Evaluation of Fluorophotometry to Assess the Vitreal Pharmacokinetics of Protein Therapeutics

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PURPOSE. In this work, we assessed the ability of fluorophotometry to measure the vitreal pharmacokinetics (PK) of fluorescently-labeled ranibizumab in the rabbit after intravitreal injection. We compared these values to those obtained using enzyme-linked immunosorbent assays (ELISA). Data obtained in this study were also compared to historical ranibizumab ocular PK data, either measured in-house or previously published.

METHODS. Three individual in vivo studies were performed in New Zealand White rabbits to assess the feasibility of using fluorophotometry to measure rabbit ocular PK of ranibizumab; explore the dynamic range of dosing fluorescently-labeled ranibizumab; and directly compare ranibizumab concentrations and calculated PK parameters measured by vitreal fluorophotometry to those measured using ELISA.

RESULTS. In direct comparisons between fluorophotometry and ELISA, the calculated clearance (CL) values were 0.26 and 0.21 mL/day, the volumes of distribution at steady state (Vss) were 0.80 and 0.94 mL, the half-lives (t1/2) were 3.1 and 2.9 days and the dose normalized areas under the curve (AUC/D) were 4.7 and 3.9 µg·day/mL·µg, respectively. These values fell within the ranges of 0.13 to 0.44 mL/day for CL, 0.5 to 1.8 mL for Vss, 2.8 to 3.5 days for t1/2, and 2.3 to 7.9 µg·day/mL·µg for AUC/D that have been either measured previously in-house or published elsewhere.

CONCLUSIONS. Although not suitable for measuring retinal concentrations, fluorophotometry is a valuable, noninvasive method to measure vitreous concentrations of protein therapeutics after intravitreal injection.

Keywords: fluorophotometry, vitreous pharmacokinetics, protein therapeutics, ranibizumab, 3Rs (replacement, reduction, refinement)

Originally developed as a clinical instrument to determine blood-retinal barrier permeability, early cataract formation, tear flow rates, and aqueous humor turnover,1–3 fluorophotometry (FPT) has become a versatile technique in oculair sciences. Ocular fluorophotometry was developed to further the understanding of ocular pharmacology, physiology, and pathology. Recently, ocular fluorophotometry has been used to assess the ocular pharmacokinetics (PK) of small molecules in preclinical models. In this method, molecules either possess inherent fluorescence or are labeled with a fluorescent dye (usually fluorescein) and administered either systemically or directly into ocular tissue with subsequent measurements of the fluorophore in the transparent ocular tissues.

Ocular fluorophotometry allows continuous measurement of fluorescence along the central axis of the eye. The dynamic range and lower limits of quantification of fluorophotometry are similar to other techniques such as mass spectrometry (MS) and ELISA. However, these methods often require invasive and/or terminal sampling. Those options result in the use of a large number of animals and the inability to measure drug concentrations longitudinally in the same animal. Ocular fluorophotometry may offer a noninvasive alternative to measure ocular concentrations and the PK of protein therapeutics and sustained delivery technologies.

Several groups have successfully used fluorophotometry to measure the ocular PK of nonprotein molecules and to investigate different methods of ocular drug delivery.4–7 Berezovsky et al.4 used fluorophotometry to evaluate transscleral delivery of sodium fluorescein and fluorescein conjugated to 40 and 70 kDa dextrans (FITC-D) in rabbits. In this study, the authors were able to measure retinal/choroidal and vitreous levels of all three molecules after sub-Tenon injection using fluorophotometry and found that the extent of penetration into ocular tissues was dependent on the molecular weight. The concentrations achieved with free fluorescein were much higher in the retina/choroid and vitreous tissues than obtained with 70 kDa FITC-D. Concentrations of the 40 kDa FITC-D were in-between those of sodium fluorescein and the 70 kDa FITC-D. In addition, the ocular concentrations of sodium fluorescein and the 40 and 70 kDa FITC-D in live and euhathanized animals were assessed. Ocular tissue concentrations of these molecules were higher in live versus euhathanized...
animals. This result highlights the necessity to conduct ocular PK assessments in vivo.

Currently, there is no literature describing the use of fluorophotometry in assessing the ocular PK of protein therapeutics such as antibodies, antibody fragments, or fusion proteins. Preliminary results assessing the utility of fluorophotometry to measure ocular PK of rhuFab V2 were presented at a past ARVO conference, but never published (Gaudreault J, et al. IOVS 2002;43:ARVO E-Abstract B798). In addition, the results of PK analysis using fluorophotometry have not been compared with those using MS or ELISA. The objectives of our current study were to address these two gaps by using fluorescently labeled ranibizumab, an anti-VEGF Fab. A comparison of measurement methods was performed to allow for a head-to-head comparison of fluorophotometry with ELISA, the “gold standard” method used for the measurement of protein therapeutics in biological matrices. In addition to this comparison between fluorophotometry and ELISA, the data obtained in this study were also compared to historical ocular PK data obtained with ranibizumab. Our data suggest that the use of fluorophotometry is a valuable, noninvasive method to measure the ocular concentrations of protein therapeutics.

**Materials and Methods**

**Generation of AlexaFluor 488-Labeled Ranibizumab**

AlexaFluor 488 (AF-488; Life Technologies, Carlsbad, CA, USA) was chosen for labeling due to its superior brightness, photostability, and pH insensitivity compared to fluorescein. The dye molecule to antibody ratio was kept as close to 1:1 as possible (as assessed by the instructions outlined in Life Technologies manual number MP 10235), although this could vary between different lots of AF-488-labeled ranibizumab (AF-488-ranibizumab). Ranibizumab (Genentech, South San Francisco, CA, USA) in 0.1M sodium bicarbonate was labeled with a 2-fold molar excess of AF-488 NHS ester (product number A-10235; Life Technologies) to create an approximate 1:1 labeling ratio. The excess dye was removed from AF-488-ranibizumab using S200 (GE Healthcare, London, UK) size-exclusion chromatography. The eluted peak was pooled, concentrated, dialyzed into PBS, and sterile filtered. Endotoxin levels were assessed using the Pierce LAL chromogenic endotoxin quantitation kit (Life Technologies, Foster City, CA, USA). Endotoxin levels were less than 2 mg/mL.

**In Vivo Dosing and Vitreous Collection**

All animal procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee in an AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals and applicable laws and regulations. New Zealand White rabbits (male and female, >2.5 kg, 3-6 months of age) were used for all in vivo studies. Up to 30 minutes prior to dosing, rabbits were placed in cat bags and topical tropicamide (1%; Akorn Pharmaceuticals, Lake Forest, IL, USA) and/or phenylephrine (2 drops, Akorn Pharmaceuticals) was applied to each eye to dilate the pupil. Rabbits were removed from the cat bags and returned to their cages. For intraocular injections, rabbits were anesthetized with xylazine (3–5 mg/kg, Akorn Pharmaceuticals) approximately 10 minutes prior to ketamine (20–40 mg/kg; Henry Schein, Dublin, OH, USA). Once anesthetized, a topical anesthetic, proparacaine, (Bausch and Lomb, Rochester, NY, USA) was applied to both eyes (2 drops) and buprenorphine (0.02 mg/kg IV bolus, Reckitt Benckiser Healthcare, Berkshire, UK) was given. The dosed eye was opened with a lid speculum and the globe fixated with a Colibri forceps. The eye was then washed with a 1:10 diluted Betadine solution (Henry Schein) and flushed with saline solution (OcuSoft, Rosenberg, TX, USA). Sterile gauze was used to absorb any pooled solution from the flush.

A 28-gauge insulin syringe with a 12-mm needle was used. The needle was inserted approximately 5 mm deep into midvitreous (inferior temporal) and a dose volume not to exceed 50 μL was delivered. Animals that exhibited any regurgitation following injection were not used further in the study, and replaced with another animal that did not display signs of regurgitation. Animals were monitored until they were able to right themselves and then returned to their cages. Rabbits were monitored twice daily while being dosed with buprenorphine. Buprenorphine was administered once prior to recovery from anesthesia, then every 12 hours for 3 days.

At each scanning time point, the animals were placed under ketamine 20 to 40 mg/kg + xylazine 3 to 5 mg/kg intramuscularly before fluorophotometry scans. For terminal time points, animals were treated with Beuthanasia (Virbac Animal Health, Fort Worth, TX, USA) 4.5 mg/mL intravenously 10 minutes prior to ocular tissue collection. Each eye was dissected, the vitreous (approximately 1.3 mL) was removed with a 1 cm³ syringe with no needle, and placed in an Eppendorf tube and frozen on dry ice until assayed.

To keep within the dynamic range of the instrument but still deliver the desired amount of protein, AF-488-ranibizumab was combined with unlabeled ranibizumab as described in each individual study (Fig. 1). The dosing solution for the first study was prepared by spiking various levels of AF-488-ranibizumab (30%, 60%, and 80% of total ranibizumab) with unlabeled ranibizumab to a total amount of 500 μg ranibizumab in PBS per eye. Dosing solutions for subsequent studies were prepared the same way as the initial study but with a 1:1 ratio of AF-488-ranibizumab to unlabeled ranibizumab. In the second study, the rabbits were dosed with different amounts of ranibizumab in each group (10, 50, 500 and 1000 μg per eye) with a single 24 hours post dosing termination point. In the third study, rabbits were dosed with the same amount of ranibizumab per eye (500 μg) but terminated at various time points (6, 24, 168, and 336 hours post dosing).

**Ocular Measurements of AF-488-Ranibizumab Using Fluorophotometry**

Rabbit eyes were dilated with both topical 1% tropicamide and at least 2 drops of phenylephrine hydrochloride USP 2.5% (with additional phenylephrine if not adequately dilated) 15 to 30 minutes before fluorophotometry measurement. Rabbits were anesthetized with inhalable isoflurane gas at least 5 minutes prior to the measurement at survival time points while at terminal time points, ketamine 20 to 40 mg/kg + xylazine 3 to 5 mg/kg and Beuthanasia 4.5 kg/mL was used as anesthetic.

After the rabbit had been anesthetized and pupil dilated, ocular measurements of AF-488-ranibizumab were done with a Fluorotron Master (OcuMetric, Inc., Mountain View, CA, USA) instrument and software. The animal eye was aligned with the optic head as assessed by the operator viewing window. The fluorescence signal measured was then converted to total ranibizumab concentration based on a standard curve of AF-488-ranibizumab test article measured using the cuvette attachment.
Vitreous Measurements of Ranibizumab and AF488-Ranibizumab Using ELISA

A qualified ligand-binding ELISA was designed to measure the concentrations of AF488-ranibizumab and/or unlabeled ranibizumab in the rabbit vitreous matrix. The assay used a recombinant human VEGF-A (Genentech, Inc., South San Francisco, CA, USA) to capture both molecules in rabbit vitreous samples. Bound molecules were detected using an antihuman F(ab’)2 conjugated to horseradish peroxidase (HRP, Jackson Immuno Research Laboratories, West Grove, PA, USA), and a peroxidase substrate, 3,3’,5,5’-tetramethylbenzidine, (TMB; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was used for color development. The drug level was quantitated using absorbance spectrophotometry using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The concentration of total ranibizumab in the study samples was calculated from a standard curve, and the minimum quantifiable concentration (MQC) in rabbit vitreous matrix was 1.5 ng/mL. We found AF488-ranibizumab and unlabeled ranibizumab behaved similarly in this assay (data not shown), and the AF488 did not interfere with binding to either VEGF-A, detection reagent or the measurement of color development using absorbance spectrophotometry.

Vitreous Chromatography

The vitreous samples collected in study 3 were analyzed by high-performance liquid chromatography (HPLC) with a size-exclusion column (300 x 7.8 mm, BioSep-SEC-s3000; Phenomenex, Torrance, CA, USA) and detected by an in-line fluorescence detector (FLD SEC-HPLC, Agilent Open Lab 1100 HPLC; Agilent Technologies, Santa Clara, CA, USA) with excitation and emission wavelengths of 495 and 517 nm, respectively. Phosphate buffered saline was used as the mobile phase with the isocratic flow rate of 0.5 mL/minute for 35 minutes. Vitreous samples were diluted with naïve rabbit vitreous to keep within the detection range of the detector, and then further diluted with an equal amount of PBS to reduce the viscosity. The resulting chromatogram profiles were compared to determine the stability of AF488-ranibizumab over the course of the study. The concentration of AF488-ranibizumab was calculated from the standard curve, and the minimum quantifiable concentration in rabbit vitreous matrix was 1.0 ng/mL. The total ranibizumab concentration was calculated from the ratio of AF488 and unlabeled ranibizumab in the study.

Pharmacokinetic and Statistical Analysis

Vitreous concentration–time profiles were used to estimate PK parameters using noncompartmental analysis (Phoenix Win-
**RESULTS**

All intravitreal injections were well tolerated and without complications or in-life findings. For all studies, ranibizumab concentration was calculated based on a standard curve that was generated on the fluorophotometer using either stock solutions or the individual dosing solutions. In previous studies (Supplementary Fig. S1), similar results were obtained with a standard curve generated in either PBS or rabbit vitreous humor. Therefore, standard curves used in studies described in this manuscript were generated in PBS.

**Initial Feasibility Study With AF488-Ranibizumab (Study 1)**

To determine the feasibility of using fluorophotometry to assess the PK of AF488-ranibizumab administered intravitreally, an initial study was performed as illustrated in Figure 1A. The total amount of administered ranibizumab remained constant between treatment groups while the level of AF488 dye varied. This allowed initial investigation of the dynamic range of the instrument while keeping the total protein load constant. An example of the raw fluorophotometry depth scans over the course of 14 days is shown in Figure 2A. The broad vitreous peak between approximately 0 to 20 mm was observed over the entire time period and for all AF488 levels (Fig. 2A, Supplementary Fig. S2). Two methods were employed to translate the raw fluorescence signal to the concentration of ranibizumab. The first was the peak method that utilized the maximum peak measured between 0 and 20 mm (Figs. 2B, 3). The second method utilized the total area under the curve (AUC) between 0 and 20 mm (Fig. 2B, Supplementary Fig. S3). As shown in Figure 3A and Supplementary Figure S4A, the fluorescence signal measured using the fluorophotometer showed a first order loss over the course of 14 days for all fluorescence levels evaluated. Signal normalized to fluorescence and to an AF488-ranibizumab standard curve (Figs. 3B, 3C, Supplementary Figs. S4B, S4C) also displayed first-order kinetics. Normalized ranibizumab concentrations were used to calculate the PK parameters (Table 1) for individual animals for both the peak and AUC methods. Animal-to-animal variability was low for all PK parameters as evidenced by the standard deviations in Table 1. Using the peak method, the calculated vitreous clearance (CL) of ranibizumab was 0.25 ± 0.02 mL/day, the volume of distribution at steady state (Vss) was 1.1 ± 0.13 mL, the half-life (t1/2) was 3.2 ± 0.25 days, and the AUC was 1944 ± 114 µg·day/mL. Using the AUC method, the calculated vitreous CL of ranibizumab was 0.35 ± 0.06 mL/day, the Vss was 1.5 ± 0.32 mL, the t1/2 was 3.3 ± 0.12 days, and the AUC was 1540 ± 372 µg·day/mL. As shown in Table 1, differences in CL, Vss, and AUC values were statistically significant (P value < 0.05) between the peak method and AUC method, whereas t1/2 values were not. However, all values calculated were within the ranges observed historically, suggesting that both the peak and AUC methods are viable approaches for normalizing the fluorescence signal obtained with fluorophotometry. For subsequent analysis, only the peak method was used with fluorophotometry.

**Exploring the Dynamic Range and Sensitivity (Study 2)**

To further explore the dynamic range and sensitivity of fluorophotometry, a second study was performed as outlined in Figure 1B. In this study, animals received 0.01 to 1.0 mg/eye ranibizumab of which 50% was fluorescein labeled. Therefore, both total protein and total fluorescence varied between treatment groups, although the ratio remained the same. After taking a fluorophotometric reading at 24 hours, animals were euthanized and the vitreous humor was collected for measurement of the concentrations of ranibizumab with ELISA. From previous experiments (Fig. 2 and data not shown), the 24-hour time point was found to be a long enough time period after dosing to achieve near homogeneous distribution of a Fab molecule throughout the visual axis. As shown in Figure 4 and Table 2, vitreous concentrations of ranibizumab measured via fluorophotometry and ELISA were comparable at the 10 and 500 µg/eye doses. Concentrations of ranibizumab were 7.14 ± 1.04 and 5.69 ± 0.80 µg/mL for the 10 µg/eye dose and 364 ± 46 and 367 ± 14 µg/mL for the 500 µg/eye dose for
using fluorophotometry and ELISA, respectively. For the 50 μg/eye dose, concentrations of ranibizumab were statistically significantly different between fluorophotometry and ELISA (P < 0.05) with the concentrations of 48.0 ± 4.4 and 37.4 ± 4.0 μg/mL, respectively. At the 1000 μg/eye dose, the concentrations of ranibizumab determined using ELISA and fluorophotometry diverged significantly. Vitreous concentrations were 532 ± 65 and 694 ± 89 μg/mL for fluorophotometry and ELISA measurements, respectively, and this difference was statistically significant (P < 0.05).

**Direct Pharmacokinetic Comparison Between Fluorophotometry and ELISA and Comparison to Historical Data (Study 3)**

For a direct comparison of PK parameters determined using fluorophotometry and ELISA, a third study was performed as outlined in Figure 1CA 500 μg/eye dose of total ranibizumab (1:1 AF488-ranibizumab to unlabeled ranibizumab) was given intravitreally. Then, ranibizumab concentrations that were measured by fluorophotometry and by ELISA were used to generate PK parameters using noncompartmental analysis. The results of this study are shown in Figure 5 and Table 3. Vitreous CL values for ranibizumab were 0.26 and 0.21 mL/day, Vss values were 0.80 and 0.94 mL, and t1/2 values were 3.1 and 2.9 days for concentrations measured using fluorophotometry and ELISA, respectively. The values of AUC were normalized by dose (AUC/D) for comparison to historical data and were 4.7 and 3.9 day μg/mL/μg for fluorophotometry and ELISA, respectively. To better assess whether the PK values obtained from the studies herein were within the expected range, they were compared to historical values that were either obtained at Genentech over the course of several years or identified from the literature. Nine total historical studies were used in this evaluation. Seven PK studies in rabbits were performed at Genentech using ranibizumab, one of which had been previously published.8 In the nine historical studies, the mean and standard deviation values for CL, Vss, t1/2, and AUC/D of ranibizumab were 0.30 ± 0.09 mL/day, 1.3 ± 0.4 mL, 3.2 ± 0.3 days, and 3.8 ± 1.9 day μg/mL/μg, respectively. The range of values for CL, Vss, t1/2, and AUC/D of ranibizumab were 0.13 to 0.44 mL/day, 0.5 to 1.8 mL, 2.8 to 3.5 days, and 2.3 to 7.9 day μg/mL/μg, respectively. The PK parameters calculated in studies 1 and 3 herein using concentrations of ranibizumab determined by fluorophotometry or ELISA were within the ranges measured in previous studies.

**Assessment of AF488-Ranibizumab Stability**

The stability of the AF488-ranibizumab was assessed from rabbit vitreous samples obtained from study 3. Exposure to the ocular environment and ambient light over time could potentially lead to instability of the Fab-dye conjugate or decreased dye sensitivity. As shown in Figure 6, AF488-ranibizumab analyzed from vitreous samples 7 or 14 days after dosing showed similar peak retention times compared to the dosing solution with no indication of free dye or unexpected protein-dye fragments. This result suggested that the Fab-dye conjugation remained intact and was stable in the vitreous. When normalized to total ranibizumab concentration using a standard curve generated by HPLC-fluorescence, concentrations were comparable to those measured using fluorophotometry and ELISA. This outcome suggested that dye sensitivity was not affected (Supplementary Fig. S5).

**DISCUSSION**

The measurement of ocular drug concentrations and assessment of ocular PK in preclinical species is an important aspect of ophthalmologic drug development. It allows for the prediction of human ocular PK and thus helps guide clinical dosing strategies. Measuring ocular drug concentrations generally relies on terminal euthanasia of the animal in order to obtain ocular matrices. Although an aqueous tap is a useful technique that can be done without killing the animal, the
invade the nature of this method precludes it from being performed more than every other week in the rabbit. Finding an alternative to highly invasive ocular sampling and collection techniques would not only advance the fundamental practice of 3Rs (reduction, refinement, and replacement), it would also improve scientific practice as longitudinal measurements could be obtained from the same animal.

Ocular fluorophotometry has the unique ability to excite a fluorophore in the eye and to measure the emission from this fluorophore. Fluorescence is only quantified along the central visual axis of the eye. Thus, it is limited to one-dimensional scans. However, fluorophotometry offers a fast, safe, and sensitive method of measurement in the eyes of live animals. Although other technologies exist, such as MRI,9,10 that can provide a three-dimensional image of eyes in vivo, they tend to be much more expensive, and have limited sensitivity and resolution with extended imaging times. Additionally, the invasive nature of this method precludes it from being performed more than every other week in the rabbit. Finding an alternative to highly invasive ocular sampling and collection techniques would not only advance the fundamental practice of 3Rs (reduction, refinement, and replacement), it would also improve scientific practice as longitudinal measurements could be obtained from the same animal.

There are caveats and assumptions that need to be addressed when using ocular fluorophotometry for assessment of PK. One assumption is that molecules will distribute homogenously through the vitreous and central visual axis such that the measurements taken via fluorophotometry are representative of concentrations throughout the entire vitreous. This assumption may not be valid for molecules or long-acting delivery technologies that may form a depot outside of the central visual axis, or for which distribution is markedly slower. Thus, the concentration measured using fluorophotometry may not be indicative of the entire vitreous. In this case, other techniques may offer a more comprehensive view of the molecular distribution and localized concentration throughout the entire eye.

Fluorescence measurements were normalized based on the percentage of AF488-labeled ranibizumab used, and total ranibizumab concentrations were calculated from a standard curve generated using the fluorophotometer. Pharmacokinetic parameters were generated using noncompartmental analysis.

**TABLE 1. Pharmacokinetic Parameters Obtained by Measuring AF488-Labeled Ranibizumab Using FPT**

<table>
<thead>
<tr>
<th>AF488, %</th>
<th>CL, mL/d</th>
<th>Vss, mL</th>
<th>t1/2, d</th>
<th>AUC, µg d/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal 1A</td>
<td>30</td>
<td>0.24</td>
<td>0.89</td>
<td>2.8</td>
</tr>
<tr>
<td>Animal 1B</td>
<td>30</td>
<td>0.24</td>
<td>1.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Animal 2A</td>
<td>60</td>
<td>0.28</td>
<td>1.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Animal 2B</td>
<td>60</td>
<td>0.24</td>
<td>1.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Animal 3A</td>
<td>85</td>
<td>0.24</td>
<td>1.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Animal 3B</td>
<td>85</td>
<td>0.24</td>
<td>1.1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Peak method average ± SD 0.25 ± 0.02 1.1 ± 0.13 3.2 ± 0.25 1944 ± 114

**AUC method**

| Animal 1A | 30 | 0.42 | 1.8 | 3.2 | 1179 |
| Animal 1B | 30 | 0.34 | 1.7 | 3.5 | 1358 |
| Animal 2A | 60 | 0.37 | 1.7 | 3.2 | 1299 |
| Animal 2B | 60 | 0.34 | 1.6 | 3.3 | 1413 |
| Animal 3A | 85 | 0.26 | 1.2 | 3.4 | 1854 |
| Animal 3B | 85 | 0.26 | 1.0 | 3.3 | 2136 |

AUC method average ± SD 0.53 ± 0.06* 1.5 ± 0.32* 3.3 ± 0.12 1540 ± 372*

Only the right eye was dosed in each animal. Fluorescence measurements were normalized based on the percentage of AF488-labeled ranibizumab used, and total ranibizumab concentrations were calculated from a standard curve generated using the fluorophotometer. Pharmacokinetic parameters were generated using noncompartmental analysis.

* P < 0.05 using two-tailed, unpaired t-test as compared to peak method.

In these studies, drug concentrations were not assessed in the aqueous humor. In theory, the same experimental techniques employed here for the vitreous humor can also be used to assess the concordance between fluorophotometry and ELISA measurements in the aqueous humor. As seen in Figure 2B, there is a fluorescence signal in the depth scan area that corresponds to the anterior chamber (approximately 22–28 mm). Parameters of PK using fluorophotometry were not

**TABLE 2. Concentrations of Total Ranibizumab Measured in Rabbit Vitreous Humor by FPT or ELISA 24 Hours After Dosing**

<table>
<thead>
<tr>
<th>Ranibizumab Dose, µg/eye</th>
<th>Ranibizumab Concentration via FPT, µg/mL</th>
<th>Ranibizumab Concentration via ELISA, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>7.14 ± 1.04</td>
<td>5.69 ± 0.80</td>
</tr>
<tr>
<td>50</td>
<td>48.0 ± 4.4*</td>
<td>37.4 ± 4.0</td>
</tr>
<tr>
<td>500</td>
<td>364 ± 46</td>
<td>367 ± 14</td>
</tr>
<tr>
<td>1000</td>
<td>532 ± 65*</td>
<td>694 ± 89</td>
</tr>
</tbody>
</table>

The total dose of ranibizumab is a 1:1 mixture of unlabeled ranibizumab and AF488-labeled ranibizumab (approximately one dye molecule per molecule of ranibizumab). Two animals were dosed bilaterally for each dose for a total of four separate measurements per dose. Values represent the mean ± standard deviation.

* P < 0.05; comparing FPT and ELISA using a 2-tailed, unpaired t-test.
assessed for aqueous humor in this study, due to low fluorescence signal in the aqueous humor by day 7. Fluorescence concentrations would thus have to be optimized in order to measure aqueous humor PK.

Caveats aside, this study demonstrated the utility of using fluorophotometry to assess vitreous PK of protein therapeutics. When using fluorophotometry, it is important to keep the fluorescence signal within the dynamic range of the instrument over the appropriate time course. The starting fluorescence level will likely be dependent on the dye molecule used (e.g., AF488, fluorescein, Oregon green), the dye to protein molecule ratio, and the protein therapeutic molecule used in the study. Although several fluorescein-like fluorophores can be detected by this particular instrument, each fluorophore has specific excitation and emission spectra, pH sensitivities, and extinction coefficients. Prestudy standard curve analysis was and should be used to guide the precise amount of labeled versus unlabeled protein used in the fluorophotometric experiments. In our current study, the dye-to-Fab ratio was kept as close to one to one as possible as signal quenching has been observed for molecules with high dye to protein ratios (data not shown). However, our data (studies 1 and 2 herein) do indicate flexibility in the extent of dye labeling and the amount of labeled molecule used. Our results from studies 2

### Table 3. Comparison of Ranibizumab PK Parameters Measured in the Current Study and Those Measured in Prior Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose, µg/eye</th>
<th>CL, mL/d</th>
<th>Vss, mL</th>
<th>t1/2, d</th>
<th>AUC/D, d µg/mL/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPT (study 1) peak method</td>
<td>500</td>
<td>0.25</td>
<td>1.1</td>
<td>3.2</td>
<td>3.9</td>
</tr>
<tr>
<td>FPT (study 1) AUC method</td>
<td>500</td>
<td>0.53</td>
<td>1.5</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td>FPT (study 3)</td>
<td>500</td>
<td>0.26</td>
<td>0.80</td>
<td>3.1</td>
<td>4.7</td>
</tr>
<tr>
<td>ELISA (study 3)</td>
<td>500</td>
<td>0.21</td>
<td>0.94</td>
<td>2.9</td>
<td>3.9</td>
</tr>
<tr>
<td>14-0995</td>
<td>1000</td>
<td>0.27</td>
<td>1.3</td>
<td>3.5</td>
<td>3.7</td>
</tr>
<tr>
<td>12-3483</td>
<td>300</td>
<td>0.31</td>
<td>1.5</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>14-0779</td>
<td>300</td>
<td>0.36</td>
<td>1.4</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>12-3371</td>
<td>500</td>
<td>0.44</td>
<td>1.8</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>02-082-1757</td>
<td>500</td>
<td>0.32</td>
<td>1.4</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>02-291-1757</td>
<td>500</td>
<td>0.28</td>
<td>1.3</td>
<td>3.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Gaudreault et al.8*</td>
<td>625</td>
<td>0.13</td>
<td>0.5</td>
<td>2.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Ahn et al.13*</td>
<td>250</td>
<td>NR</td>
<td>NR</td>
<td>2.8</td>
<td>NR</td>
</tr>
<tr>
<td>Bakri et al.14</td>
<td>500</td>
<td>NR</td>
<td>NR</td>
<td>2.9</td>
<td>NR</td>
</tr>
<tr>
<td>Range of historical and published studies</td>
<td>300–1000</td>
<td>0.13–0.44</td>
<td>0.5–1.8</td>
<td>2.8–3.5</td>
<td>2.3–7.9</td>
</tr>
<tr>
<td>Average ± SD of historical and published studies</td>
<td>NA</td>
<td>0.30 ± 0.09</td>
<td>1.3 ± 0.4</td>
<td>3.2 ± 0.3</td>
<td>3.8 ± 1.9</td>
</tr>
</tbody>
</table>

Unless indicated, PK parameters were calculated using noncompartmental analysis. With the exception of the current study, all ranibizumab concentrations were measured via ELISA.

* Fit to a one compartmental model; NR, not reported; NA, not applicable.
and 3 indicate that there is good agreement between the concentrations measured by fluorophotometric and ELISA techniques when the level of fluorescence is kept within the dynamic range of the instrument. In study 2, the main divergence between fluorophotometric and ELISA values appeared at fluorescence concentrations that were above the dynamic range of the instrument for this particular fluoro-phore-protein combination.

Based on Table 3, it appears that the PK parameters calculated using the peak method and $AUC$ method gave comparable results. Because the peak method showed less variability in calculated $CL$, $V_{ss}$, and $AUC$ values, it was used for subsequent analyses. The reason for the increased variability using the $AUC$ method is currently not fully understood, and the limited data comparing the two methods make any theory difficult to support. However, as seen in Figure 2 and Supplementary Figure S2, the fluorometric peak shapes tend to be quite variable at the early time points (up to 48 hours). This may be due to the exact location of administration and subsequent diffusion of the molecule through the vitreous, parameters that are likely to be slightly different from eye to eye.

Ranibizumab PK parameter estimates from studies 1 and 3 are also in good agreement with those that were measured previously at Genentech or were published. Clearance, $V_{ss}$, $t_{1/2}$, and $AUC/D$ values fell within the range of those measured in previous studies. Although published PK parameters for ranibizumab measured by groups outside of Genentech are limited, the $t_{1/2}$ values reported in these studies are in line with those measured in the current studies using both ELISA and fluorophotometry. One limitation of the current investigation is the lack of study-to-study variability using fluorophotometry with direct comparison to ELISA. While the average of several studies is likely a better comparison to use against the historical data, the authors felt that it did not justify the use of more animals. We do believe, however, that nonterminal PK studies using fluorophotometry in higher order species such as mini-pig or cynomolgus monkey are worth investigating as these species are generally considered to be better ocular models for humans. In summary, the present study indicates that fluorophotometry can be a useful tool for assessing the PK of protein therapeutics in the rabbit vitreous. Although the initial

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934564/ on 10/30/2018)

**Figure 5.** Direct comparison of FPT and ELISA measurements from study 3. Concentration versus time profiles over the course of 14 days comparing FPT and ELISA data. After FPT readings were obtained, vitreous humor was immediately collected for total ranibizumab measurement using an ELISA as outlined in the Materials and Methods section. Time points represent average and standard deviation of $n = 4$ eyes (two animals dosed bilaterally). There was no statistically significant difference between the ELISA and FPT measurements at each individual time point as assessed using an unpaired $t$-test.

![Figure 6](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934564/ on 10/30/2018)

**Figure 6.** Stability of AF488-labeled ranibizumab. Ranibizumab-AF488 was measured via high performance liquid chromatography fluorescence as described in the Materials and Methods section. The initial dosing solution was diluted 8-fold in PBS for this comparison to be within the dynamic range of the detector and the expected concentration of the vitreous samples. Vitreous samples from days 7 and 14 were each diluted 1:1 with PBS for this analysis. The chromatogram is representative of all animals in study 3.
cost of instrument purchase (approximately $65,000) could be a limiting factor for some labs, the cost savings in both animal usage and work time could likely offset this. The need for time-consuming assay development, especially at the drug discovery stage, would be completely negated. Fluorophotometry also allows for longitudinal measurements in live animals and can reduce animal usage by up to 70% for standard ocular PK studies.

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References