Poster Note 71930

Targeted nano LC-MS Quantification of Rituximab in Human Bio-Matrix with High-Resolution Accurate Mass MS

Alexander Boychenko, Martin Samonig, Frank Steiner, Remco Swart
Thermo Fisher Scientific, Germering, Germany

Overview

Purpose: to establish a targeted quantification of rituximab in complex human bio-sample using nano LC and high-resolution accurate mass MS.

Methods: DDA, tSIM and PRM quantification methods with using Thermo Scientific™ UltiMate™ 3000 RSLCnano system with novel ProFlow™ technology for direct nano flow control coupled with Thermo Scientific™ Q Exactive™ HF with Thermo Fisher™ EASY-Spray™ ion source and Thermo Fisher™ EASY-Spray ™ 75 μm x 50 cm Acclaim™ PepMap™ column.

Results: Nano LC HR MS targeted Selected Ion Monitoring (tSIM) and Parallel Reaction Monitoring (PRM) allows to develop sensitive and selective methods for quantification of monoclonal antibody (mAb) in complex bio-matrix.

Introduction

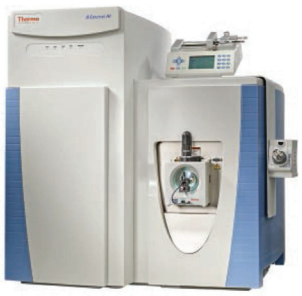
Monoclonal antibodies (mAb) used as biotherapeutics are of great importance for the treatment of various diseases such as cancer, autoimmune disorders and chronic inflammation. The precise quantification of antibodies in complex human matrices such as biofluids or tissue samples is essential for pharmacodynamics and pharmacokinetics studies as well as for understanding drug penetration and target engagement. Due to a low therapeutic mAb level and high similarity with human proteins their quantification is a challenging task for immunoaffnity as well as liquid chromatography mass-spectrometric (LC-MS) techniques.

Rituximab is the first commercial mAb that was approved for clinical use in treatment of non-Hodgkin's lymphoma or chronic lymphocytic leukemia. In this study it was used to assess a new method for analysis of biotherapeutics in complex matrixes.

Nano flow LC in combination with targeted analysis using a quadrupole-Orbitrap mass spectrometry provides high sensitivity and low limits of detection in complex matrices. In this study we developed tSIM and PRM methods and compared them with DDA detection of rituximab. The new ProFlow technology for UltiMate 3000 RSLCnano system provided high retention time precision. This allowed to multiplex targeted assays by using narrow retention time windows and obtain highly reproducible results.

Methods

Liquid Chromatography
The UltiMate 3000 RSLCnano system with ProFlow flow meter was used to separate rituximab tryptic peptides spiked into a HELA cell lysate digest. The separations have been performed on a Thermo Scientific™ EASY-Spray™ Acclaim™ PepMap™ C18 100Å (2 μm, 75 μm i.d. x 50 cm) column and the flow rate was kept constant at 300 nL/min during the runs.



Q Exactive HF

EASY-Spray column

UltiMate 3000 RSLCnano

Sample Preparation
Rituximab samples were digested using the Thermo Scientific™ SMART Digest™ kit to obtain tryptic peptides. Disulfide bonds were reduced by incubation for 30 minutes at 60° C with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The working solutions of rituximab with concentrations: 20, 4, 0.2, $2{\cdot}10^{-2}$, $2{\cdot}10^{-3}$ ng/μL were prepared by subsequent dilution of 400 nL/min standard solution. Pierce™ HeLa Protein Digest Standard was dissolved in 0.1% formic acid (FA) solution and diluted to 100 ng/μL. HeLa cell lysate digest was spiked with rituximab tryptic digest in ratios 1 to: 100 (Sample 1), $1{\cdot}10^3$ (Sample 2), $5{\cdot}10^3$ (Sample 3), $1{\cdot}10^4$ (Sample 4) , $2.5{\cdot}10^4$ (Sample 5), $5{\cdot}10^4$ (Sample 6), $7.5{\cdot}10^4$ (Sample 7), 10^5 (Sample 8).

Mass Spectrometry
The Thermo Scientific Q Exactive HF mass spectrometer was used for rituximab detection and quantification. The instrument was operated in data-dependent acquisition mode (DDA), targeted Selected Ion Monitoring (tSIM) and Parallel Reaction Monitoring (PRM) modes. DDA MS settings were: TOP20, MS1 resolution 60,000, Maximum IT 50 ms; MS2 resolution 15,000, Maximum IT 50 ms, MS1 range 350-2000 m/z; dynamic exclusion 10 s. Targeted SIM settings were: 120,000, Maximum IT 100 ms, isolation window 2 m/z. PRM settings were: MS2 60,000, Maximum IT 150 ms, Isolation window 2 m/z, AGC target $2{\cdot}10^5$.

Table 1. Nano LC gradient for peptide separation

30 min gradient	
Time, min	Solvent B, %
0	10
4	10
34	30
35	50
37	90
40	90
40	10
60	10

Separation conditions
The sample has been loaded onto an Thermo Fisher™ Acclaim™ PepMap™ μ-Guard Column (300 μm i.d. x 5 mm) with the micro flow pump at a flow rate of 5 μL/min. 5 μL of HeLa cell lysate digest (100 ng/μL) spiked with rituximab tryptic digest were injected using μL-pickup injection mode to avoid sample loss. Peptides were separated with a 30 min gradient (Table 1). Solvent A was water with 0.1% FA and solvent B was acetonitrile (ACN) with 0.1% FA.

Data Acquisition and Analysis
Data was acquired with Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2 and exported as .raw files for data analysis. Thermo Scientific™ Proteome Discoverer™ 2.0 software was used for DDA analysis and data base search with the Sequest™ HT algorithm (false discovery rate (FDR) < 0.1). Full MS data was processed with Chromeleon 7.2, tSIM and PRM data was processed with Thermo Scientific™ Pinpoint™ 1.4.0 and Skyline 3.1 software. Thermo Scientific™ PepFinder™ 2.0 was used for identification of rituximab tryptic peptides with de novo sequencing.

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Results

Selection of rituximab peptides for nano LC-MS analysis

The choice of the appropriate peptides is extremely important to develop a sensitive and robust targeted quantification method. Rituximab is a chimeric mouse/human mAb that consists of two light and two heavy chains [1]. The heavy chain of rituximab contains 451 amino acids and incorporates human protein Ig gamma-1 chain C region (P01857, 330 amino acid residues [2]). The light chain of rituximab contains 213 amino acids and has a high sequence homology with human proteins, e.g. Q6PJF2. This results in a limited number of unique peptides that could be used for rituximab quantification in human matrices. In order to select a set of unique tryptic peptides, rituximab tryptic digest has been profiled with DDA (Figure 1).

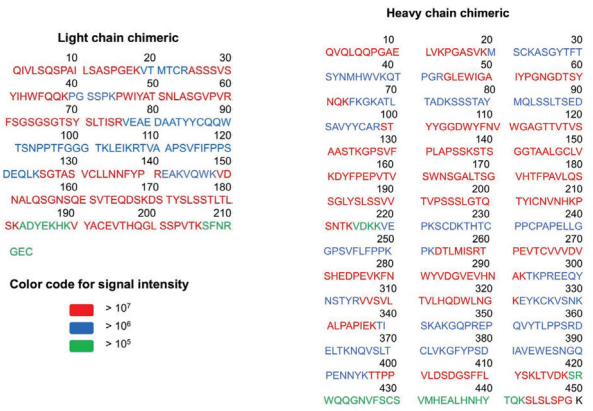
TABLE 2. The unique peptides of rituximab with corresponding m/z for singly, doubly, and triply charged peptide forms and retention time for each peptide (30 min gradient; 60 min total runtime). The red color indicates the peptide forms with highest intensity

	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	Rt, min
Heavy chain chimeric				
GLEWIGAIYPGNGDTSYNQK	2183.0410	1092.0244	728.3522	35
QVQLQQPGAELVKPGASVK	1977.1133	989.0608	659.0606	27
ASGYTFTSYNMHWVK	1791.8165	896.4122	597.9440	27
STYYGGDWYFNVWGAGTTVTVSAASTK	2889.3372	1445.1725	963.7843	39
SSSTAYMQLSSLTSEDSAVYYCAR	2677.1848	1339.0963	893.0668	32
Light chain chimeric				
FSGSGSGTSYSLTISR	1606.7635	803.8896	536.2623	23
QIVLSQSPAILSASPGEK	1825.0071	913.0075	609.0076	36
VEAEDAATYYC[CAM]QQWTSNPPTFGGGTK	2878.2716	1439.6397	960.0958	31

Data dependent profiling of the spiked HeLa samples

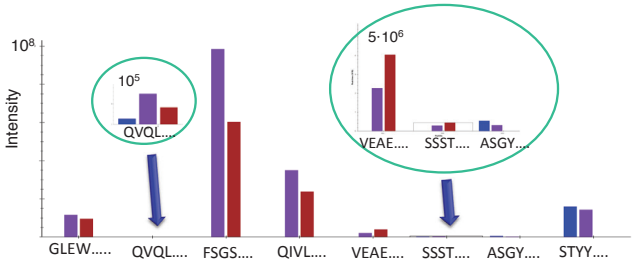
HeLa samples spiked with rituximab at ratio from 1:100 to $1:10^5$ were subjected to nano LC-MS analysis in DDA acquisition mode in order to detect mAb. Around 2800 protein groups and 15.000 peptides were identified at 1% FDR with 500 ng HeLa lysate injected onto column.

FIGURE 1. Light and heavy chain sequence coverage of rituximab. Color code is for the signal intensity of peptides.



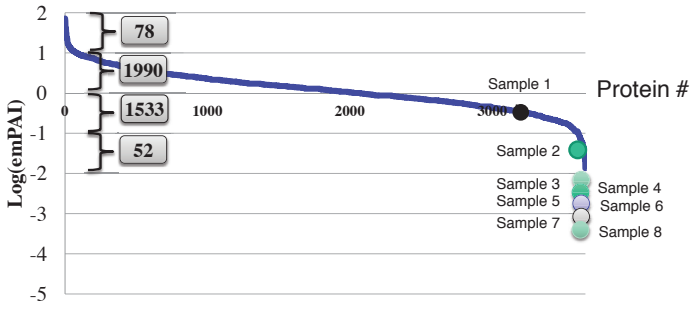
The database search against SwissProt database and rituximab fasta file revealed a set of unique peptides that could be used for mAb quantification in human matrices (Table 2; Figure 2). Among the selected peptides the highest relative response was observed for FSGSGSGTSYSLTISR and QIVLSQSPAILSASPGEK from the light chain of rituximab.

Figure 2. Relative abundance of unique peptides selected for rituximab quantification (most abundant isotopes for each precursor ion are shown)



In DDA the relative abundance of the identified proteins was estimated by exponentially modified protein abundance index (emPAI). 96% of the identified proteins have a relative abundance within 2 orders of magnitude and the overall deepness of proteome profile reached 4 orders of magnitude (Figure 3). The relative abundance of the spiked rituximab is indicated on the distribution curve with color dots and is spread over 4 orders of magnitude at the lower end of the detectable protein concentrations of the HeLa cells lysate (Figure 3). The concentration levels of spiked rituximab are significantly lower than the typical protein abundance range covered by shotgun proteomics with short separation gradients. Thus, targeted quantification methods are required to quantify low levels of rituximab in a complex matrix.

FIGURE 3. Intensity distribution of identified peptides according to emPAI scoring. The spiked samples 1-8 are at the lower end of the intensity scale.

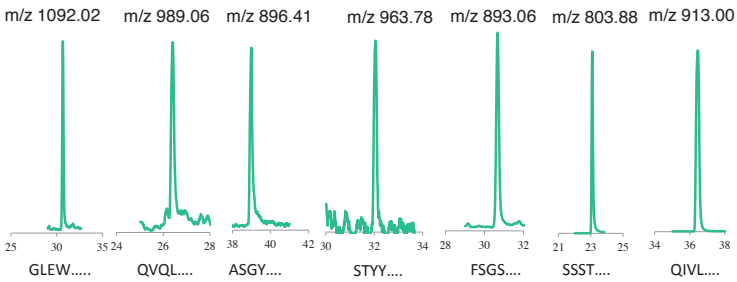


Rituximab heavy chain has been identified in four out of eight samples and rituximab light chain has been identified only in Sample 1. The lowest amount of rituximab loaded onto column and detected with DDA acquisition method corresponds to 30 amol of pure protein. However, identified peptides DSTYSLSSTLTLSK, GPSVFPLAPSSK, ALPAPIEK are not uniquely attributed to rituximab sequence and could not be used for quantification of therapeutic antibody in human samples. PRM and tSIM methods were developed for unambiguous quantification of rituximab in complex matrices with nano LC-MS.

Targeted SIM method for rituximab quantification

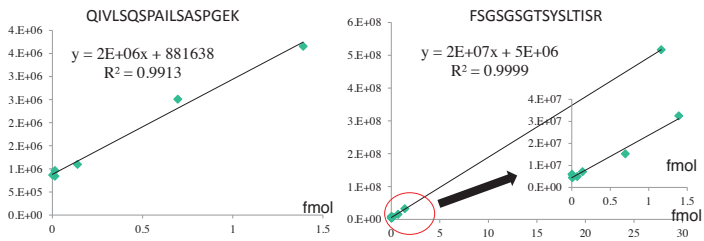
The quantitative analysis of proteins in complex biological samples requires selective methods that can discriminate targeted analytes from interferences. Robust nano LC separations on long 50 cm column was achieved with ProFlow technology that allowed to precisely control the flow rate of A and B channels in real time. High efficiency of Ultra High Performance nano LC separations resulted in narrow peaks with peak width at half high as low as 3 sec (Figure 4). In order to collect sufficient number of data points per each of 8 targeted peptides multiplexed tSIM has been applied with a MS resolution of 120,000.

FIGURE 4. Extracted ion of eight peptides uniquely attributed to rituximab



The highest sensitivity was observed for FSGSGSGTSYSLTISR and QIVLSQSPAILSASPGEK. The linearity range covered four orders of magnitude from amol to fmol level (Figure 5).

FIGURE 5. Calibration plots for rituximab quantification with tSIM analysis

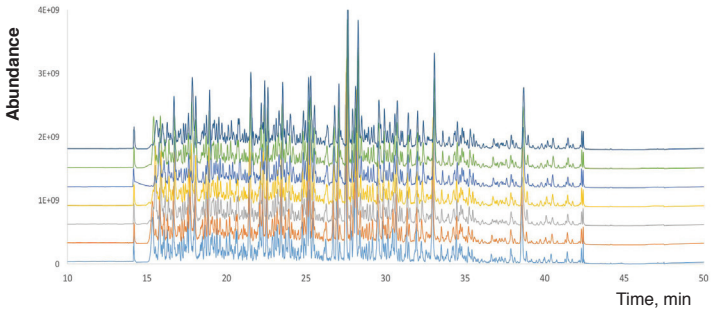


In complex matrices several factors affect the sensitivity of peptide quantification. The most important are signal suppression caused by co-eluting compounds and reduced selectivity due to overlap with peptides with similar m/z values. tSIM mode observed the two most intense peptides in Samples 1 and 2 (Table 2) with a ratio 1:100 and 1:1000 of rituximab to HeLa total protein amount. However, a lower concentration could not be detected. Thus, a PRM method was established to achieve lower quantification limits for rituximab in complex matrices.

Product Reaction Monitoring for rituximab quantification

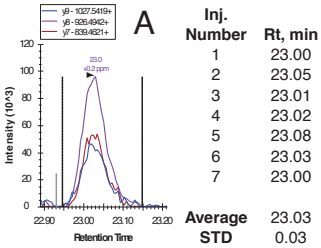
Nano LC-MS in PRM mode is the preferred option for peptides quantification in complex matrices due to an additional level of selectivity provided by detection of MS/MS fragments with high resolution and accurate mass. In order to multiplex PRM measurements and obtain sufficient number of data points per chromatographic peak, the selection of retention time windows for peptide monitoring is important. We investigated the retention time precision for seven replicates and found it to be less than 0.2% RSD for most of the peaks.

FIGURE 6. Overlaid base peak chromatograms of HeLa cell lysate digest spiked with digested rituximab



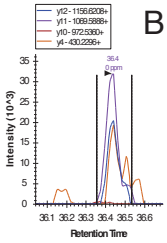
The PRM method allowed to detect rituximab based on FSGSGSGTSYSLTISR and QIVLSQSPAILSASPGEK peptides at 1 : 7.5*10⁴ rituximab to total protein ratio. Multiplexed PRM and narrow retention time windows (Figure 7) for targeting of eight selected unique rituximab peptides allowed collection of a sufficient number of data points for extremely narrow peaks to proceed with accurate quantification.

FIGURE 7. PRM transitions for FSGSGSGTSYSLTISR (A) and QIVLSQSPAILSASPGEK (B) for Sample 4 (1:10⁴ rituximab to HeLa total protein amount) and retention time repeatability.



A

Inj. Number	Rt, min
1	23.00
2	23.05
3	23.01
4	23.02
5	23.08
6	23.03
7	23.00
Average	23.03
STD	0.03
RSD, %	0.12



B

Inj. Number	Rt, min
1	36.47
2	36.46
3	36.45
4	36.45
5	36.44
6	36.42
7	36.42
Average	36.44
STD	0.019
RSD, %	0.052

Conclusions

Robust and highly efficient nano LC separations in combination with targeted high resolution accurate mass quantification achieve high selectivity for quantification of rituximab in complex biomatrices.

- The UltiMate 3000 RSLCnano system with ProFlow technology enables high precision of retention times that allowed to develop a scheduled targeted nano LC-MS method
- FSGSGSGTSYSLTISR and QIVLSQSPAILSASPGEK are uniquely peptides of rituximab and provide highest sensitivity for its quantification in complex matrix
- DDA allow multiple protein profiling based on thousands of identified peptides; tSIM provides very high sensitivity in simple samples; PRM quantification gives highest selectivity and amol sensitivity for quantification of rituximab and could be successfully applied for simultaneous quantification of multiple peptides in a single run

References

1. Samonig M., Swart R. LC-UV-MS Peptide Mapping Development for Easy Transfer to LC-UV QA/QC. Application Note 1134. Thermo Fisher Scientific. 3 p.
2. UniProtKB - P01857 (IGHG1_HUMAN). http://www.uniprot.org/uniprot/P01857

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