. Samonig, R. Swart. Sensitive, Fast and Robust Quantification of Antibodies in Complex Matrices by Capillary Flow UHPLC and High-Resolution Accurate-Mass MS. ASMS2016, Poster Note, PN64787 hyperlink
2. A. Boychenko, S. Meding, W. Decrop, M. Baynham, M. Ruehl, J.-M. T. Wong, M. Markus, and R. Swart. Deep and Reproducible Human Proteome Profiling with Novel Nano Flow LC Technology and HRAM Mass-Spectrometry. Pittcon2017, PO72267 hyperlink
3. P. Bults, R. Bischoff, H. Bakker, J.A. Gietema, N.C. van de Merbel. LC-MS/MS-Based Monitoring of In Vivo Protein Biotransformation: Quantitative Determination of Trastuzumab and Its Deamidation Products in Human Plasma. *Anal. Chem.*, **2016**, 88 (3), pp 1871–1877 hyperlink

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Yue Xuan and Dr. Tabiwang Arrey from Thermo Fisher Scientific, Bremen for help with measurements on Q Exactive HF-X mass spectrometer.

TRADEMARKS/LICENSING