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應用手冊

Simple, Standardized, and Sensitive Quantification of Bevacizumab (Avastin) Using ProteinWorks eXpress Digest Kits

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Abstract

This application note describes the fast, sensitive quantification of bevacizumab from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol.

Benefits

Simple, standardized approach to protein quantification; broadly applicable optimized digest kit eliminates method development for discovery studies; samples are ready for LC-MS analysis in 4–6 hours; high sensitivity detection limit of 100 ng/mL for bevacizumab was achieved

Introduction

During the period of 2013-2020, many of the world's top selling antibody-based drugs come off patent,[1] including

bevacizumab, an almost $6 billion dollar drug, expiring in 2020. In addition, as of 2013, there were 338 new monoclonal antibody drugs in development.[2] This represents the largest class of biologics in the pipelines. As patents for bevacizumab and other important antibody therapies expire and additional antibody drugs are developed, the need for streamlined LC-MS protein bioanalysis methods and approaches continues to grow. This is particularly true as bioanalysis studies using LC-MS were historically dominated by small molecule therapies with far simpler and more straightforward sample prep and analysis methods. Bevacizumab (Avastin, Genentech/Roche) is a tumor angiogenesis inhibitor, which selectively binds to and neutralizes the biologic activity of human vascular endothelial growth factor (VEGF) and is an adjunct IV therapy for colorectal, lung, cervical and kidney cancer, amongst others.[3,4] Typical dosing is in the 5–15 mg/kg range every 2–3 weeks, depending on indication. It was reported that doses >1 mg/kg produced serum levels of bevacizumab ≥10 µg/ml for at least 14 days.[5] This information suggests that a quantification method with a detection limit of ≥100 ng/mL and an upper limit of quantification (ULOQ) of several hundred µg/mL would be more than sufficient. While this would be a trivial exercise for a small molecule bioanalyst, the lack of expertise in biological molecule handling and the techniques associated with protein quantification via the surrogate peptide approach make it challenging for those same individuals to readily obtain high quality bioanalytical data in support of antibody drug programs. A generic, yet standardized approach to protein bioanalysis using LC-MS which is broadly applicable to large molecule drug development would enable novice scientists to successfully support discovery studies. In addition, one such universal proven methodology could facilitate transfer of methods between sites and ensure reproducibility of results. This application note describes the fast, sensitive quantification of bevacizumab (Figure 1) from rat plasma using the ProteinWorks eXpress Digest Kit and Protocol. A single universal sample prep method using pre-weighed, lot-traceable reagents and a carefully developed, yet generic set of simple step-wise instructions produced an LLOQ of 100 ng/mL bevacizumab from 35 µL of rat plasma.

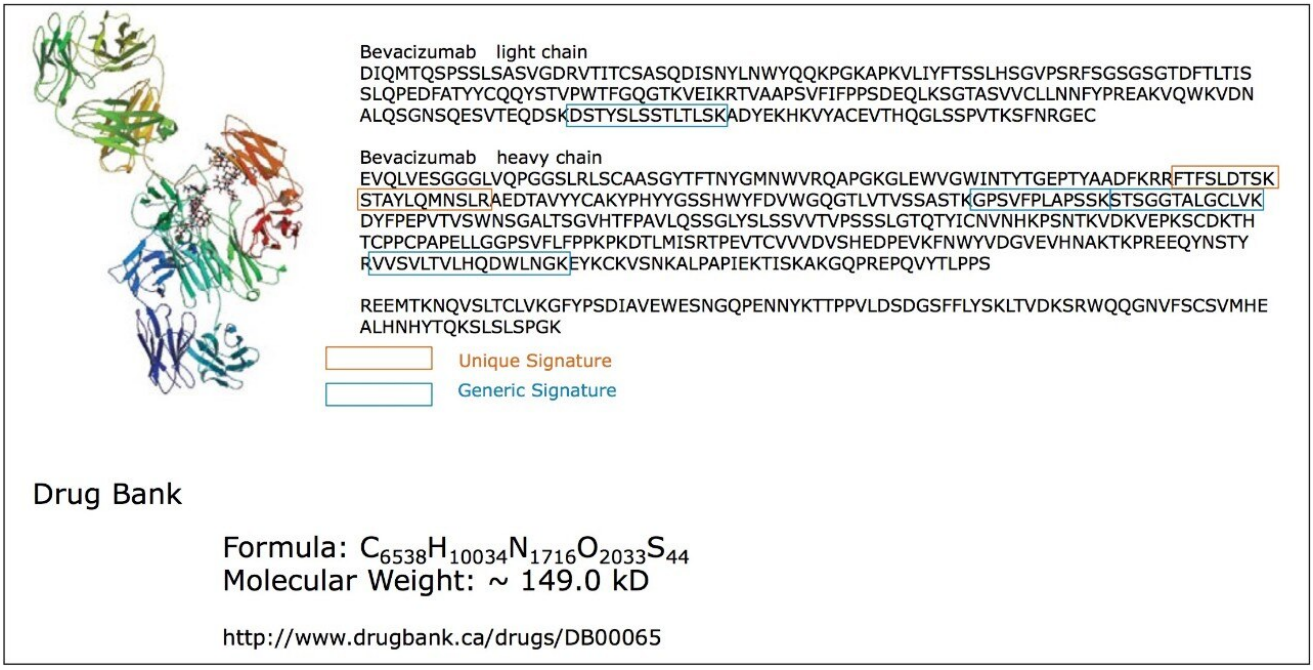

Figure 1. Bevacizumab (Avastin) Protein Structure.

Experimental

Sample description

Bevacizumab was first immuno-purified from 35 µL rat plasma using a 96-well Protein A agarose-based plate. Samples were then prepared for LC-MS analysis using the ProteinWorks eXpress Digest Kit and protocol. Finally, signature peptides were cleaned-up using the ProteinWorks µElution SPE Clean-up Kit and Protocol.

Method conditions

LC system:	ACQUITY UPLC
Detection:	Xevo TQ-S Mass Spectrometer, ESI+

Column:	ACQUITY UPLC Peptide BEH C_{18}, 300Å, 1.7µm, 2.1 mm x 150 mm
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Data management:	MassLynx (v4.1)

Gradient:

Flow rate (mL/min)	Time (min)	Profile		Curve
		%A	%B	
0.3	0	100	0	6
0.3	1	100	0	6
0.3	7	50	50	6
0.3	8	10	90	6

MS conditions

Capillary (kv):	3
Cone (V):	30
Source offset (V):	50
Source temp. (°C):	150
Desolvation temp. (°C):	600
Cone gas flow (L/hr):	150
Desolvation gas flow (L/hr):	1000
Collision gas flow (mL/min):	0.15
Nebuliser gas flow (Bar):	7

Peptide	MRM transition	Cone voltage (V)	Collision energy (eV)
FTFSLDTSK*	523.30>797.48	16	14
STAYLQMNSLR*	642.30>748.45	36	20
DSTYSLSSTLTLSK	751.88>836.47	31	24
STSGGTAALGC[+57.0]LVK	661.34>576.32	31	25
NTQPIMDTDGSYFVYSK (ISTD)	983.95>397.21	32	26

**Unique Signature Peptide*

Table 1. MRM conditions for bevacizumab peptides and the internal standard peptide.

Results and Discussion

With the bevacizumab US patent expiration date of 2020 drawing ever closer, the focus on this important drug in CRO's as well as biosimilar research labs has increased. However, typical workflows are incredibly complex, with a multitude of choices and options. This makes the development of high sensitivity LC-MS methods for this and other monoclonal antibody-based drugs particularly challenging. In this application note, we have used the ProteinWorks eXpress Digest Kit to simplify and streamline the process. Bevacizumab samples were affinity purified, digested, and peptides extracted using SPE in under 6 hours total. This enabled same day data acquisition, with several 96-well plates being run by the following morning. Multiple unique signature peptides as well as a generic human peptide were simultaneously monitored for use in quantification. The best sensitivity and specificity were achieved using the unique peptide FTFSLDTSK from the variable region of the heavy chain, while additional generic (STSGGTAALGC+57LVK, heavy chain) and specific (STAYLQMNSLR, heavy chain) bevacizumab peptides were monitored for confirmation. A signature peptide (NTQPIMDTDGSYFVYSK) from a common murine mAb standard (p/n 186006552) was used as the internal standard.

Using the optimized protocol and reagents provided in the kit, only 35 µL of plasma was needed to achieve a quantification limit of 100 ng/mL for bevacizumab (Figure 2). Linearity and accuracy of the standard curves arising from each peptide are summarized in Table 2. The primary, and most sensitive and specific quantitative peptide, FTFSLDTSK, was linear over 3.5 orders of magnitude with a mean accuracy of 100% for all points on the curve. The additional three peptides were linear over 3–3.5 orders of magnitude with average accuracies >99% for all curve points.

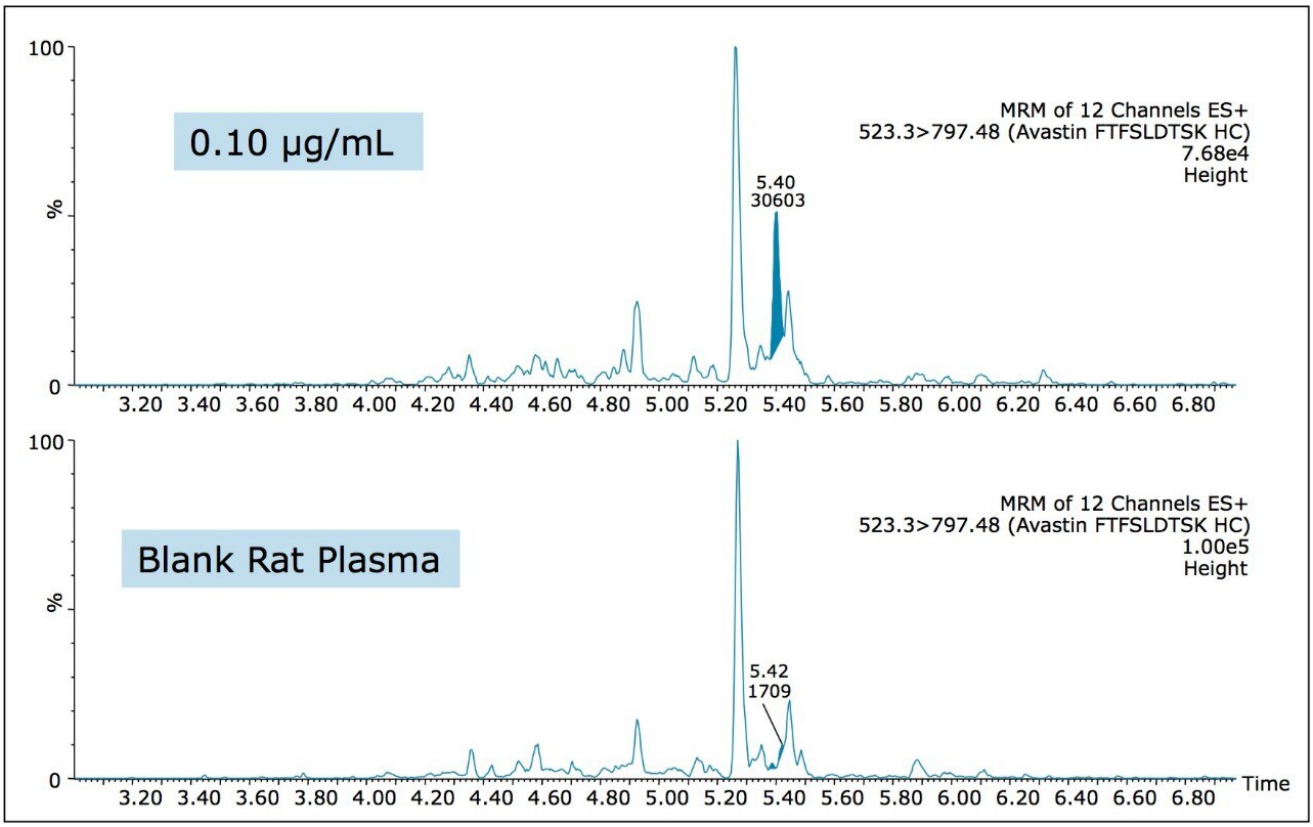

Figure 2. Chromatogram showing 100 ng/mL of bevacizumab in rat plasma, as compared to blank rat plasma. The unique peptide FTFSLDTSK is shown.

Peptide	Std. curve range (µg/mL)	Weighting	Linear fit (r^2)	Mean % accuracy of all points
FTFSLDTSK*	0.05–250	1/X	0.998	100.00
STAYQMNSLR*	0.50–500	$1/X^2$	0.997	100.02
DSTYSLSSTLTLSK	0.25–500	$1/X^2$	0.996	100.00
STSGGTAALGC+57LVK	0.05–250	$1/X^2$	0.996	100.60

**Unique signature peptide*

Table 2. Linear dynamic range and standard curve statistics for signature peptides used to quantify bevacizumab in rat plasma.

In addition, the accuracy and precision for the QC samples was excellent with %CVs all ≤7%. This is summarized in Table 3. In fact, the average %CV for QC samples from the FTFSLDTSK peptide was 4%. Similarly, the average %CV for QC samples from the STAYLQMNSLR peptide was only 3%. Furthermore, the precision of the low QC across all peptides was 3%. Mean accuracies for all peptides hovered close to 100%.

Peptide	QC conc (µg/mL)	Mean cal. conc (µg/mL)	Std. dev.	%CV	Mean accuracy
FTFSLDTSK*	0.035	–	–	–	–
	0.350	0.342	0.013	3.82	97.7
	3.500	3.553	0.126	3.56	101.5
	35.000	32.386	0.611	1.89	92.5
	350.000	290.135	21.024	7.25	82.9
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
STAYLQMNSLR*	0.035	–	–	–	–
	0.350	0.345	0.004	1.12	98.6
	3.500	3.819	0.110	2.89	108.3
	35.000	35.065	1.262	3.60	100.2
	350.000	335.347	15.208	4.54	95.8
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
DSTYSLSSTLTLSK	0.035	–	–	–	–
	0.350	0.351	0.016	4.61	100.3
	3.500	3.397	0.045	1.32	97.1
	35.000	32.082	0.469	1.46	91.7
	350.000	320.836	9.141	2.85	91.7
Peptide	**QC conc (µg/mL)**	**Mean cal. conc (µg/mL)**	**Std. dev.**	**%CV**	**Mean accuracy**
STSGGTALGC+57LVK	0.035	–	–	–	–
	0.350	0.366	0.012	3.36	104.4
	3.500	3.509	0.063	1.79	100.2
	35.000	33.015	1.406	4.26	94.3
	350.000	305.140	3.259	1.07	87.2

Table 3. Statistics for QC samples from all bevacizumab peptides used for quantification.

We consistently achieved single digit accuracy and precision during bevacizumab analysis through a combination of three primary factors: high fidelity chromatographic data, an optimized, well controlled protocol, and the use of a set of standardized, pre-measured reagents. These three critical components were realized

through application of the ProteinWorks eXpress Digest Kit, which facilitated low level detection, separation from residual endogenous interferences, and the very high accuracy and precision. This performance is highlighted in the QC chromatograms from representative signature peptides in Figures 3 and 4.

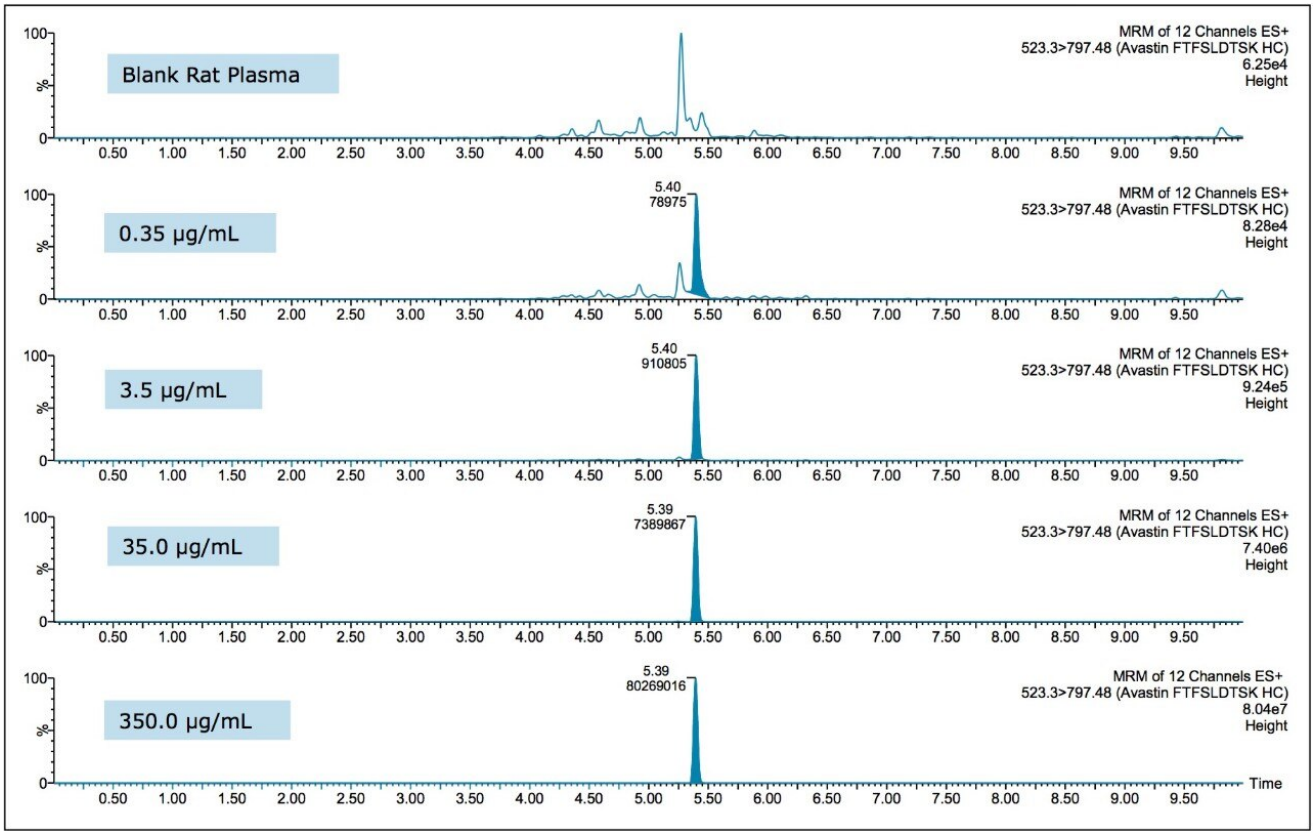

Figure 3. Bevacizumab QC Chromatograms for the FTFSLDTSK Unique Signature Peptide.

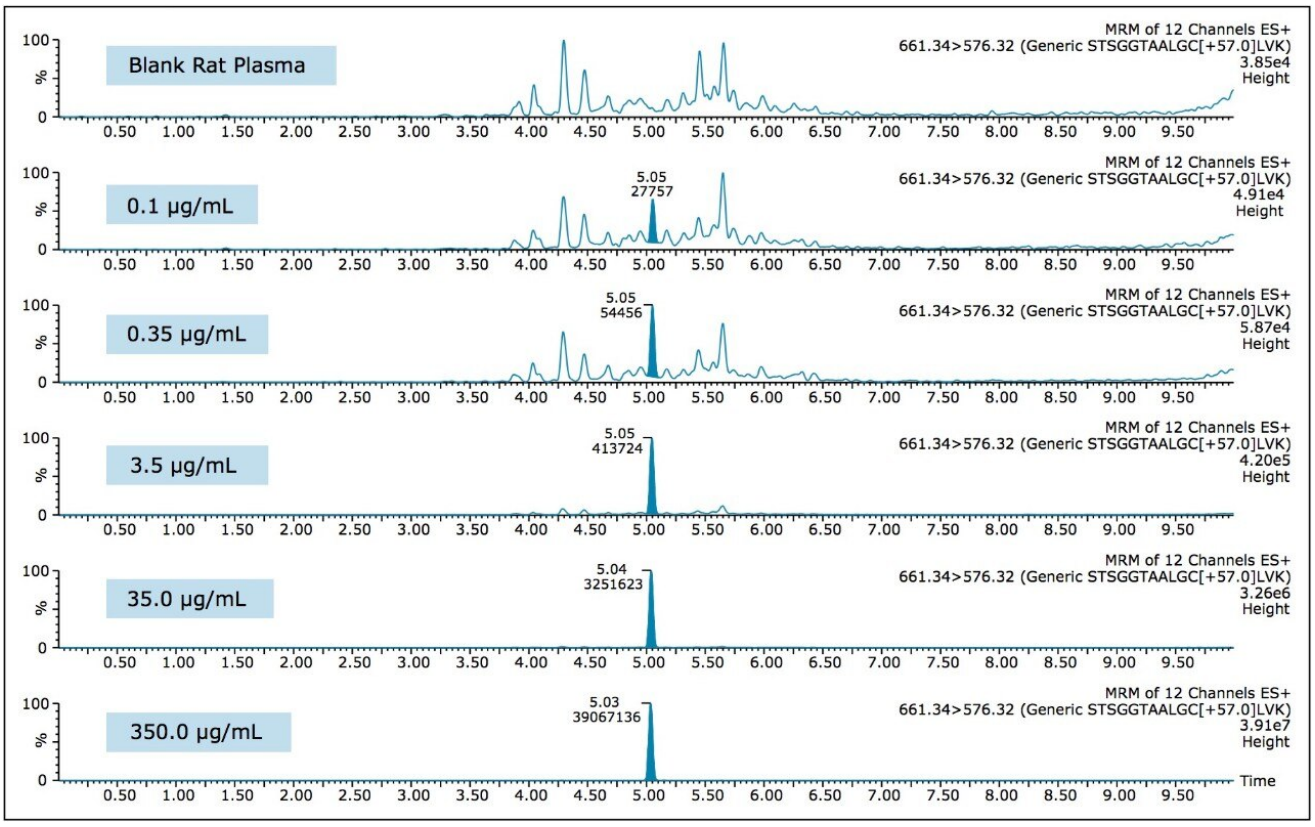

Figure 4. Bevacizumab QC Chromatograms for the STSGGTAALGC+57LVK Generic Signature Peptide.

Conclusion

The ProteinWorks eXpress Digest Kit was successfully used to purify bevacizumab from a typical set of standard curve and QC samples in rat plasma. A limit of quantification of 100 ng/mL was readily achieved from only 35 µL rat plasma, while maintaining excellent linearity and single digit precision. The total sample prep time including an affinity purification step was under 6 hours. The total digest prep time was just over 2 hours. The universal, kit-based approach allows novice users to achieve low detection limits with a simple step-wise protocol and a set of standardized, pre-measured reagents, ensuring both the sensitivity required and the transferability desired of such methods.

In addition, the kit is optimized and flexible enough to enable simultaneous, sensitive quantification of both

unique and generic signature peptides from monoclonal antibodies. This is important as confirmatory data from multiple peptides is critical in supporting the confident use of LC-MS for protein bioanalysis workflows.

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