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Microvolume Analysis of Aflibercept in Aqueous Humor Using Mass Spectrometry

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Purpose: To develop a microvolume analytical method for measurement of the aflibercept concentration in human intraocular fluid and plasma.

Methods: We analyzed trace amounts of aflibercept in human aqueous humor using Fab-selective proteolysis and nano-surface and molecular-orientation limited (nSMOL) proteolysis, coupled with liquid chromatography–tandem mass spectrometry (LC-MS/MS). Patients with age-related macular degeneration or diabetic macular edema were recruited. Just after an injection of 50 µL of aflibercept, regurgitate from needle holes was collected with a micropipette pressed to the side of the injection hole within 10 seconds. The median amount of regurgitate was 4 µL (range, 1–18 µL).

Results: In human plasma, the aflibercept concentration ranged between 0.195 and 50 µg/mL when using the quantitative signature peptide IIWDSR (aa. 56–61) present on the vascular endothelial growth factor receptor 1 domain of aflibercept. The method was validated by evaluating its linearity, carryover, selectivity, accuracy and precision, dilution effect, and sample/processing stability. As only a minimal amount of regurgitate through needle holes can be sampled, we performed and verified the aflibercept assay using patient samples after 1:10 dilution with control human plasma, a recognized diluent. The median concentration of aflibercept in the regurgitate was 240 µg/mL (range, 13–4300 µg/mL).

Conclusions: Our findings indicate that the aflibercept assay using human intraocular fluid can be reliably performed using nSMOL coupled with LC-MS/MS.

Translational Relevance: This technique for quantifying aflibercept in the regurgitate suggests that the amount of drug lost post-injection can be ignored, even in patients with a relatively large leak after vitreous injection. This new methodology suggests possible therapeutic responses and may be employed as a general analytical method for trapping many biologics, such as vascular endothelial growth factor, in various types of clinical samples, unaffected by proteinaceous or small organic pharmaceuticals.

Introduction

Anti-vascular endothelial growth factor (VEGF) drugs[1,2] are widely used to treat major retinochoroidal disorders,[3–5] including neovascular age-related macular degeneration (AMD),[6] macular edema associated with retinal vein occlusion, diabetic macular edema (DME)[7,8] and retinopathy of prematurity. Application of these drugs is expanding for the treatment of proliferative and pre-proliferative diabetic retinopathy, as well. Aflibercept[9] is a commonly used anti-VEGF drug that neutralizes not only VEGF but also placental growth factor signaling.[10–12] Aflibercept consists of the extracellular binding domains of VEGF receptors 1 and 2 fused to an immunoglobulin Fc domain.[13,14] From the standpoint of drug efficacy and side effects, the pharmacokinetics of aflibercept in humans is of vital importance; however, despite the widespread clinical use of this drug, the dose actually administered during intravitreal injection therapy has not been assayed because of technical difficulties relative to its measurement.[15–18] We hypothesized that measuring the aflibercept concentration of the regurgitate from needle holes (liquid leaking after intravitreal injection; see Supplemental Photographs S1 and S2) is also important, as the presence of aflibercept in this liquid would reduce the actual dosage. While the drug solution is injected intravitreally, the solution forms a drop at the tip of the needle, but when the needle is withdrawn the drop does not remain in place and is pulled toward the sclera with the needle. Therefore, it is possible that the regurgitate might have a high aflibercept concentration.

There are two important problems that must be addressed in this regard. First, measurements relying on molecular interactions, such as enzyme-linked immunosorbent assay (ELISA) and Biacore are prone to errors when the experimental sample contains molecules that interfere with recognition of the target protein. Previously, we reported that VEGF assays performed in the presence of the anti-VEGF drugs aflibercept, bevacizumab, and ranibizumab were underestimated 2- to 100-fold when compared with commercially available ELISA reagents. Concentrations were probably underestimated due to the various VEGF forms, free and bound to anti-VEGF drugs at the receptor-binding domain assumed during the ELISA reaction. Therefore, it is necessary to develop an accurate assay method that is unaffected by the biological matrix that can be employed in clinical research.[19]

Second, except for immunoassays, the detection and quantification of specific proteins in small sample volumes has been challenging. We have been focusing on aqueous humor as an important biosample in which to monitor retinochoroidal diseases and have found that aqueous humor reflects intraocular immune conditions during eye diseases and is associated with cytokine levels in plasma.[20] Measurement of the aflibercept concentration in aqueous humor, however, is challenging, as the amount of aqueous humor that can be sampled is usually limited (10–20 μL).[21] Previous studies have used ELISA or multiplex assays for protein measurements, but prior to the current study there has been no reliable assay method to measure aflibercept in small liquid volumes.

Liquid chromatography–mass spectrometry (LC-MS/MS), in which signature peptides of tryptic fragments are quantified, allows quantitative measurement of proteins in a small volume. Use of nano-surface and molecular-orientation limited (nSMOL) proteolysis enables precise measurement of target proteins (such as bevacizumab) in the presence of molecules such as VEGF that interfere with other types of assays.[22] Furthermore, ensuring selectivity among endogenous proteins is essential for quantifying biopharmaceuticals in biological samples.[16,23] In this regard, we have developed a validated bioanalytical method for therapeutic antibodies, independent of other diverse antibodies, by combining structure-specific cleavage with structural separation by LC-MS/MS.[24] In brief, Fc-fusion proteins and immunoglobulins (IgGs) are collected with a Protein A resin with 100 nm pores via the Fc chain. Fab and the fused domain orient toward the reaction solution. Collected proteins are hydrolyzed by immobilized trypsin bound to the surfaces of the 200 nm nanoparticles. Trypsin nanoparticles physicochemically limit access to substrate protein because of the difference of the two resins, thereby decreasing the peptide number while maintaining the structural specificity of fused-domain peptides and/or complementarity-determining region peptides. Peptide quantitation is performed by multiple reaction monitoring (MRM) using triple-quadrupole LC-MS/MS.[25–27] In the present study, we developed a novel assay for aflibercept and measured its concentration in regurgitate from needle holes in anti-VEGF therapy.

Materials and Methods

Chemicals

An nSMOL Antibody BA kit was purchased from Shimadzu (Kyoto, Japan). Aflibercept was purchased from Santen Pharmaceutical (Osaka, Japan).

Individual male and female control human plasma samples were obtained from Kohjin Bio (Saitama, Japan). P14R (14-Pro and Arg) internal standard synthetic peptides were purchased from Sigma-Aldrich (St. Louis, MO). Ultrafree-MC GV centrifugal filters (0.22 µm pore size) were procured from EMD Millipore (Billerica, MA). Other reagents, buffers, and solvents were purchased from Sigma-Aldrich and FUJIFILM Wako Pure Chemicals (Osaka, Japan).

Structural Confirmation of Signature Peptides

Aflibercept Fc-fusion protein was denatured and reduced in 8 M urea and 2 mM neutralized Tris(2-carboxyethyl)phosphine at room temperature for 30 minutes. Next, the sample solution was diluted 10-fold using 25 mM Tris–HCl (pH 8.0) and digested using modified trypsin at 37°C for 16 hours. The proteolytic reaction was quenched by adding trifluoroacetic acid to a final concentration of 0.5%. The peptide solution was purified using a MonoSpin C18 reversed-phase column (GL Science, Tokyo, Japan). The eluting solution was evaporated in a centrifugal evaporator, and peptides were reconstituted in 0.1% formic acid (FA). Structures of tryptic aflibercept peptides were analyzed by high-resolution liquid chromatography–quadrupole ion-trap time-of-flight (QTOF) MS (Nexera Mikros high-performance microflow liquid chromatography and LCMS-9030; Shimadzu). LC-MS conditions were as follows: solvent A, 0.1% aqueous FA; solvent B, 80% acetonitrile with 0.1% FA; trap column, L-column2 ODS analytical columns, 0.3 × 5 mm, 3-µm resin, 10-nm pore (Chemicals Evaluation and Research Institute, Tokyo, Japan); separation column, L-column2 ODS analytical columns, 0.3 × 150 mm, 2 µm resin, 10 nm pore (Chemicals Evaluation and Research Institute); column temperature, 40°C; and flow rate, 5 µL/min. The gradient program was as follows: 0 to 10 minutes, %B = 0; 10 to 95 minutes, %B = 0 to 40 gradient; 95 to 105 minutes, %B = 40 to 100 gradient; 100 to 115 minutes, %B = 100; 115 to 130 minutes, %B = 0. MS and MS/MS spectra were obtained using a desolvation line, interface, and heat block at 200°C, 100°C, and 250°C, respectively. Nebulizer nitrogen gas flow was set to 1 L/min. The flow rate of heating gas was 3 L/min. The electrode of the electrospray ionization (ESI) interface was set to 3 kV. Pulse times for MS and MS/MS were 194 µs and 154 µs, respectively. Ion accumulation time was set to 100 ms for MS and 80 ms for MS/MS. MS/MS analysis was performed using the intensity-dependent top-12 MS/MS per scan based on data-dependent acquisition. The precursor MS was set from *m*/*z* 300 to 800, and fragments were set from *m*/*z* 200 to 1200. Ion valency was set to range between +2 and +6. The electrode of the collision-induced dissociation (CID) cell was set at -25 ± 5 V, with argon gas pressure at 250 kPa. Precursor and fragment ions were assigned using Mascot Distiller 2.6.2 peak processing software (Matrix Science, London, UK), and PEAKS Studio Xplus software (Bioinformatics Solutions, Waterloo, Canada), using the in-house FASTA database of aflibercept and other monoclonal antibody sequence information. The allowance of peptide *m*/*z* tolerance was set to within 50 ppm for precursor ions and 50 mDa for fragment ions.

Prediction of Signature Peptides

The amino acid sequence of aflibercept was obtained from the Kyoto Encyclopedia of Genes and Genomes (entry no. D09574) and Drug Bank (accession no. DB08885) in Figure 2. Multiple sequence alignment analyses were performed using the ClustalW algorithm in GENETYX software (GENETYX, Tokyo, Japan) and the amino acid sequences of human VEGF receptors 1 and 2 (VGFR1 and VGFR2). In this analysis, theoretical tryptic peptides, having no overlap with the sequence of immunoglobulin Fc, hinge regions, or cysteine residue and S-S bonding positions, or potential glycosylation residues, were aligned and selected as suitable signature peptides.

Conditions for Multiple Reaction Monitoring of Each Signature Peptide

Peptide quantitation was analyzed using an LC–electrospray ionization–MS with triple quadrupole (Nexera X2 and LCMS-8050, Shimadzu). LC-MS conditions were as follows: solvent A, 0.1% aqueous FA; solvent B, acetonitrile with 0.1% FA; column, Shim-pack GISS C18, 2.1 × 50 mm, 1.9 µm, 20 nm pore (Shimadzu); column temperature, 50°C; flow rate, 0.4 mL/min. The gradient program was as follows: 0 to 1 minute, %B = 1; 1 to 6 minutes, %B = 1 to 35 gradient; 6 to 8 minutes, %B = 95; 8 to 9 minutes, %B = 1. MS spectra were obtained with the ESI probe temperature, desolvation line, and heat block at 300°C, 250°C, and 400°C, respectively. Nebulizer, heating, and drying gas flow rates were set to 3 L/min, 10 L/min, and 10 L/min, respectively. The dwell time was set at 10 ms for each transition. For peptide fragments, multiple reaction monitoring (MRM) ions were determined from the measured values of the structure-assigned fragments by QTOF-MS analysis. In the Q2 cell, the CID argon partial pressure was set to 270 kPa.

Table 1. Signature Peptide Sequences and MRM Transitions for Aflibercept

Peptide Sequence	Position	MRM Transition	Purpose
1. IIWDSR	aa.56–61 (VGFR1)	395.30>563.25	Quantitation
		395.30>262.15	Structure
		395.30>227.20	Structure
2. GFIISNATYK	aa.63–72 (VGFR1)	557.40>909.50	Structure
		557.40>796.40	
		557.40>683.35	
3. QTNTIIDVVLSPSHGIELSVGEK	aa.97–119 (VGFR1–2)	812.85>940.00	Structure
		812.85>883.65	
		812.85>776.50	
4. FLSTLTIDGVTR	aa.166–177 (VGFR2)	661.85>1062.60	Structure
		661.95>761.40	
		661.95>432.25	
5. P14R	—	512.10>292.30	Internal standard
		512.10>389.30	
		512.10>660.40	

Candidate MRM transition *m*/*z* was computationally determined, and the electrode voltage of Q1 pre-bias, collision cell Q2, and Q3 pre-bias, with abundant *m*/*z* of precursor and fragment ions, were determined using all tryptic peptides of aflibercept with optimization support software (LabSolutions; Shimadzu). Signature peptide candidates identified by QTOF MS and MS/MS analysis are shown in Table 1, with MRM transitions and their uses for quantitation and structural confirmation.

nSMOL Proteolysis Coupled With LC-MS/MS

The current analytical method is based on our previously reported bioanalytical validation for the Fc-fusion proteins etanercept and abatacept in human serum using the nSMOL method.[25] The protocol is illustrated in Figure 1. A 5 µL aliquot of aflibercept-spiked human plasma was diluted 10-fold in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% *n*-octyl-β-D-thioglucopyranoside (OTG) to avoid non-specific binding to the resin and plastic materials. The IgG fraction from the plasma sample was collected using 25 µL of a PBS-substituted immunoglobulin collection resin slurry in 95 µL of PBS containing OTG, with gentle vortexing at 25°C for 5 minutes. The collection resin was harvested onto an Ultrafree filter and washed twice with 200 µL of PBS containing OTG to remove other serum proteins, except IgGs, followed by 200 µL PBS to remove detergents that inhibit column separation, carryover, and ionization of peptides in the ESI interface. Each washing substitution was performed by centrifugation (10,000*g* for 1 minute) on the filter devices. After these washing steps, the collection resin was replaced with 80 µL of the nSMOL reaction solution. nSMOL proteolysis was performed using 5 µg trypsin on FG beads with gentle vortexing at 50°C for 5 hours in a saturated vapor atmosphere to allow uniform contact between the collection resin and FG beads nanoparticles. After nSMOL proteolysis, the reaction was quenched by adding FA at a final concentration of 0.5%. The peptide solution was collected by centrifugation (10,000*g* for 1 minute) and magnetic separation to remove the collection resin and trypsin FG beads. These analytes were transferred into low-protein-binding polypropylene vials (TORAST-H Bio Vial; Shimadzu) and then analyzed by LC-MS.

Validation

nSMOL proteolysis coupled with the LC-MS/MS method was validated in accordance with the Guideline on Bioanalytical Method Validation in Pharmaceutical Development from Notification 0711-1 of the Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau, the Ministry of Health, Labour and Welfare, dated May 24, 2018. The objective of a full validation is to demonstrate assay performance within 20% accuracy at the lower limit of quantification (LLOQ) and 15% at other concentrations—that is, selectivity, LLOQ, low concentration of quality control, middle concentration of quality control, high concentration of quality control, calibration curve, accuracy and precision, matrix effect, carryover, dilution integrity, and stored and processed sample

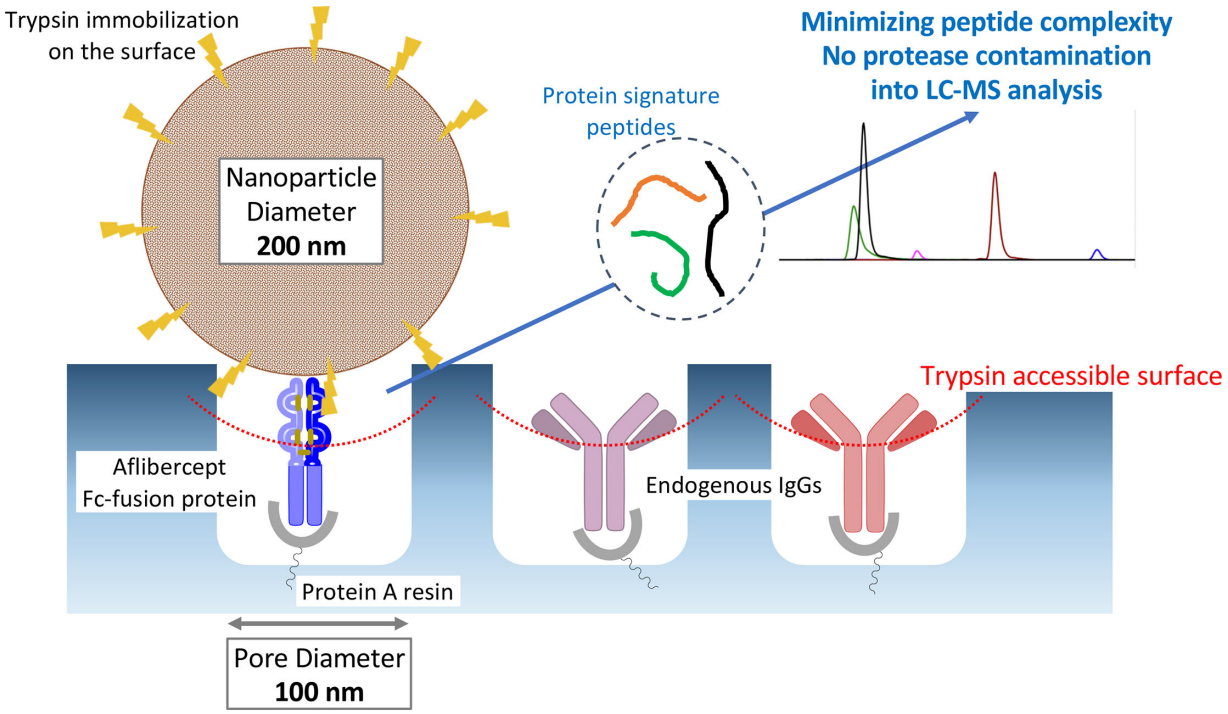

Figure 1. nSMOL workflow for aflibercept analysis. Aflibercept (blue) and endogenous IgGs (purple and red to show diversity) were collected from pores of Protein A resin preceding site-selective tryptic proteolysis by the nSMOL reaction, as immobilized trypsin enzyme has limited access to the substrate in the reaction solution due to physicochemical size differences between the two resins. Peptide fragments derived from fused domain and Fab do not require further purification and can be directly analyzed by LCMS.

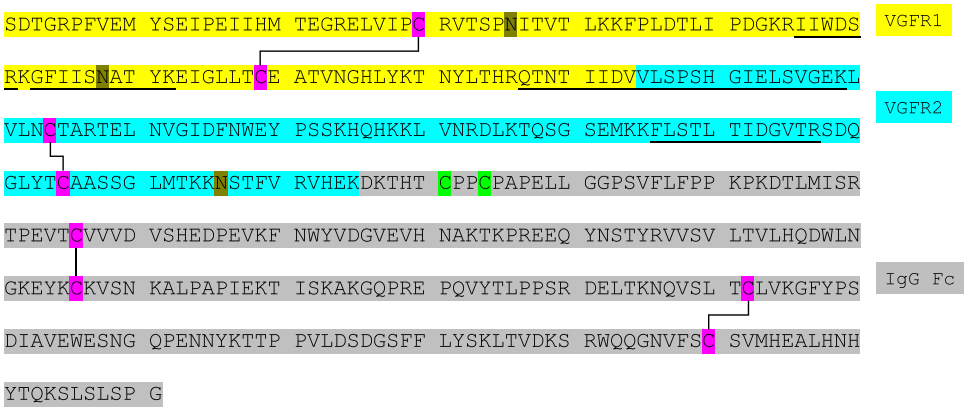

Figure 2. Aflibercept structure. Aflibercept possesses a dimeric structure, including a core hinge, DKTHTCPPCPAPELLGG, with two disulfide bridges. Yellow and blue represent the VGFR1 and VGFR2 regions, respectively. Gray represents the immunoglobulin Fc domain. Four intra-chain disulfide bridges (30–79, 124–185, 246–306, and 352–410) and two inter-chain disulfides (211 and 214) are highlighted in pink and green, respectively. Potential glycosylation sites are represented in gray. Underlined sequences were selected as signature peptides of aflibercept. VEGFR, vascular endothelial growth factor receptor.

stability. All validation sample sets were prepared and stored at –80°C for 24 hours or longer before each validation assay.

Selectivity

To assess selectivity, individual plasma samples from three males and three females were used.

Matrix Effect

Individual plasma samples were analyzed to assess the matrix effect at 1 µg/mL and 15 µg/mL. The results presented an excellent response accuracy, within 6%.

Carryover

Carryover was evaluated by analyzing three replicates of aflibercept immediately after the upper limit of quantification (50 µg/mL). Carryover was calculated as the percentage response in blank samples when compared with the LLOQ (0.195 µg/mL).

Calibration Linearity

Assay linearity was evaluated by analyzing nine calibration standards (blank sample and 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25.0, and 50.0 µg/mL) and three replicates using a linear regression model. Regression weighting was performed using the $1/area^2$ method.

Inter- and Intra-Assay Accuracy and Precision

Accuracy and precision were determined by analyzing the human plasma validation set at 0.195, 1.00, and 15.0 µg/mL of aflibercept. Intra-day and inter-day assays were performed by analyzing five replicates on 3 days.

Dilution Integrity

The effect of dilution on the aflibercept concentration was analyzed by preparing validation human plasma samples at concentrations of 250 and 300 µg/mL. The diluent was a mixture of plasma and PBS (9:1).

Clinical Sample Information

This study was conducted in accordance with the tenets of the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Jichi Medical University (CU20-C007). In the macular clinic of Jichi Medical University, all patients are recruited to various studies that use aqueous humor. Intraocular fluid samples from patients with AMD ($n = 12$) or DME ($n = 6$) who were recruited from May 2019 to March 2020 were used. In all cases, the intraocular fluid sample that leaked out through the injection hole was aspirated using a micropipette. We collected regurgitate from the needle hole at the pars plana, not from the anterior chamber tap. As such, the samples are deemed to represent vitreous humor. To minimize contamination from the ocular surface fluid, such as tear, the tip of the micropipette was pressed to the injection hole immediately after (within 10 seconds) the injections of 50 µL aflibercept. The median amount of the regurgitation was 4 µL (range, 1–18).

Collection of Regurgitate From Needle Holes Following a 2-mg, 50-µL Aflibercept Injection

Following injection of 2 mg of aflibercept in 50 µL to the vitreous using a 30-gauge (G) needle (with a standard protocol, with the needle inserted vertically to the sclera), 1 to 18 µL (median, 4 µL) of regurgitate was obtained from the needle hole, which was 2% to 36% (median, 8%) of the injected aflibercept solution. Because the amount of regurgitate from needle holes was very small and was very difficult to collect, it was collected only during treatment of coauthor HT. Collection was attempted with 18 consecutive treatments. Regurgitates from the needle holes of patients with AMD and DME treated with aflibercept were collected using a 20 µL pipette immediately after removing the 30 G aflibercept injection needle (Supplemental Photograph S2). Some of the regurgitate from needle holes was vitreous and could not be aspirated. Samples were immediately stored at −80°C until use. Assay verification of aflibercept in regurgitates was performed by diluting samples 10-fold using pooled human plasma. The starting volume of samples was set at 0.5 µL.

Results

Validated Assay Method for Aflibercept Using nSMOL Coupled With LC-MS/MS

The selection of signature peptides was performed by considering sequence specificity for other endogenous components, high sensitivity or high response, appropriate hydrophobicity, and avoiding the N- or C-terminal peptides, as far as possible. We generally selected signature peptides with 5 to 20 amino acids, according to validation studies. The current method showed high selectivity. For the control human plasma sample, almost no interference peak was observed in the MRM chromatogram of aflibercept peptide IIWDSR with a retention time of 3.85 minutes (Fig. 3). Overall, the results of the validation test met the criteria. The matrix effect revealed response accuracies of 96.2% and 106%, at 1 and 15 µg/mL, respectively (Supplementary Table S1). The mean carryover response was 13.7% of the LLOQ data (Supplementary Table S2). Calibration linearity demonstrated that the accuracy was 91.0% to 90.0% at LLOQ and 91.0% to 111% at other concentration points. Supplementary Table S3 summarizes the linear quantitation range and optimized MRM conditions for each signature

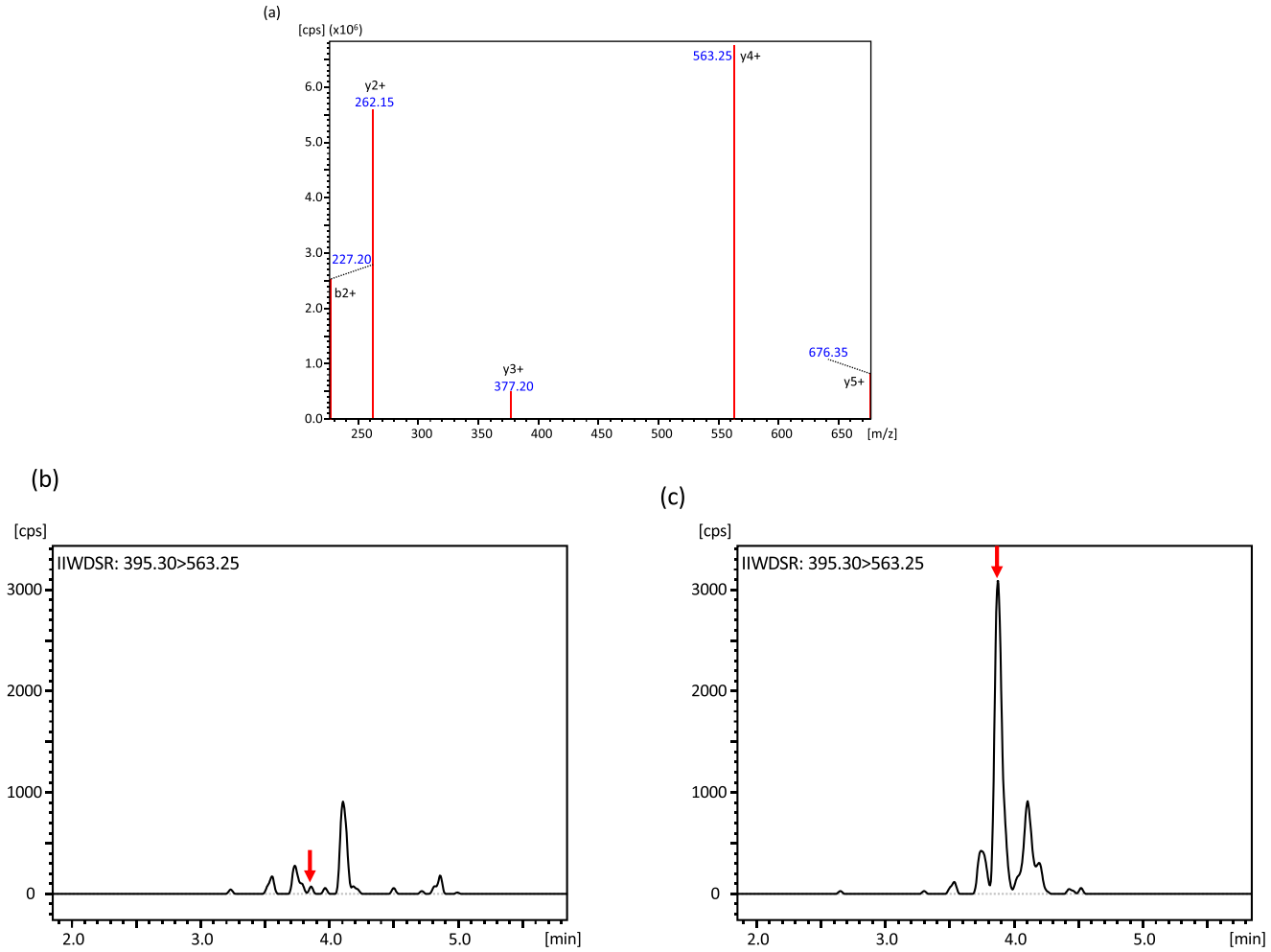

Figure 3. Representative MRM chromatogram of the signature aflibercept peptide IIWDSR. (a) Structure assignment of fragment ions from the peptide IIWDSR. The highest transition of *m/z* 395.30 to 563.25 (y4+) was selected for quantitative analysis. Representative chromatogram of transition *m/z* 395.30 to 563.25 for aflibercept using (b) blank sample and (c) LLOQ (0.195 µg/mL). The peak at 3.85 minutes (indicated with an arrow) shows the response of the signature peptide transition.

aflibercept peptide in human plasma samples. Inter-assay accuracies were 91.9%, 96.8%, and 95.8% at 0.195, 1.00, and 15.0 µg/mL, respectively. An average intra-assay accuracy of 98.0% was recorded at other concentration points (Table 2). The accuracy and precision following the 10-fold dilution from 250 µg/mL and 20-fold dilution from 300 µg/mL were 101% and 4.12% and 90% and 3.23%, respectively, indicating the absence of any interference due to the dilution process (Supplementary Table S4).

Sample Stability Prior to Sample Treatment

Short-term stability at room temperature for 4 hours, stability following five freeze–thaw processing cycles at −80°C with 12-hour frozen periods, and processed sample stability at 5°C for 24 and 48 hours were evaluated at concentrations of 1.00 µg/mL and 15.0 µg/mL, presenting an accuracy of 93.109% (Supplementary Tables S5–S8), suggesting high stability under these conditions.

Aflibercept in Patients with AMD and DME

In total, 18 needle hole regurgitates were diluted 10-fold using pooled human plasma and then analyzed using the nSMOL-validated assay (Tables 3 and 4). Aflibercept levels in regurgitates ranged between 13.4 µg/mL and 4.30 mg/mL (median, 237.5 µg/mL) (Fig. 4). Two samples (2550 and 4300 µg/mL) were over the upper limit of quantification and were calculated using an extended standard curve. This verification indicates that the nSMOL assay coupled with

Table 2. Accuracy and Precision of Quantifying the Aflibercept Peptide IIWDSR Using the nSMOL Method

	Set Concentration (µg/mL)		
	0.195	1.00	15.0
Run 1			
Calculated concentration (µg/mL)	0.198	0.901	14.9
	0.176	0.939	14.6
	0.188	0.857	14.9
	0.169	0.998	14.3
	0.166	0.951	14.9
Average (µg/mL)	0.179	0.929	14.7
Accuracy (%)	91.9	92.9	98.0
Precision (%)	7.49	5.72	1.83
Run 2			
Calculated concentration (µg/mL)	0.201	0.984	15.3
	0.193	1.10	15.4
	0.190	1.05	15.0
	0.182	1.07	15.9
	0.179	1.02	16.1
Average (µg/mL)	0.189	1.05	15.5
Accuracy (%)	96.8	105	104
Precision (%)	4.66	4.26	2.91
Run 3			
Calculated concentration (µg/mL)	0.174	0.998	14.8
	0.207	0.994	14.6
	0.188	1.02	14.6
	0.185	1.03	14.3
	0.182	1.01	14.5
Average (µg/mL)	0.187	1.01	14.6
Accuracy (%)	95.8	101	97.1
Precision (%)	6.54	1.48	1.24
Averaged accuracy (%)	94.8	99.5	99.6
Averaged precision (%)	6.23	3.82	1.99

LC-MS/MS can be utilized in clinical pharmacokinetic assessments of aflibercept in intraocular fluid.

Concentration of Aflibercept in Regurgitates From Needle Holes

The leaked aflibercept concentration was 0.034% to 11% (median, 0.45%) of the original aflibercept injection; therefore, 99.55% of the ejected liquid was vitreous liquid. The leaked aflibercept concentration ranged between 0.052 and 19.6 µg (median, 1.33 µg), which was 0.0026% to 0.98% (median, 0.066%) of the injected aflibercept (Table 3). There was no correlation among the amount and concentration, disease, age, sex, or axial length. However, the amount of regurgitate from needle holes tended to be large in eyes with thin sclera with long axial length ($P = 0.18$). The concentration of aflibercept in regurgitate tended to be small in eyes with liquefied vitreous of aged patients ($P = 0.16$, Table 5).

Discussion

To the best of our knowledge, this is the first study to evaluate aflibercept in aqueous humor regurgitate using nSMOL coupled with mass spectrometry, and this method can be applied to assay biologics for various eye diseases in the future. Compared with blood, amounts of aqueous humor, spinal fluid, and joint fluid available for sampling are severely limited. Although cytokine distribution and profiles have been well investigated to diagnose disease activity, localized inflammatory findings through pharmacokinetic

Table 3. Evaluation of Leakage Following an Injection of 2 mg of Aflibercept in 50 µL Using a 30 G Needle

Concentration (µg/mL)	Concentration From the Original (%)	Sample Amount (µL)	Aflibercept (ng)	Ratio of Leaked Aflibercept (%)
13.4	0.0335	4	53.6	0.00268
24.7	0.06175	4	98.8	0.00494
52.2	0.1305	1	52.2	0.00261
68.7	0.17175	2	137.4	0.00687
85.8	0.2145	3	257.4	0.01287
90.3	0.22575	4	361.2	0.01806
117	0.2925	6	702	0.0351
149	0.3725	18	2682	0.1341
214	0.535	6	1284	0.0642
261	0.6525	6	1566	0.0783
342	0.855	4	1368	0.0684
755	1.8875	3	2265	0.11325
1174	2.935	1	1174	0.0587
1637	4.0925	12	19644	0.9822
1938	4.845	6	11628	0.5814
1956	4.89	3	5868	0.2934
2549	6.3725	6	15294	0.7647
4296	10.74	1	4296	0.2148

Table 4. Patient Demographics

Demographic	Value
Cases (*N*)	18
Age (y), mean ± SD	67.8 ± 13.1
Male, *n* (%)	11 (61)
AMD/DME, *n* (%)	12 (67)/6 (33)
Axial length (mm), mean ± SD	23.7 ± 1.1

assessment of biologics have not yet been fully determined.[10,11] Here, we report a robust, high-sensitivity method by diluting a small amount of aqueous humor regurgitate from needle holes diluted 10-fold with pooled human plasma. The amount of leaked aflibercept was at most 1% of the injected aflibercept. Therefore, the amount of drug lost can be ignored even in patients with a relatively large leak after vitreous injection. The current minimum detectable aflibercept concentration is 0.2 µg/mL, which is almost equal to the calculated aqueous aflibercept concentration 2 months after injection.[19,28] Therefore, the duration of aflibercept efficacy may not be compromised, even if aflibercept leakage is substantial. Hence, the current method is a valuable tool to assess pharmacokinetics in the eye.

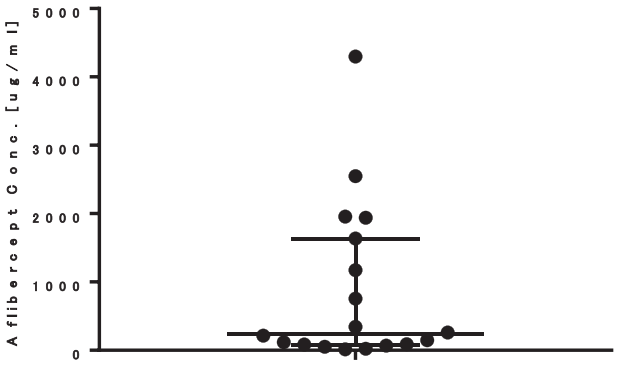

Figure 4. Clinical verification of the aflibercept assay using 18 patient samples. The left axis presents the aflibercept concentration (µg/mL) in regurgitate from needle holes, and the median with a 95% confidence interval is shown as a solid line.

Immunoglobulins possess a unique tetrameric structure of two heavy chains composed of Fv, followed by constant regions CH1 and Fc (CH2 and CH3) and two light chains composed of Fv and CL1. Aflibercept possesses a dimeric structure composed of extracellular domains of VGFR1 and VGFR2 fused to Fc, which differs significantly from that of immunoglobulins. However, a previous report revealed that the Fc domain is bound in resin pores via Protein A ligand and that the fused domain is oriented toward the reaction surface. This molecular orientation can be successfully aligned with the principle of the domain-selective nSMOL chemistry.[25] Accordingly, we developed an assay for aflibercept bioanalysis based on the same platform.

The pharmacokinetic (PK) correlation of protein drugs with intraocular and systemic circulation is

Table 5. Coefficients and *P* Values of the Multivariate Statistical Analysis

	Coefficient (*P*)	
	Amount of Regurgitation Through Needle Holes (µL)	Concentration of Aflibercept in Regurgitation Through Needle Holes (µg/mL)
Age (y)	0.027 (0.79)	−47 (0.16)
Sex (male)	0.46 (0.66)	89 (0.78)
Disease type (AMD)	0.85 (0.55)	320 (0.46)
Axial length (mm)	1.4 (0.18)	−18.8 (0.95)

affected by the blood–eye barrier and viscosity heterogeneity due to aging, and dosing has not been fully optimized. Recent studies suggest that undertreatment by the administration of fewer injections than required is an important factor in poor visual care.[29,30] Our approach has the advantage of using plasma-diluted samples of regurgitate from needle holes as a starting material, which can be measured in both aqueous humor and plasma, thus providing overall PK information. Quantification of PK in aqueous humor is expected to provide an index of clinical efficacy. Furthermore, clarification of the relationship between systemic PK in circulation and intraocular PK may provide a simpler method to measure drug efficacy.

From studies on ranibizumab of an Fab fragment and bevacizumab of a full-length IgG, it has been suggested that the same VEGF-trap protein may show significant differences in drug behavior, such as half-life and trough level, due to differences in the most stabilized shape with VEGF, electrostatic interactions, clearance from the vitreous, entry into the bloodstream, effects on systemic recirculation mediated by neonatal Fc receptors, and administration rate into the vitreous.[14,31,32] Currently, antibody shapes such as (Fab)2, single-chain Fv, and polymer conjugates are being studied in new therapeutic antibodies. The potential to accurately analyze drug behavior using our mass spectrometry technique, including drug half-lives and clearance times, will also be useful for the structural design of proteins with effective clinical outcomes.

One note about our current approach is that we measured intact aflibercept and did not distinguish between free and VEGF-bound aflibercept.[27] In the assay principle from our previous studies, nSMOL quantitative data do not affect binding of substrates or anti-drug antibodies, and the presence of VEGF is not expected to affect quantification of aflibercept. Cytokines, such as VEGF or interleukins, are bioactive substances that function locally, and it is believed that there are significant differences between systemic concentrations and levels at disease sites.[33–35] Cytokine determinations are currently performed by ligand binding-based multiplex assays, but from ELISA assays it is well known that quantitative values deviate significantly in the presence of inhibitory substances. MS-based assays simultaneously measure free and VEGF-bound aflibercept, and, in principle, an additional determination of VEGF signature peptides is required. However, it is possible to add an MRM transition of VEGF peptide and to separate aflibercept bound to VEGF signal from unbound aflibercept. We should assume that there is a large concentration difference between cytokine expression levels and administered antibodies, and it is necessary to optimize LC-MS/MS conditions to linearly quantify these molecules. The most useful method would be to quantify bound aflibercept and VEGF using a stable isotope–labeled peptide and to set up an analytical method.[36]

In conclusion, we have reported a novel assay method for quantifying trace amounts of injected aflibercept in regurgitate from needle holes by dilution with human plasma using nSMOL coupled with LC-MS/MS. In addition to the clinical pharmacokinetics of aflibercept in aqueous humor, our technique is applicable to many protein pharmaceuticals and can be employed for local pharmacokinetic analysis of disease lesions, correlation of cytokine profiles, and assays for therapeutic antibodies and short-chain antibodies administered by intravitreal injection.

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