Supporting Information

Direct and precise measurement of Bevacizumab levels in human plasma based on controlled methionine oxidation and multiple reaction monitoring

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Table of Contents

Experimental procedures

Reagents were obtained from Sigma Aldrich (Oakville, Canada), if not stated otherwise. Trypsin (Sequencing grade) was purchased from Worthington (Lakewood, NJ, USA); Microcon-30 kDa Centrifugal Filters were from Millipore-Sigma (Oakville, Canada); S-Trap^{TM} mini-columns, purchased from Protifi (NY, USA); LC-MS grade acetonitrile (ACN) and water were purchased from VWR International (Montreal, Canada) and Honeywell B&J Brand (Muskegon, USA), respectively. Bevacizumab (Avastin, 25.4 mg/mL) was kindly provided by Apobiologix, Canada. STAYLQMNSLR unlabeled 'light' (native, NAT) and STAYLQMNSL**R**+10 Da SIS peptides were synthesized, purified, and quantified using amino acid analysis at the University of Victoria Genome BC Proteomics Centre.

Selection of proteotypic peptides for MRM. 100 µg of Bevacizumab (Avastin, 25.4 mg/mL) was diluted in lysis buffer (1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.8), reduced with 10 mM dithiotreitol (DTT) for 30 min at 56^{o}C, and alkylated with 30 mM iodoacetamide for 30 min at room temperature. The sample was diluted with freshly prepared [1] 8 M urea, 100 mM Tris-HCl at pH 8.5 to give a final concentration of 6 M urea, and the solution was transferred to 30 kDa Molecular Weight Cut-off (Merck, Millipore) centrifugal filters (Pall) for filter-aided sample preparation (FASP).[2-4] The liquid was passed through the filter at 13,500 x g (at 25°C) and the same centrifugation conditions were used for all the following FASP steps. The filter devices were washed three times with 100 µL of 8 M urea, 100 mM Tris-HCl at pH 8.5 and three times with 100 µL of 50 mM ammonium bicarbonate (ABC), pH 7.8. The protein sample was digested in 100 µL of 50 mM ABC pH 7.8, 0.2 M guanidine hydrochloride (GuHCl), 1 mM $CaCl_2$ and trypsin (Worthington) in a 10:1 (protein:enzyme) ratio at 37°C overnight. The digests were collected by centrifugation, followed by a rinse with 50 µL of 50 mM ABC that was collected in the same reaction tube. The digests were acidified with 0.1 % formic acid (FA) and were dried under vacuum. The generated peptides were analyzed by DDA on a Triple TOF 6600 (AB Sciex), and the raw data was converted into the mgf format using Proteowizard.[5] Peak lists were searched using Proteome Discoverer 2.3 (Thermo Fisher Scientific) against a human Uniprot protein database to which sequences from 17 mAbs including Bevacizumab[6] (20,410 forward sequences; downloaded October 9, 2018), had been added. Only unique peptides identified with high confidence were considered as potential target peptides, and were ranked according to their number of PSMs and signal intensities.

MRM assay development. STAYLQMNSLR NAT and SIS peptides were used to determine the best transitions for MRM, to optimize the collision energies (CEs) per transition, and to develop calibration curves and determine the assay metrics (linearity, precision, accuracy, sensitivity). LC-MRM analyses were conducted on an Agilent 6495 triple-quadrupole mass spectrometer, coupled to an Agilent 1290 Infinity UHPLC system via an ESI source with Agilent Jet Stream technology, in the positive ion mode. The capillary and nozzle voltages were set at 3500V and 300V, respectively. Peptides were separated using an Agilent RRHD Eclipse Plus C18 column (2.1 mm inner diameter x 150 mm length, 1.8 μm particle size), maintained at 50°C, using a binary gradient at a flow rate of 0.4 mL/min. Mobile phases A and B consisted of 0.1% formic acid (FA), and 0.1% FA in acetonitrile (ACN), respectively, and the gradient was as follows: 0 min: 2% B, 1 min: 3% B, 8 min: 35% B, 9.5 min: 90% B, 10 min: 90% B, 10.5 min, 2% B. MRM data was analyzed using Skyline (version 20.1.0.155).[7] The transitions with the highest signal intensities showing interference in some cases (used as qualifier ions: y6, y5) or no interference (quantifier ion: y7) in plasma samples were selected. Optimized CEs for the individual MRM transitions were m/z 650.3217 → m/z 877.4560 (y7; CE 13), m/z 650.3217 → m/z 764.3719 (y6; CE 17), and m/z 650.3217 → m/z 636.3134 (y5; CE 8). To induce complete Met oxidation, samples were incubated for 30 min in 0.5% H_2O_2 and 0.5% TFA at 30°C, immediately before LC-MRM analysis, while controls were incubated without the addition of H_2O_2.

Proteolytic Digestion of Bevacizumab in plasma using S-Trap™ mini-columns. Bevacizumab was diluted and spiked into commercial plasma at following concentrations: 1.09, 2.19, 4.38, 8.75, 17.5, 35, 70, 140 μg of drug per mL of plasma. For each concentration, six aliquots of 2.25 μL of plasma containing Bevacizumab were diluted with lysis buffer (5% SDS, 150 mM NaCl, 50 mM Tris, pH 7.8), heated to 95°C for 15 min, reduced with 20 mM tris-2-carboxyethyl-phosphine (TCEP) for 30 min at 60°C, and alkylated with 25 mM iodoacetamide for 30 min at room temperature. The samples were acidified to a final concentration of 1.2% phosphoric acid, and then diluted seven times in binding buffer (100 mM ammonium bicarbonate in 90% methanol). The solution was transferred to a S-Trap™ mini-column (Protifi) and centrifuged for 60 s at 4000 x g. The column was rinsed three times with 150 μL of binding buffer, and centrifuged as above. Proteins were digested in 120 μL of 50 mM ammonium bicarbonate supplemented with trypsin (Worthington) in a 10:1 (protein:enzyme) ratio at 47°C for two hours. The digests were

collected by centrifugation at 1000 x g for 60 s, and the columns were successively rinsed with 80 µL of 50 mM ABC, with 80 µL of 0.1% FA and with 80 µL of 50% ACN, 0.1% FA, all collected in the same reaction tube. The digests were dried under vacuum, and resuspended in 30 µL H_2O containing 30 fmol/µL of SIS peptide, which was used as normalizer. For each Bevacizumab concentration, three 30-µL digest aliquots were treated with H_2O_2 for Met oxidation, as described above, while three aliquots were treated with H_2O instead, and served as controls. Per sample 10 µL (corresponding to 50 µg of plasma) were injected on-column and analyzed by LC-MRM. Data was analyzed using Skyline (version 20.1.0.155)[7] and is shown in Figure 1C.

MRM assay validation. Calibration curves were prepared in five replicates by spiking a constant level of SIS (final concentration of 60 fmol/µL) and increasing amounts of NAT (final concentrations of 2.9; 4.4; 5.8; 8.8, 11.7; 17.5; 35; 70, 140, and 280 fmol/µL) into a tryptic plasma digest with a total protein concentration of 20 µg/µL. Per sample, 5 µL containing 100 µg of total plasma protein digest-were injected on column and analyzed by LC-MRM. A divert valve was used so that only peptides eluting during minutes 5-7, where the target peptides and their non-oxidized variants eluted, were allowed to enter the mass spectrometer. Scheduled MRM transitions for the STAYLQM(ox)NSLR NAT and SIS peptides and their non-oxidized variants (monitored for quality control of the oxidation reaction) were acquired during minutes 5-7.

References

(1) Kollipara, L., Zahedi, R. P. (2013) Protein carbamylation: in vivo modification or in vitro artefact? *Proteomics*, *13*, 941-944.
(2) Wiśniewski, J. R., Zougman, A., Nagaraj, N., Mann, M. (2009) Universal sample preparation method for proteome analysis. *Nature methods*, *6*, 359-362.
(3) Liebler, D. C., Ham, A.-J. L. (2009) Spin filter–based sample preparation for shotgun proteomics. *Nature methods*, *6*, 785-785.
(4) Manza, L. L., Stamer, S. L., Ham, A. J. L., Codreanu, S. G., Liebler, D. C. (2005) Sample preparation and digestion for proteomic analyses using spin filters. *Proteomics*, *5*, 1742-1745.
(5) Holman, J. D., Tabb, D. L., Mallick, P. (2014) Employing ProteoWizard to convert raw mass spectrometry data. *Current protocols in bioinformatics*, *46*, 13.24.1-13.24.9.
(6) Consortium, U. (2019) UniProt: a worldwide hub of protein knowledge. *Nucleic acids research*, *47*, D506-D515.
(7) MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., MacCoss, M. J. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, *26*, 966-968.