A Hybrid Immunocapture-LC-MS/MS Method for the Determination of Humanized Monoclonal Antibody in Rat Plasma

H. Kharbouche, S. Denham and P. Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

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Introduction

LC-MS/MS bioanalytical methods using signature peptides as stoichiometric representations have emerged for quantification of monoclonal antibodies (mAbs) in biological matrices. While promising, the selection of sensitive and selective signature peptides remains a critical step in the development of a robust bioanalytical method to deliver high-quality data for mAb quantitation.

The aim of this study was to develop a LC-MS/MS method for the determination of Bevacizumab, a humanized anti-VEGF monoclonal antibody, in rat plasma. This approach combines specificity of immunocapture for sample preparation and selectivity of mass spectrometry for detection. This poster describes the strategy used to select and optimize MRM signature peptides, and to identify the most sensitive and selective signature peptides of a Bevacizumab digest in rat plasma using Skyline software.

Method

Analyte	Bevacizumab (Avastin®, GENENTECH/ROCHE) recombinant humanized monoclonal antibody to VEGF
Matrix	Rat plasma
Sample volume	100µL
Immunocapture method	Streptavidin immobilized-tips (Thermo Scientific) Biotin conjugated anti-mAb-Fc (Human) antibody (CaptureSelectTM)
Enzymatic digestion	Trypsin, MS Grade
LC system	Waters Acquity UPLC I-Class System (SM-FTN)
LC Method	UPLC reverse phase
Analytical column	BEH C18, 2.1mm x 50mm, 1.7µm particles
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Flow rate, elution mode	300µL/min, linear gradient
MS system	Sciex 5500QTRAP
Acquisition mode, polarity	MRM, positive ESI
Injection volume	2µL

Selection and optimization of signature peptides suitable for quantification

Candidate tryptic peptides were selected according to defined criteria. Details on the method used to select and optimize signature peptides with Skyline are shown in Fig.1.

Candidate tryptic peptide selection criteria	
Included	■ 7 to 25 amino acid-containing peptides, multiple positively charged precursor ions (+2 and +3 charge states), b and y product ions (+1 and +2 charge states). ■ Product ions with m/z higher than the precursor ions.
Excluded	■ Peptides containing residues susceptible to posttranslational or artefactual modifications. ■ Peptides containing sequence that commonly results in missed cleavage.

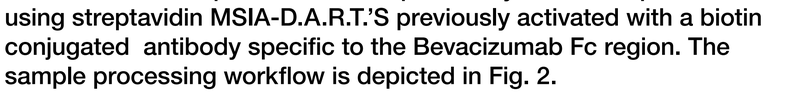

Figure 1. Method used for identification and selection of the best peptide MRM transitions and optimization of CE and DP parameters of selected MRM transitions. Unscheduled MRM methods (diagram A) were generated to identify peptide's retention times and to select the best MRM transitions for each tryptic peptide. Scheduled MRM methods (diagram B) were used to optimize CE and DP parameters.

Sample Preparation and Tryptic Digestion Workflow

Bevacizumab was purified from rat plasma by immunocapture using streptavidin MSIA-D.A.R.T.'S previously activated with a biotin conjugated antibody specific to the Bevacizumab Fc region. The sample processing workflow is depicted in Fig. 2.

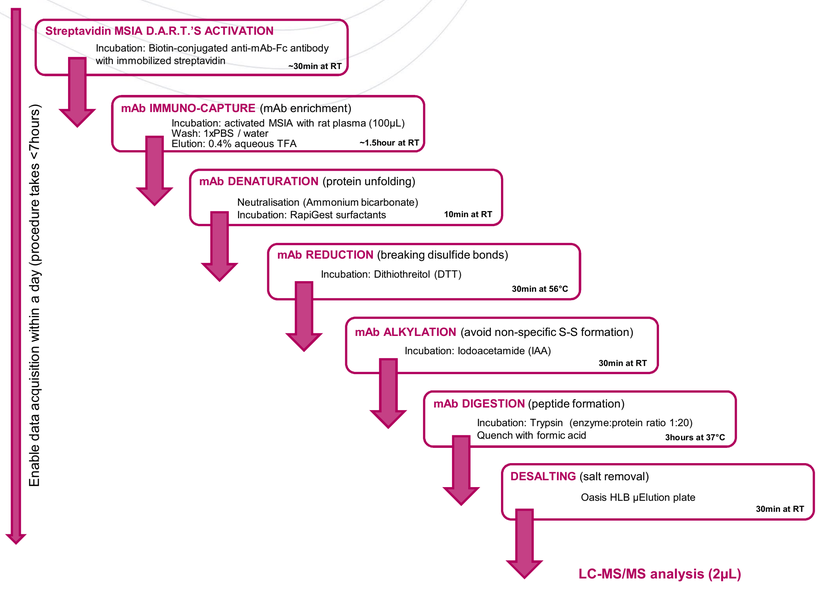

Figure 2. Sample Processing Workflow Used for Bevacizumab Enrichment and Digestion.

Results

Selection and Optimization of Signature Peptides

MRM experiments of a Bevacizumab digest were performed on 5500 QTRAP to screen for the 21 candidate tryptic peptides generated:

- The most sensitive precursor ion charge state was selected for each peptide.
- The relative abundance of the MRM transitions was evaluated to identify the 2 best MRM transitions for each peptide.
- The CE and DP values were optimized for each of the selected MRM transitions.

An overview of the strategy used for the selection and optimization of tryptic peptides is shown in Fig. 3.

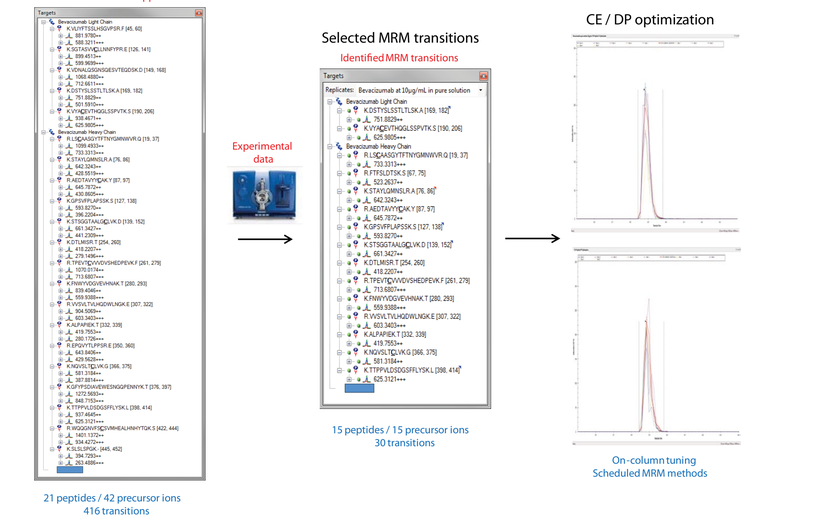

Figure 3. Selection and optimization of signature peptides using Skyline. Skyline allowed to reduce the list of MRM transitions down to the 15 signature peptides and to select the optimal instrument settings for each of the 30 selected MRM transitions. The optimized DP and CE values were recorded and used in the final MRM method.

Final MRM Method Tested on Bevacizumab Digest (pure solution)

The final MRM method with optimized conditions was tested on a Bevacizumab tryptic digest. The chromatogram obtained is shown in Fig. 4.

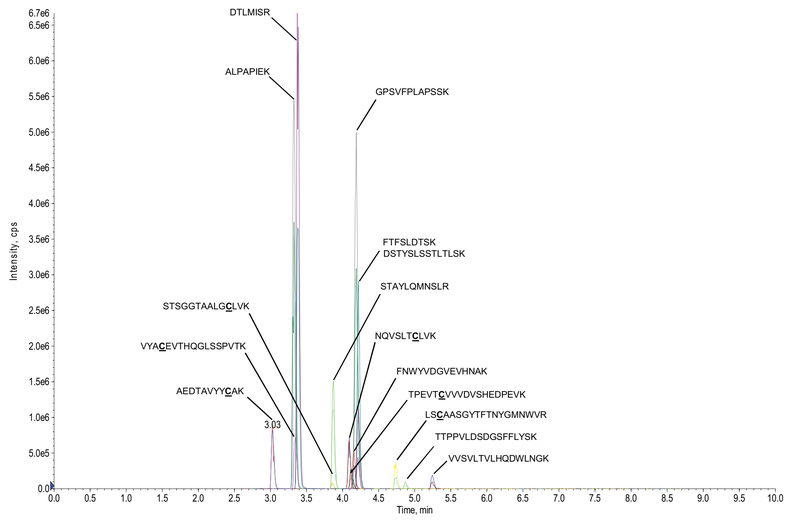

Figure 4. Representative LC-MS/MS chromatogram of Bevacizumab digest using the optimized MRM method created from Skyline software. Bevacizumab was spiked at 10µg/mL in pure solution, processed and analyzed by LC-MS/MS. Note: no immunocapture enrichment was performed.

Identification of the Most Sensitive Signature Peptides in Rat Plasma

The final MRM method was tested on Bevacizumab in rat plasma. The 15 signature peptides were simultaneously monitored to identify peptides that produced the best sensitivity. The best sensitivity was achieved using the signature peptides shown in Fig. 5. The key characteristics of the selected signature peptides are shown in Table 1.

Light chain
DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
EDFATYYCQQYSTVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGN
SQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Heavy chain
EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKS
TAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY
FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Selected and optimized signature peptides
Signature peptides being the most sensitive

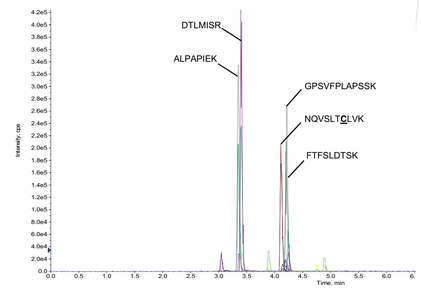

Figure 5. Amino acid sequence information of the light and heavy chain of Bevacizumab. The blue-underlined sequences represent the 15 optimized signature peptides. The red-squared sequences represent the most sensitive signature peptides.

Position	Selected peptide Sequence	MRM transition	MW	pI	# of residues	Region in Bevacizumab
254 - 260	DTLMISR	418.2 (2^+) → 619.4 (y_5^+) 418.2 (2^+) → 506.3 (y_4^+)	834.99	7.4	7	Fc
332 - 339	ALPAPIEK	419.8 (2^+) → 654.4 (y_6^+) 419.8 (2^+) → 486.3 (y_4^+)	838.01	6.4	8	Fc
127 - 138	GPSVFPLAPSSK	593.8 (2^+) → 846.5 (y_8^+) 593.8 (2^+) → 699.4 (y_7^+)	1186.37	10.1	12	Fab
366 - 375	NQVSLTC[+57]LVK	581.3 (2^+) → 919.5 (y_8^+) 581.3 (2^+) → 820.5 (y_7^+)	1161.38	9.0	10	Fc
67 - 75	FTFSLDTSK	523.3 (2^+) → 797.4 (y_7^+) 523.3 (2^+) → 650.3 (y_6^+)	1045.16	6.4	9	Fab

Table 1. Characteristics of the selected signature peptides. The three peptides DTLMISR, ALPAPIEK and NQVSLTC[+57]LVK are generated from the constant region (Fc) of the Bevacizumab and can thus be used as generic signature peptides since they are conserved among different human mAb (IgG) subclasses. The two other peptides GPSVFPLAPSSK and FTFSLDTSK are generated from the variable region (Fab) of Bevacizumab and can thus be used as unique signature peptides since they are only present in Bevacizumab.

The background noise levels were compared for the 5 selected signature peptides, in rat plasma and in pure solution for selectivity assessment. The immuno-enrichment procedure, combined to mass spectrometry, allowed to remove endogenous interferences from rat plasma, and resulted in good selectivity (Fig. 6).

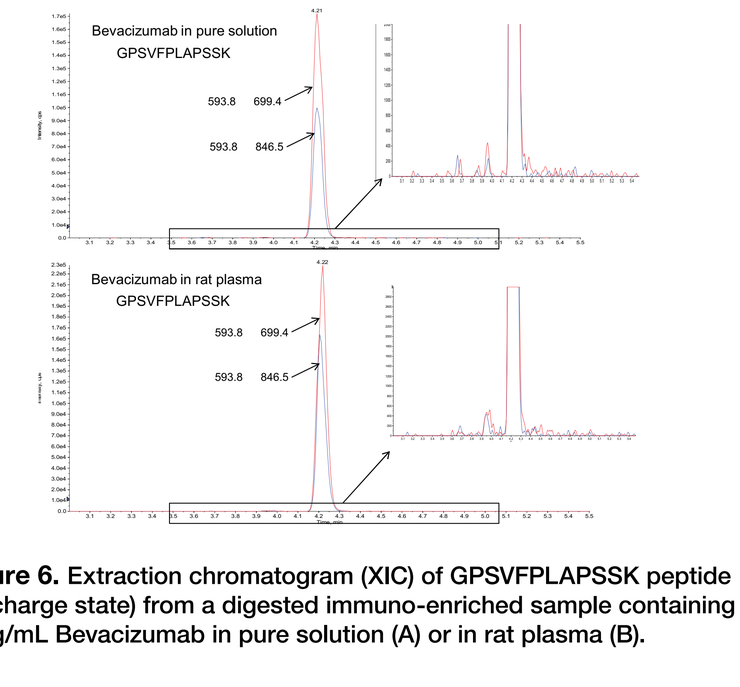

Figure 6. Extraction chromatogram (XIC) of GPSVFPLAPSSK peptide (+2 charge state) from a digested immuno-enriched sample containing 10µg/mL Bevacizumab in pure solution (A) or in rat plasma (B).

Conclusions

Our approach demonstrated its utility in identifying the most effective signature peptides for quantitation of Bevacizumab in rat plasma. The method offers the advantages to:

- Quantify other mAbs in preclinical studies by monitoring the 3 signature peptides which were found to be conserved among different human mAbs.
- Quantify Bevacizumab in clinical studies by monitoring the 2 signature peptides which were found to be unique.

This method will be tested, together with a heavy isotope labeled mAb internal standard, for bioanalytical assay performance.

Poster presentation at EBF 2016

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