SHIMADZU

WP 487

LC-MS/MS method development of aflibercept using Fab-selective proteolysis nSMOL technology

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1. Introduction

Aflibercept is a biopharmaceutical drug inhibiting of vascular endothelial growth factor (VEGF) signaling and composed of the extracellular domains of human VEGF receptors 1 and 2 that are fused to the Fc portion of the human IgG1 immunoglobulin. It is important to determine an appropriate dose for medical optimization, but little amount of intraocular fluid is able to collect as a specimen in the treatment of retina. To offer the quantitative assessment method of aflibercept in biological matrix, the primary structure was confirmed using a quadrupole time-of-flight (Q-TOF) mass spectrometer LCMS-9030, and quantitative analysis was performed with a triple quadrupole mass spectrometer LCMS-8060 by using nSMOL (nano-surface and molecular orientation limited proteolysis) technology.

2. Methods and Materials

The tryptic digest of aflibercept was analyzed by MS using a Q-TOF, and MS/MS analysis of the VEGF receptor region was performed to select transitions for multiple reaction monitoring (MRM) assay. The observed fragments were selected for the quantitative analysis using a triple quadrupole LC-MS/MS. A serial dilutions of aflibercept were prepared with human plasma and 5 uL was used for each assay. Using nSMOL Antibody BA Kit, aflibercept and endogenous IgGs were captured in resin pore via Protein A and selectively proteolyzed with trypsin immobilized on the surface of nanoparticles to collect the signature peptides for aflibercept.

2-1. Sample treatment for sequence confirmation of aflibercept peptides

Aflibercept (10 μg) was dissolved in 10 μL of 2 M urea containing 1 mM TCEP, reacted at room temperature for 30 minutes, then 100 μL of 20 mM tris-HCl buffer (pH 8.0) and trypsin 1 μg were added, and the mixture was treated at 37°C for 16 hours.

2-2. MS and MS/MS analysis conditions

<Nexera X3 system>

Column : Shim-pack GISS-HP C18 (100 mm x 2.1 mm I.D., 3 μm)
Mobile phase A : 0.1% Formic acid / Water
Mobile phase B : 0.1% Formic acid / Acetonitrile Flow rate : 0.2 mL/min
Time program : B conc. 1% (0-5 min) → 40% (40 min) → 95% (40.01-50 min) → 1% (50.01-60 min)
Column temp. : 40 ºC Injection vol. : 5 μL

<LCMS-9030>

Ionization : ESI, Positive mode MS range : *m/z* 100-1300
CID : 10-30 V DL temp. : 250 ºC
Interface temp. : 300 ºC Heat block temp. : 400 ºC
Nebulizer gas : 3.0 L/min Heating gas : 10 L/min
Drying gas : 10 L/min

2-3. LC-MS condition for MRM assay

<Nexera X2 or Nexera X3 system>

Column : Shim-pack GISS C18 (50 mm x 2.1 mm I.D., 1.9 μm)
Mobile phase A : 0.1% Formic acid / Water
Mobile phase B : 0.1% Formic acid / Acetonitrile
Flow rate : 0.4 mL/min (0-5.51, 6.72-7.5 min), 1 mL/min (5.53-6.7 min)
Time program : B conc. 1% (0-2 min) → 40% (5.5 min) → 95% (5.52-6.33 min) → 1% (6.35-7.5 min)
Column temp. : 50 ºC Injection vol. : 10 μL

<LCMS-8060>

Ionization : ESI, Positive mode DL temp. : 200 ºC
Interface temp. : 400 ºC Heat block temp. : 350 ºC
Nebulizer gas : 3.0 L/min Heating gas : 10 L/min
Drying gas : 10 L/min

2-4. nSMOL sample preparation

The nSMOL kit was used for pretreatment for quantification of aflibercept in human plasma. After collection of aflibercept from 5 uL of sample with immunoglobulin collection resin, peptide fragments were obtained by trypsin immobilized on nanoparticles. The protocol scheme is described Figure 1.

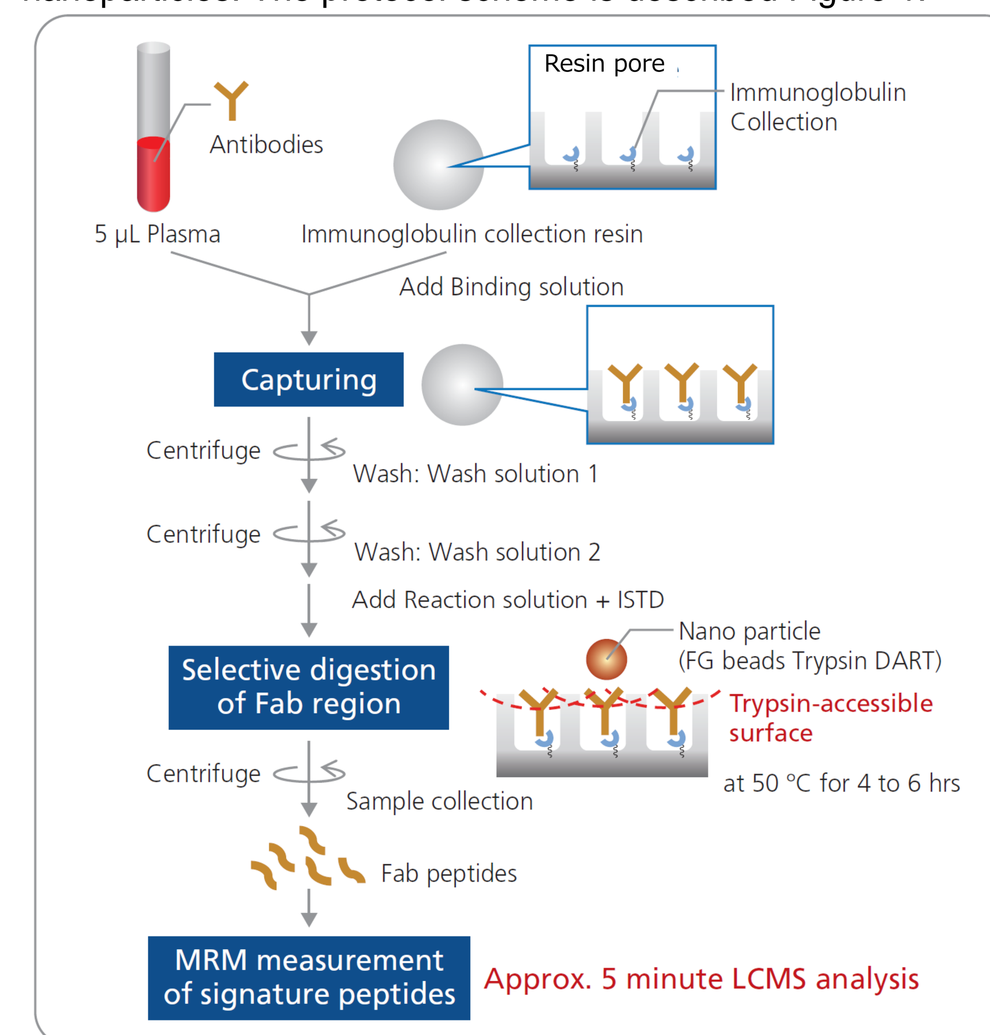

Figure 1. Scheme of nSMOL method.

3. Result

3-1. Prediction of aflibercept signature peptides

Figure 2 shows the peptide sequence of aflibercept. Aflibercept is composed of human VEGFR1, human VEGFR2 and IgG Fc region. The signature peptide candidates are underlined.

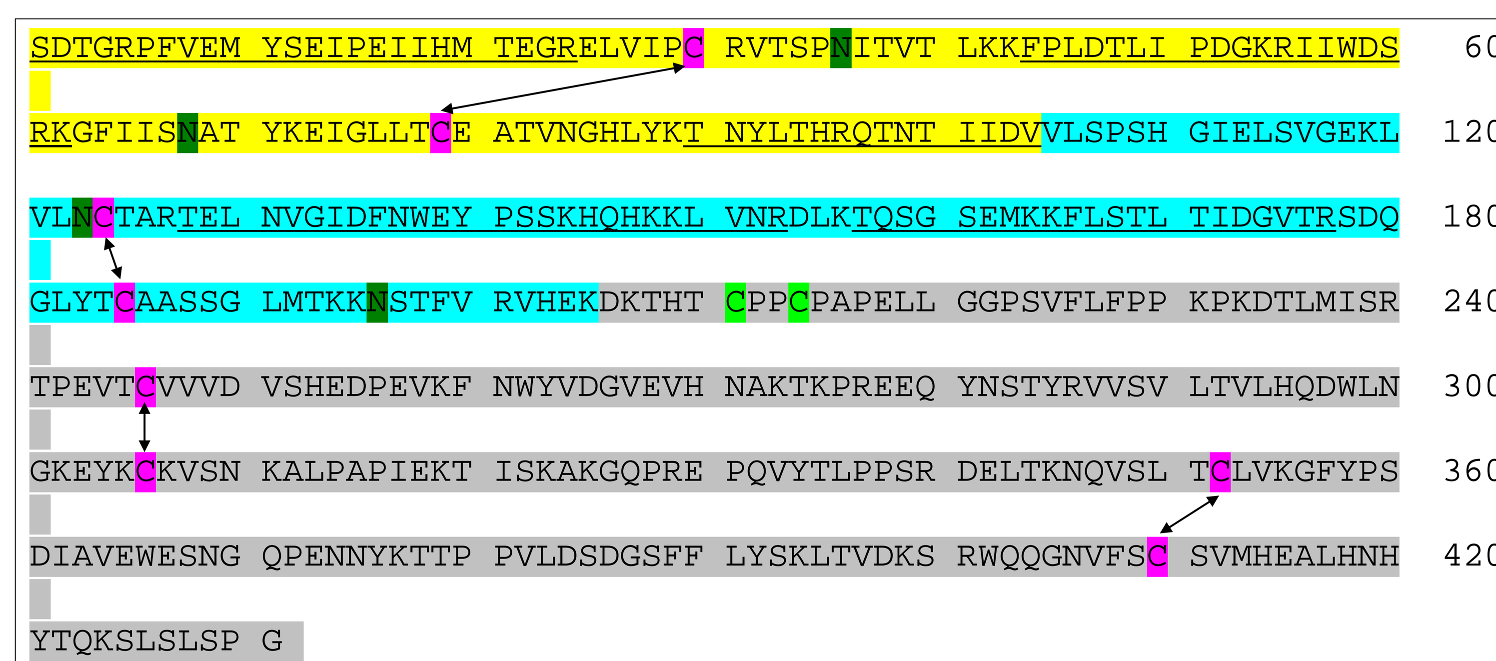

Figure 2. Peptide sequence of aflibercept.
VEGFR1 region: 1-104, VEGFR2 region: 105-205, IgG Fc region: 206-431,
Disulfide bridge: Intrachain) 30-79, 124-185, 246-306, 352-410, Interchain) 211-211', 214-214'
Glycochain addition (potential): 36, 68, 196. The arrows show disulfie bonding.

Table 1. MS analysis of aflibercept.

Region	Peptide	RT [min]	Observed *m/z*	Charge	Calculated *m/z*	Delta [ppm]	Area [cps]
166-177	FLSTLTIDGVTR	27.6	661.8693	+2	661.8694	-0.0801	2,170,787
56-61	IIWDSR	21.4	395.2163	+2	395.2163	-0.0076	1,444,997
97-119	QTNTIIDVVLSPSHGIELSV GEK	33.5	812.7707	+3	812.7709	-0.2596	979,639
1-24	SDTGRPFVEMYSEIPEIIHM TEGR	34.8	699.3345	+4	699.3349	-0.5834	897,543
90-96	TNYLTHR	15.6	302.1591	+3	302.1594	-0.8638	480,001
157-164	TQSGSEMK	6.8	434.1976	+2	434.1975	0.1382	461,589
150-153	LVNR	7.7	251.1608	+2	251.1608	0.1155	399,945
44-54	FPLDTLIPDGK	29.6	608.3343	+2	608.3346	-0.4208	382,417
128-144	TELNVGIDFNWEYPSSK	31.3	666.9858	+3	666.9863	-0.7826	350,038
63-72	GFIISNATYK	24.0	557.3005	+2	557.3006	-0.1992	288,647
6-24	PFVEMYSEIPEIIHM TEGR	35.2	760.0335	+3	760.0343	-1.1000	228,865
145-148	HQHK	1.2	183.7680	+3	183.7679	0.3646	94,214
157-165	TQSGSEMKK	5.0	332.4987	+3	332.4991	-1.2000	50,805
44-55	FPLDTLIPDGKR	27.9	457.9255	+3	457.9259	-0.8953	44,064
145-149	HQHKK	1.2	226.4666	+3	226.4662	1.7221	17,632

3-2. MS analysis

Aflibercept peptide was identified using Q-TOF mass spectrometer LCMS-9030 and the identified peptides (with no cysteine residue containing) are shown in Table 1 in descending order of sensitivity.

3-3. MS/MS analysis and partial validation

MRM was optimized using the full digest of aflibercept and transitions were selected by MS/MS analysis using Q-TOF. Furthermore, a serial dilutions of aflibercept with human plasma were prepared and treated by nSMOL kit for evaluating the prepared MRM. There are two optimized buffers for enzyme reaction of nSMOL, thus we examined using each of them. The concentration of lower limit of detection of each peptide within 0.24 to 250 μg/mL were shown Table 2, and we have selected 3 candidate signature peptide (IIWDSR, TNYLTHR, FLSTLTIDGVTR) for aflibercept quantitation. Because retention time of LVNR peptide is unstable, it was excluded.

Table 2. Limit of detection of each peptide of aflibercept.

Region	Peptide	Limit of detection [μg/mL]	
		reaction sol.	Enhanced reaction sol.
56-61	IIWDSR	<0.24	<0.24
90-96	TNYLTHR	<0.24	0.49
166-177	FLSTLTIDGVTR	<0.24	0.49
150-153	LVNR	<0.24	0.98
44-55	FPLDTLIPDGKR	0.12	0.49
63-72	GFIISNATYK	0.12	15.6
128-144	TELNVGIDFNWEYPSSK	0.24	0.49
157-164	TQSGSEMK	0.98	15.6
157-165	TQSGSEMKK	0.98	62.5
145-148	HQHK	31.25	31.25
1-24	SDTGRPFVEMYSEIPEIIHM TEGR	31.25	125
145-149	HQHKK	125	15.63
97-119	QTNTIIDVVLSPSHGIELSV GEK	125	62.5
44-54	FPLDTLIPDGK	125	-
6-24	PFVEMYSEIPEIIHM TEGR	-	-

3-4. Lower limit of quantification, CV% and accuracy

The partial validation was in accordance with the Guideline on Bioanalytical Method Validation in Pharmaceutical Development of the Ministry of Health, Labor and Welfare. Because the lower limit of detection with reaction solution of these three selected peptides were 0.049 μg/mL, partial validation was verified at 0.049 to 200 μg/mL.

Table 3. MRM transition of aflibercept.

Peptide	Region	MRM transition	Collision [V]	Role
TNYLTHR	90-96	452.75 >689.35 (y5+)	-19	Quantitation
		452.75 >413.25 (y3+)	-22	Structure
		452.75 >526.30 (y4+)	-20	Structure
IIWDSR	56-61	395.20 >563.25 (y4+)	-14	Quantitation
		395.20 >262.15 (y2+)	-21	Structure
		395.20 >227.20 (b2+)	-13	Structure
FLSTLTIDGVTR	166-177	661.85 >761.40 (y7+)	-23	Quantitation
		661.85 >1062.60 (y10+)	-24	Structure
		661.85 >432.25 (y4+)	-35	Structure
P14R	Internal standard	512.10 >292.30 (b3+)	-20	Quantitation
		512.10 >660.40 (y6+)	-17	Structure
		512.10 >563.30 (y5+)	-17	Structure

Table 4. Summary of CV% and accuracy (average, n = 7) of aflibercept.

Run	Set Concentration [μg/mL]	Peptide					
		TNYLTHR		IIWDSR		FLSTLTIDGVTR	
		CV%	Accuracy%	CV%	Accuracy%	CV%	Accuracy%
1	0.049	77.35	44.53	11.38	97.00	14.97	67.76
	0.15	31.64	69.58	2.76	87.13	12.07	76.53
	2.34	3.61	97.45	2.96	97.53	2.56	102.97
	160	3.52	96.37	3.49	101.17	3.22	96.79
2	0.049	42.72	64.43	6.11	91.84	34.08	78.23
	0.15	16.43	53.00	4.51	91.57	6.15	101.64
	2.34	9.31	96.10	6.50	98.43	6.19	102.44
	160	3.08	95.09	2.96	104.70	3.13	97.41
3	0.049	89.42	24.81	8.13	89.61	9.24	99.66
	0.15	16.15	69.06	9.74	86.24	10.41	92.89
	2.34	5.01	94.41	3.75	97.76	4.43	95.59
	160	2.88	95.37	2.62	102.32	3.16	95.84

As the QC sample, LLOQ, LOQ, MOQ, and HOQ were set as 0.049, 0.15, 2.34, and 160 ug / mL, respectively. The MRM transition, CV value and average of accuracy (n = 7) of each peptide are shown in Tables 3 and 4. From the table 5, since CV% and Accuracy% were achieved with IIWDSR, this was designated as the signature peptide. Figure 3 shows the IIWDSR MS chromatogram and calibration curve.

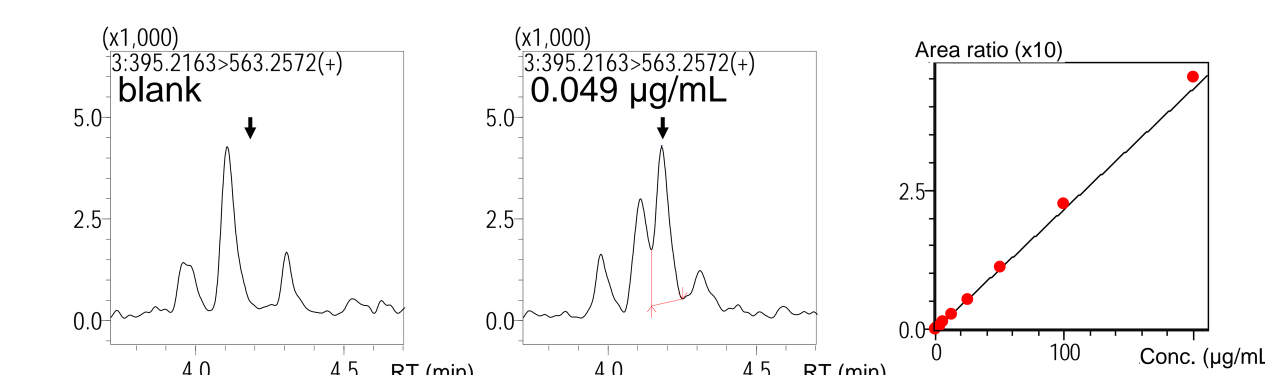

Figure 3. MS chromatogram and calibration curve of signature peptide (IIWDSR).

4. Conclusions

- We established a method to analysis of aflibercept with high sensitivity and high stability.
- Aflibercept can be quantified in the range of 0.049 to 200 μg/mL with a small amount of specimen using IIWDSR as the signature peptide.
- It was confirmed that the signature peptide does not overlap with the endogenous protein and does not compete with the biological matrix.

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