Intraocular Pharmacokinetics of Intravitreal Aflibercept (Eylea) in a Rabbit Model

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Purpose. We determined the intraocular pharmacokinetic properties of intravitreally injected aflibercept (Eylea) in a rabbit model.

Methods. Aflibercept was injected intravitreally in 21 eyes from New Zealand White rabbits. The eyes were enucleated 1, 24, 48, 120, 216, 360, and 720 hours (1, 2, 5, 9, 15, and 30 days, respectively) after injection and immediately frozen at −80°C. The concentrations of aflibercept in the vitreous, aqueous humor, and retina/choroid were determined by performing an indirect enzyme-linked immunosorbent assay, and analyzed to understand the pharmacokinetic properties of the drug.

Results. The maximum concentration of aflibercept was observed 1, 48 (2 days), and 24 (1 day) hours after intravitreal administration in the vitreous, aqueous humor, and retina/choroid, respectively. The one-compartment model was selected as the final model for all three ocular tissues. In the vitreous, aqueous humor, and retina/choroid, the estimated half-lives of aflibercept were 94.1, 48.0, and 58.2 hours, and the estimated mean residence times (MRTs) were 135.8, 69.2, and 84.0 hours, respectively. The area under curve from time 0 to the end point (AUC_{last}) was 135,810.6 hours × μg/mL for the vitreous, 13,889.7 hours × μg/mL for the aqueous humor, and 2453.1 hours × μg/g for the retina/choroid.

Conclusions. In rabbits, the vitreous half-life of aflibercept is 94.1 hours (3.92 days). This is shorter than that of bevacizumab (6.99 days), and longer than that of ranibizumab (2.51 days) and VEGF-Trap (3.63 days).

Keywords: anti-vascular endothelial growth factor agent, aflibercept, eylea, intraocular pharmacokinetics

The introduction of anti-VEGF treatment has changed the paradigm of standard treatment for and improved visual prognosis of VEGF-related ocular diseases, such as exudative age-related macular degeneration (AMD), diabetic macular edema, and retinal vein occlusion. Intravitreal ranibizumab (Lucentis; Genentech, Inc., San Francisco, CA, USA) has been used widely as a first-line treatment for these vision-threatening conditions, along with the off-label use of intravitreal bevacizumab (Avastin; Genentech, Inc.). Recently, aflibercept (VEGF Trap-Eye, Eylea; Regeneron, Inc., Tarrytown, NY, USA and Bayer Healthcare Pharmaceuticals, Berlin, Germany) was approved for therapeutic use and has since been used widely. Aflibercept has greater binding affinity to VEGF than does ranibizumab or bevacizumab, which indicates longer duration of action for aflibercept in the eyes.[1–3] However, unlike that for ranibizumab and bevacizumab, data for the intraocular pharmacokinetics (PK) of aflibercept are scarce. A rabbit model–based PK study reported intravitreal PK properties of intravitreally administrated I-124–labeled aflibercept by the use of positron emission tomography/computed tomography (PET/CT) imaging; the intravitreal half-life of I-124–aflibercept was 4.58 days, which was similar to the manufacturer data of 4.79 days.[2,4] Meanwhile, another PK study using a conventional immunoassay-based macaques model showed aqueous humor PK properties of intravitreally administrated aflibercept in comparison with ranibizumab; intravitreally administrated aflibercept and ranibizumab have similar half-lives in aqueous humor (2.2 and 2.3 days, respectively).[5] However, to our knowledge, no study has addressed detailed, comparable intraocular PK properties of aflibercept by the use of a conventional immunoassay-based rabbit model as did the previous PK studies for ranibizumab and bevacizumab.[6–12] This resulted in our recent study investigating the detailed intraocular PK properties of intravitreally administrated VEGF-Trap, a prototype of VEGF Trap-Eye, by the use of a conventional immunoassay-based rabbit model.[3] However, although the sequences of VEGF-Trap and VEGF Trap-Eye are similar and show differences in only 5.3% (25 of 476) of the amino acids, these differences

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might result in differences in their intraocular PK properties.[1,3] Moreover, the commercially available VEGF Trap-Eye, Eylea, (which is widely used in clinical practice) passes through several manufacturing processes, which also might affect its intraocular PK properties. Hence, in this study, we investigated the intraocular PK properties of aflibercept (Eylea) in the same experimental setting described in our previous PK studies for bevacizumab, ranibizumab, and VEGF-Trap to provide comparable PK data of aflibercept with that of bevacizumab, ranibizumab, and VEGF-Trap.[3,6,7]

Methods

Animal Experiments

The present study was approved by the Seoul National University Bundang Hospital Institutional Animal Care and Use Committee, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. We also confirm adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

A total of 21 eyes were obtained from 21 healthy New Zealand White rabbits weighing 1.5–2 kg. The experimental design for assessing intraocular PK of intravitreally injected aflibercept was similar to that in our previous studies.[3,6,7] Briefly, the animals were anesthetized with an intramuscular injection of 15 mg/kg Zoletil (a mixture of tiletamine hydrochloride and zolazepam hydrochloride; Virbac Laboratories, Carros, France) and 5 mg/kg xylazine hydrochloride (Bayer Korea, Ltd., Seoul, South Korea). Topical anesthesia (1% proparacaine hydrochloride ophthalmic eye drops; Alcaine; Alcon Laboratories, Inc., Fort Worth, TX, USA) also was administered after dilation of the eyes with phenylephrine hydrochloride and tropicamide eye drops (Mydrin-P; Santen Pharmaceutical Co., Osaka, Japan). Povidone iodine (5%) was placed on the periocular region and conjunctiva of the right eye. Next, aflibercept (1.2 mg/0.03 mL; Eylea; Regeneron and Bayer Healthcare Pharmaceuticals) was intravitreally injected into the right eye, 1 mm behind the surgical limbus in the superotemporal quadrant, using a 30-gauge needle and a Hamilton syringe. Three rabbits were killed at each of the following time points: 1, 24, 48, 120, 216, 360, or 720 hours (1, 2, 5, 9, 15, or 30 days, respectively) after injection. The right eyes were enucleated and immediately frozen at –80°C until the immunoassay was performed. Before analysis, the frozen eyes were separated into three parts: vitreous, aqueous humor, and retina/choroid. The aqueous humor samples were defrosted and their volumes measured. The vitreous samples were defrosted and solubilized overnight in 1.0 mL of PBS containing 1% BSA on a rotator at 4°C. The samples then were centrifuged at 387*g* for 10 minutes[3,8] and their volumes measured. The frozen retina/choroids were weighed and the samples were defrosted and homogenized (CellLytic MT, C3228; Sigma-Aldrich Corp., St. Louis, MO, USA).[3,11]

Aflibercept Immunoassay

We measured the concentrations of aflibercept in each of the three parts of the eyes by an indirect ELISA.[3,6–8] Briefly, the human 165-amino-acid variant of recombinant (r) VEGF was diluted to 1.0 μg/mL in 50 mM carbonate buffer (pH 9), and was immobilized in 96-well flat bottom plates (100 μL/well; NUNC, Roskilde, Denmark). The plates were incubated overnight at 4°C, washed with 1× PBS, and blocked for 2 hours at 4°C with 1% BSA in 1× PBS. After final washes, the plates were stored at 4°C until dry. Next, the vitreous, aqueous humor, and retina/choroid samples were diluted with 0.1% BSA in 1× PBS so as to be within the range of the assay, aliquoted into the rVEGF-coated plates (100 μL/well), and incubated overnight at 4°C. In each plate, known aflibercept concentrations (0.039–10 ng/mL) were included to generate a standard curve to estimate the aflibercept concentration in each sample. Bound aflibercept was detected with goat anti-human IgG/Fc secondary antibody labeled with horseradish peroxidase (ab97225; Abcam, Cambridge, UK). The diluted secondary antibody (1:20,000) was added to the rVEGF-coated wells and the plates were incubated for 1 hour at room temperature with agitation, followed by washing. Optical density was measured by detecting the absorbance after treating the 3,3′,5,5′-tetramethyl benzidine substrate with hydrogen peroxide. We created the standard curves on SoftMax Pro 5.4.1. (Molecular Devices, Sunnyvale, CA, USA), a software capable of generating a four-parameter logistic curve fit. The aflibercept concentrations in the retina/choroid samples were calculated by dividing the weights of aflibercept (μg) by that of the retina/choroid tissues (g).

Pharmacokinetic Data Analysis

The concentrations of aflibercept in the vitreous, aqueous humor, and retina/choroid samples were analyzed by one- and two-compartment models. In addition, a noncompartmental analysis was performed on the Phoenix WinNonlin software version 6.4 (Certara, Princeton, NJ, USA).

The following equation was used for the one-compartment model:

$$C(t) = \frac{\text{Dose}}{Vz/F} \times e^{-kt},$$

where $C(t)$ denotes concentration (μg/mL) at time t, Vz/F is the apparent volume (mL) of distribution, and k (1/h) indicates the elimination rate constant. For the vitreous, F was assumed to be 1. However, for the aqueous humor and retina/choroid, F was neither assumed nor estimated.

The following equation was used for the two-compartment model:

$$C(t) = A \times e^{-\alpha t} + B \times e^{-\beta t},$$

where A and B (both in μg/mL) are the back-extrapolated intercepts of the distribution and elimination phases, respectively, and α (1/h) and β (1/h) represent the rate constants for the distribution and elimination phases, respectively. Half-life ($t_{1/2}$ in hours), mean residence time (MRT in hours), maximum concentration (C_{max} in μg/mL), area under the concentration-time curve (AUC, hours × μg/mL), apparent volume of distribution (Vz/F in mL), and apparent clearance (CL/F in mL/hours) were estimated by post hoc analysis. After analysis, either the one- or two-compartment model was selected based on the following criteria: (1) the Akaike Information Criterion (AIC), (2) precision of parameter estimates, and (3) graphical analysis. The AIC was computed using the weighted residual sum of squares of model (WRSS), and the number of observations and parameters (N and P, respectively) during the modeling were given as follows:

$$\text{AIC} = N \times \log(\text{WRSS}) + 2P.$$

The AIC, precision-of-parameter estimate (standard error, presented as the coefficient of variation [CV]), and goodness-of-fit plot including the predicted versus observed concentrations were compared between the two models. In addition, the C_{max}, time to C_{max} (T_{max}), and AUC_{last} in the vitreous, aqueous

Table 1. Concentrations and Estimated Amounts of Aflibercept (Eylea) in the Vitreous, Aqueous Humor, and Retina–Choroid of Eyes From New Zealand White Rabbits 1 Hour, and 1, 2, 5, 9, 15, and 30 Days After Intravitreal VEGF-Trap-Eye Injection

	Vitreous		Aqueous Humor		Retina	
Time	Concentration, μg/mL	Amount, μg	Concentration, μg/mL	Amount, μg	Concentration, μg/g	Amount, μg
1 h	989.04 ± 342.35	1483.56 ± 513.53	12.87 ± 17.59	2.57 ± 3.52	14.24 ± 4.51	0.34 ± 0.11
1 d	567.17 ± 215.73	850.76 ± 323.60	66.07 ± 51.90	13.21 ± 10.38	21.86 ± 9.13	0.52 ± 0.22
2 d	577.57 ± 113.92	866.36 ± 170.88	108.89 ± 44.64	21.78 ± 8.93	7.33 ± 3.57	0.18 ± 0.09
5 d	394.04 ± 58.97	591.06 ± 88.46	33.33 ± 11.34	6.67 ± 2.27	7.26 ± 2.76	0.17 ± 0.07
9 d	209.04 ± 9.66	313.56 ± 14.49	18.40 ± 13.39	3.68 ± 2.68	4.52 ± 0.93	0.11 ± 0.02
15 d	91.43 ± 6.30	137.15 ± 9.45	7.13 ± 1.22	1.43 ± 0.24	1.00 ± 0.98	0.02 ± 0.02
30 d	6.44 ± 2.64	9.66 ± 3.96	0.84 ± 0.42	0.17 ± 0.08	0.08 ± 0.04	–

The volumes of the vitreous and aqueous humor of the rabbits were considered 1.5 and 0.2 mL, respectively, and the weight of the retina–choroid was considered 0.024 g.

humor, and retina–choroid samples also were calculated by following a noncompartmental method.

Results

Data were collected for 21 eyes from 21 rabbits. There was no evidence of ocular inflammation or other adverse events following drug treatment. Changes in concentrations and estimated amounts of aflibercept in the vitreous, aqueous humor, and retina–choroid samples over time are provided in Table 1. The concentration of aflibercept in the vitreous was highest 1 hour after intravitreal administration ($C_{max} = 989.0$ μg/mL), and decreased through the subsequent time points. Approximately 40% of the amount of aflibercept at 1 hour remained at 120 hours (5 days), approximately 10% remained at 360 hours (15 days), and 0.7% remained at 720 hours (30 days) after intravitreal administration. In the aqueous humor, the highest concentration of aflibercept was observed 48 hours (2 days) after intravitreal administration (C_{max} = 108.9 μg/mL), while in the retina–choroid, the highest concentration was observed at the 24-hours (1-day) time point ($C_{max} = 21.9$ μg/g).

The one-compartment model was selected as the final model for all three eye tissues. The one- and two-compartment models fit the aqueous humor. However, the one-compartment model had higher AIC values than the two-compartment model in the aqueous humor (42.4 vs. 39.6), and the estimated PK parameters of the one-compartment model had lower CVs than those of the two-compartment model. Therefore, the one-compartment model was considered more precise and its explanation clearer. In the vitreous and retina–choroids, the AIC values for the one-compartment model were 79.2 and 46.5, respectively, while the two-compartment model failed to fit both these tissues. Basic goodness-of-fit plots are presented in Figures 1A through 1F for the one-compartment model for each tissue.

The estimated one-compartment models for the vitreous, aqueous humor, and retina–choroids were as follows:

$$C(t) = \frac{1200}{1.4} \times e^{-0.0073t} \qquad C(t) = \frac{1200}{7.9} \times e^{-0.0145t}$$

$$C(t) = \frac{1200}{58.5} \times e^{-0.0119t}.$$

In the vitreous, aqueous humor, and retina–choroids, the estimated half-lives of aflibercept were 94.1, 48.0, and 58.2 hours, respectively, while the corresponding MRTs were 135.8, 69.2, and 84.0 hours. Further, the respective calculated $AUCs_{last}$ were 135,810.6 hours × μg/mL, 13,889.7 hours × μg/mL, and 2453.1 hours × μg/g. Detailed PK parameters for aflibercept are provided in Table 2. The observed concentration–time data for aflibercept at seven time points, as well as the models that fit the vitreous, aqueous humor, and retina–choroids are provided in Figure 2.

Discussion

The present study investigated the intraocular distributions of intravitreally administrated aflibercept in vitreous, aqueous humor, and retina–choroid tissues, as well as the corresponding PK properties of the drug, by a conventional immunoassay in a rabbit model using New Zealand White rabbits. The results showed that the half-life of intravitreally administrated aflibercept was 94.1 hours (3.92 days) in the vitreous, indicating that intravitreal aflibercept clears through the aqueous humor and retina–choroid. Substantial amounts of aflibercept could be measured just 1 hour after intravitreal administration in the aqueous humor and retina–choroid. Moreover, the aflibercept

Table 2. PK Parameters of Aflibercept (Eylea) in the Vitreous, Aqueous Humor, and Retina–Choroid of Eyes From New Zealand White Rabbits

PK Parameters	Vitreous	Aqueous Humor	Retina–Choroid
$T_{1/2}$, h*	94.1 ± 21.4	47.9 ± 7.1	58.2 ± 76.9
MRT, h*	135.8 ± 30.9	69.2 ± 10.2	84.0 ± 110.9
C_{max}, μg/mL†	989.0	108.9	21.9
T_{max}, h†	1	48	24
AUC_{last}, h × μg/mL†	135,810.6	13,889.7	2453.1
V/F, mL*	1.4 ± 0.1	–	–
CL/F, mL/h*	0.01 ± 0.001	–	–

$T_{1/2}$, half-life; MRT, mean residence time; C_{max}, observed maximum concentration; T_{max}, time to C_{max}; AUC_{last}, area under curve from time 0 to the end point; V/F, apparent volume of distribution; CL/F, apparent clearance.

* One-compartmental analysis; data presented as parameter estimate ± standard error.

† Noncompartmental method.

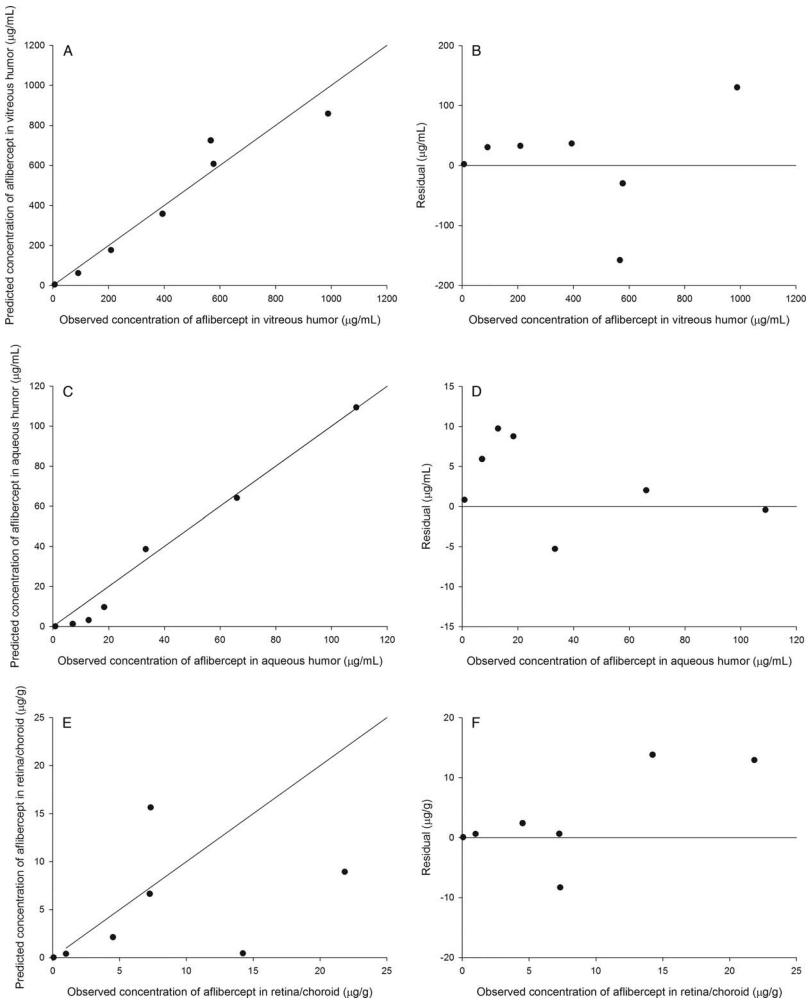

Figure 1. Plots show basic goodness-of-fit plot for one-compartment model for the vitreous, aqueous humor, and retina–choroid of eyes from New Zealand White rabbits. (**A**, **B**) Represent the vitreous. (**C**, **D**) The aqueous humor. (**E**, **F**) The retina–choroid.

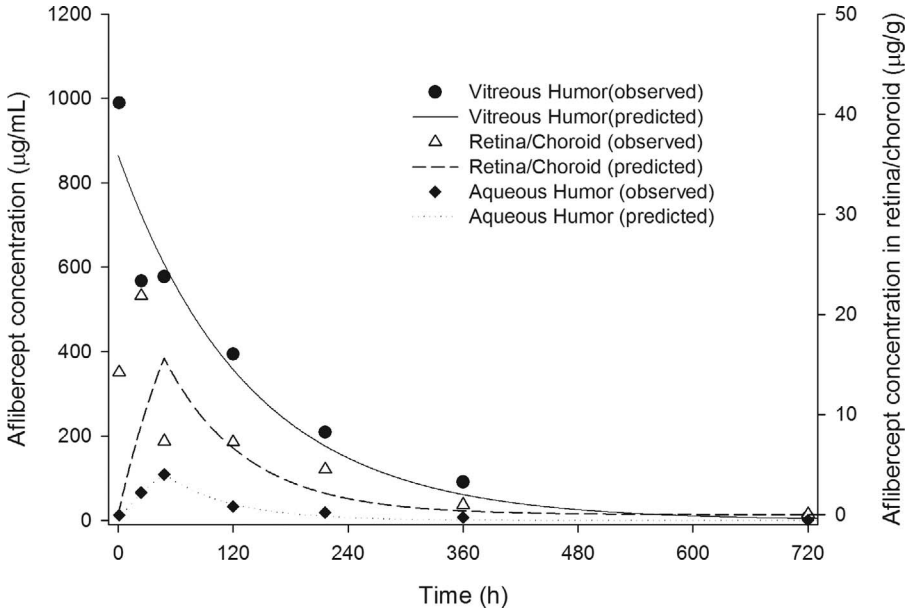

Figure 2. Time-concentration plots for aflibercept (Eylea) concentrations in the eyes of New Zealand White rabbits after intravitreal administration. *Points* represent the observed concentrations, and *lines* represent the estimated concentrations determined by the one-compartmental model.

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Table 3. Comparison of the Vitreous PK Parameters of Aflibercept (Eylea), Ranibizumab (Lucentis), Bevacizumab (Avastin), and VEGF-Trap in Eyes From New Zealand White Rabbits

PK Parameters	Aflibercept	Ranibizumab[7]	Bevacizumab[6]	VEGF-Trap[3]
Molecular weight, kD	145	49	148	145
$T_{1/2}$, h*	94.1	52.4	181.4	87.1
MRT, h*	135.8	75.5	233.8	125.7
V/F, mL*	1.4	2.7	3.06	3.8
CL/F, mL/h*	0.01	0.04	0.01	0.03

Pharmacokinetic parameters were reestimated based on the concentrations at each time point in the published data. $T_{1/2}$, half-life; MRT, mean residence time; V/F, apparent volume of distribution; CL/F, apparent clearance.

* One-compartmental analysis for aflibercept, ranibizumab, and VEGF-Trap, and two-compartmental analysis for bevacizumab.

concentrations increased, and peaked 2 days after intravitreal administration in the aqueous humor and 1 day after that in the retina–choroid. Subsequently, the concentrations decreased through the rest of the study period in all the three eye compartments. These results indicate that intravitreally-administrated aflibercept distributes rapidly to the aqueous humor and retina/choroid and remains in these tissues for considerable periods.

The vitreous half-life of aflibercept in the present study (3.92 days) was relative short in comparison with that given by the drug manufacturer (4.79 days)[2] and that reported in the PET/CT study on the I-124–labeled drug (4.58 days),[4] although the head-to-head comparison is not tractable due to the differences in measurement methodologies (PET/CT imaging versus conventional immunoassay) and rabbit breeds (Dutch-belted rabbit versus New Zealand White rabbit) between the PET/CT study and the present study. When compared to PK properties from the same experimental settings, this vitreous half-life is equivalent to, or even slightly longer than, that of VEGF-Trap in our previous study (3.63 days).[3] Similarly, the half-lives of aflibercept in the aqueous humor and retina–choroids also were slightly longer than those for VEGF-Trap (47.95 vs. 36.8 hours in aqueous humor; 58.24 vs. 35.0 hours in retina/choroid for aflibercept and VEGF-Trap, respectively). These improvements in intraocular longevity might be attributed to the development of the drug from VEGF-Trap to VEGF Trap-Eye, although the differences in amino acid sequences are minor.[3] We previously had conducted PK studies for bevacizumab[6] and ranibizumab[7] under the same experimental setting of the present study; the vitreous half-lives were 7.06 and 2.75 days, respectively. Since the applied PK models differed from each other (two-compartment model in studies for bevacizumab[6] and ranibizumab[7] and one-compartment model in the VEGF-Trap study[3] and the present study), the MRT might be a better PK property to compare drug longevity, in the vitreous, for different decoy antibodies.[3] The estimated MRTs were 9.74 days for bevacizumab, 3.15 days for ranibizumab, 5.24 days for VEGF-Trap, and 5.66 days for aflibercept.[3] Aflibercept showed a considerably longer MRT than ranibizumab, indicating longer duration of the intraocular pharmacologic effect (Table 3).

Intraocular PKs are determined by several physicochemical properties of a drug, including molecular weight, lipophilicity, and dose number.[13] Among them, molecular weight is the strongest determining factor of intraocular half-life of a drug. We observed the molecular weight of aflibercept (Eylea) to be 145 kDa (unpublished data), which is larger than the 115 kDa that is mentioned in the Eylea product information. The similar molecular weights as well as physicochemical properties of aflibercept and VEGF-Trap (molecular weight = 145 kDa) might be the reason for their similar intraocular half-lives.[3] The longer half-life of aflibercept than that of ranibizumab can be explained by the larger molecular weight of aflibercept (molecular weight of ranibizumab [Lucentis] is 49 kDa). The longer half-life of bevacizumab (Avastin; molecular weight = 148 kDa) than that of aflibercept, despite similar molecular weights, can be partly explained by bevacizumab's tendency to form conglomerates in the vitreous.[3,14]

However, comparison of the results in this study with those in our previous PK studies on different drugs should be done cautiously, because the immunoassay and PK data analysis were slightly modified in this study. First, a different secondary antibody was used in the present study. This secondary antibody showed higher efficiency in detecting the decoy antibodies. Assuming the volume of rabbit vitreous to be 1.5 mL (based on previous studies), the estimated amount of VEGF-Trap 1 hour after intravitreal administration of 300 μg of VEGF-Trap was approximately 129 μg (43%) in our previous study[3]; that of aflibercept 1 hour after intravitreal administration of 1.2 mg of aflibercept was approximately 1.4 mg. Secondly, we updated the means of standard-curve generation with the immunoassay—the curve was generated by a four-parameter logistic curve fit in this study, while in the previous studies, a simple regression curve was used.[6,7] We also observed large standard errors of half-life and MRT in a one-compartment model of retina–choroid. Measurement of concentrations in retinal tissue may be variable because of the small amount and low concentration. In addition, variable aflibercept-VEGF complex in retinal tissue may complicate the disposition kinetics, resulting in the model fitting with high standard error.

In conclusion, the present study presents, for the first time to our knowledge, detailed PK properties of intravitreally administrated, commercially available aflibercept (Eylea), by conventional immunoassay in a rabbit model. We expect that our results will aid aflibercept treatment strategies in the clinic, and will provide a foundation for future studies on such treatments.

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