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Development of a novel homogenous electrochemiluminescence assay for quantitation of ranibizumab in human serum

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ABSTRACT

A solution-phase electrochemiluminescence assay (ECLA) was developed to quantify ranibizumab in serum from patients treated with this biotherapeutic for neovascular age-related macular degeneration. Ranibizumab, a recombinant humanized Fab ("fragment, antigen binding"), binds with high affinity and specificity to vascular endothelial growth factor A (VEGF-A), inhibiting its activity. Fab molecules contain the amino acid sequence that binds antigen and are composed of one constant and one variable domain from each heavy and light chain of the antibody. High assay sensitivity was required to enable pharmacokinetic (PK) evaluation of ranibizumab-dosed patients in clinical trials. Our assay's lower limit of quantitation is 300 pg/mL ranibizumab in neat serum, achieving a 67-fold improvement in sensitivity relative to a conventional ELISA-based PK method. In this assay, ruthenium-labeled affinity-purified rabbit anti-ranibizumab antibodies and biotinylated rhVEGF are added to serum samples. During overnight incubation, these two labeled molecules bind to ranibizumab, and the resulting immune complex is then captured by streptavidin-coated paramagnetic beads and analyzed for electrochemiluminescence. The ranibizumab PK ECLA has a reporting range of 300–24,000 pg/mL, based on accuracy and precision parameters. It showed high precision for both intra- and inter-assay analyses. Recovery of ranibizumab from 10 individual donors averaged between 100% and 119% of nominal concentration. There was no cross-reactivity observed in the assay to other recombinant humanized antibodies (whole molecules or monoclonal antibody fragments) or human IgG. To our knowledge, this report represents the first description of development and validation of an ECLA-based PK assay for a recombinant humanized Fab therapeutic agent.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in adults over 50 years of age [1,2]. Approximately 200,000 new cases of neovascular (or "wet") AMD are diagnosed each year in North America [3]. The wet form of AMD accounts for the majority of cases of severe vision loss [4]. Vascular endothelial growth factor A (VEGF-A) has been implicated in the pathogenesis of neovascular AMD, in which increased angiogenesis and vascular permeability under the central part of the retina ultimately lead to scarring and damage to the macula [5–7].

Ranibizumab (LUCENTIS®, Genentech, Inc., South San Francisco, CA), a recombinant humanized monoclonal antibody fragment (Fab) that binds to and inhibits VEGF-A [8], was approved by the US Food and Drug Administration in 2006 for the treatment of neovascular AMD. In phase 3 clinical trials, ranibizumab improved the visual acuity in approximately 25% of treated patients and maintained visual acuity in ≥90% of treated patients over a period of 2 years [9–11].

Assessment of pharmacokinetics (PK) in drug development is essential to determine exposure and to interpret drug safety and efficacy data. Ranibizumab has the potential to inhibit systemic VEGF if the drug disseminates beyond the vitreous chamber following intraocular injection. Because VEGF performs critical endogenous functions in regulating normal vasculature, interference with VEGF activity could potentially result in adverse events [12]. Therefore, the ability to monitor systemic levels of ranibizumab following intravitreal injection with a highly sensitive and specific PK assay was critical for drug development. Monitoring serum levels of ranibizumab provide assurance that systemic drug levels remain below the concentration required to inhibit the biological activity of VEGF-A [13].

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However, the assessment of PK parameters for a humanized Fab therapeutic agent administered intravitreally poses several distinctive challenges: (1) periodic sampling of vitreous fluid in humans is impractical; (2) systemic exposure following intravitreal administration is low [14], and expected to be below the limits of detection of conventional ELISA-based PK methods for recombinant therapeutic antibodies; (3) traditional ligand-binding-based assays are often affected by serum interference, which may lead to suboptimal assay specificity or selectivity [15]; (4) bridging- or sandwich-type immunoassays require that the molecule of interest contain multiple noncompeting antigenic binding sites, which may not be available on Fab molecules, given their relatively small size; (5) conventional ELISA-based methodologies often require multiple washing steps, which might potentially wash away bound therapeutic molecules that have low binding affinity to capture molecules; and (6) naturally occurring anti-Fab antibodies have been reported in the literature [16–18], and these may confound our ability to accurately quantify ranibizumab in some patients. These challenges provided the impetus for developing an improved method for PK analysis of ranibizumab.

Electrochemiluminescence (ECL) offers a highly sensitive, reproducible, homogenous, and serum-tolerant alternative to ELISA [19]. Because of the simplicity and sensitivity of the assay, ECL methods provide a unique platform to support PK studies [19–23]. This article describes the development and validation of a solution-phase ECL assay (ECLA) for accurate quantification of ranibizumab in human serum following intravitreal administration.

2. Materials and methods

2.1. Reagents

Ranibizumab was produced at Genentech, Inc. (South San Francisco, CA), using recombinant DNA techniques [8]. A normal human serum (NHS) pool was purchased from Sigma–Aldrich (St. Louis, MO), and individual serum samples were collected during a ranibizumab phase 1B clinical study [24]. Recombinant human VEGF (rhVEGF) was produced at Genentech, Inc. Assay diluent consisted of a PBS-based buffer with 0.5× PBS, 0.5% BSA, 0.25% polysorbate-20, 0.05% ProClin 300, 5 mM EDTA, 0.35 M NaCl, pH 6.35.

The following reagents were used in specificity testing: (1) recombinant humanized monoclonal antibodies including anti-CD11a (Fab fragments and full length), anti-IgE (Fab fragments and full length), anti-tissue factor $F(ab')_2$ fragments, and anti-CD18 $F(ab')_2$ fragments (all produced at Genentech, Inc.); (2) human IgG Fab; (3) human IgG $F(ab')_2$; and (4) full-length human IgG (the last three reagents were purchased from Jackson ImmunoResearch, West Grove, PA).

2.2. Preparation of biotinylated rhVEGF

Biotin-LC-Sulfo-NHS ester (Biotin; BioVeris, Gaithersburg, MD) was conjugated to rhVEGF at a nominal molar ratio of 5:1, according to manufacturer's instructions. Briefly, 18.2 μL biotin (2 mg/mL in water) was mixed with a 0.5 mL aliquot of rhVEGF (1 mg/mL in PBS) and incubated for 60 min at room temperature. The reaction was quenched by addition of 20 μL of 2 M glycine. Free biotin label and glycine were removed by size exclusion chromatography using a NAP-5 column (Pharmacia Biotech, Wikstroms, Sweden); biotinylated rhVEGF was stored in PBS at 2–8 °C.

2.3. Preparation of BV-TAG-conjugated rabbit anti-ranibizumab

Ruthenium BV-TAG-NHS ester (BV-TAG; BioVeris) was conjugated to rabbit anti-ranibizumab, purified from hyperimmunized rabbit anti-sera (Genentech, Inc.) at a nominal molar ratio of 10:1, according to manufacturer's instructions. Briefly, 500 μL of purified rabbit anti-ranibizumab (1 mg/mL in PBS) was mixed with 23.5 μL of BV-NHS ester (1.5 μg/μL in dimethyl sulfoxide) and incubated for 60 min at room temperature. The reaction was stopped by the addition of 20 μL of 2 M glycine. Uncoupled BV-TAG, dimethyl sulfoxide, and glycine were removed using a NAP-5 column. BV-TAG-conjugated antibody was stored in PBS at 2–8 °C.

2.4. ECLA

During early-stage development, the ECLA was performed by mixing 50 μL of standards (ranging from 0.1 to 16,000 pg/mL, in-well concentrations), matrix controls, or serum samples with 50 μL of a conjugate solution containing 0.25 μg/mL of biotinylated rhVEGF and 1 μg/mL of BV-TAG-conjugated rabbit anti-ranibizumab in the wells of an uncoated round-bottom polypropylene plate (Corning Costar, Corning, NY). After incubation with agitation for 16–20 h at room temperature, a 25 μL solution containing 250 μg/mL streptavidin-coated Dynabeads M280 (BioVeris) was added to each well, and the mixture was incubated in the dark for 2 h. After incubation, 125 μL of assay diluent was added to each well, and the electrochemiluminescent (ECL) signal was measured at 620 nm using a BioVeris M-Series M384 Analyzer. All steps were performed at room temperature. The ranibizumab standard curve was generated using a four-parameter logistic curve-fit program; the concentration of ranibizumab present in serum samples was then quantified from the standard curve.

In its final, optimized format, the ECLA was performed by mixing 50 μL of standards (ranging from 16.5 to 12,000 pg/mL, in-well concentrations, during validation), matrix controls, and serum samples with 50 μL of a conjugate solution containing 0.1 μg/mL of biotinylated rhVEGF and 0.8 μg/mL of BV-TAG-conjugated rabbit anti-ranibizumab in the wells of a round-bottom polypropylene plate (Corning Costar). After incubation with agitation for 16–20 h, a 50 μL solution containing 250 μg/mL streptavidin-coated Dynabeads M280 was added to each well, and the mixture was incubated in the dark for 2 h. After incubation, 100 μL of assay diluent was added to each well, and ECL was measured at 620 nm using a BioVeris M-Series M384 Analyzer. All steps were performed at room temperature. The ranibizumab standard curve was generated using a four-parameter logistic curve-fit program; the concentration of ranibizumab present in serum samples was then quantified from the standard curve.

2.5. ELISA

Maxisorp microtiter plates (Nunc, Rochester, NY) were coated with 0.4 μg/mL full-length rhVEGF in 0.05 M carbonate/bicarbonate buffer at 2–8 °C for 12–72 h. The in-well concentrations of the standards ranged from 78 to 5000 pg/mL ranibizumab, prepared in assay diluent (1× PBS, 0.5% BSA, 0.05% polysorbate-20, 0.05% ProClin 300, 5 mM EDTA, 0.35 M NaCl, pH 6.35). Controls and serum samples were diluted 1:100 in assay diluent prior to testing. Standards, controls, and samples were added to rhVEGF-coated microtiter plates and incubated for 2 h. This was followed by addition of goat anti-human IgG $F(ab')_2$ conjugated to horseradish peroxidase (at 1:10,000 dilution) (Jackson ImmunoResearch, West Grove, PA) and subsequent incubation for 1 h at 37 °C. Plates were developed by addition of tetramethyl benzidine (TMB) (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and color development was stopped by addition of 1 M phosphoric acid (Genentech, Inc.) after 10 min of incubation. Plates were read at 450 nm for detection and 620–630 nm for reference in a colorimetric plate reader (Biotek, Winooski, VT). The standard curve was generated using a four-parameter logistic curve-fit program, and the concentration of

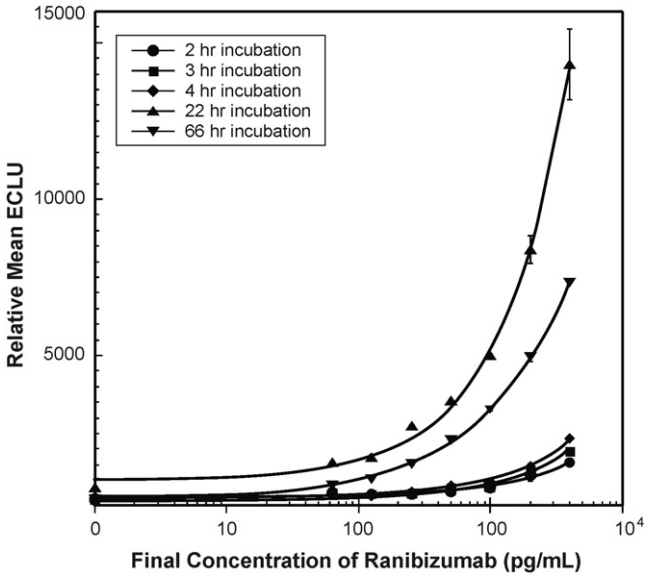

Fig. 1. Effect of incubation time on assay signals from the ranibizumab standard curve during assay development. Values are represented by relative mean electrochemiluminescence units (ECLU). Error bars represent ±1 standard deviation of the mean from triplicate determinations.

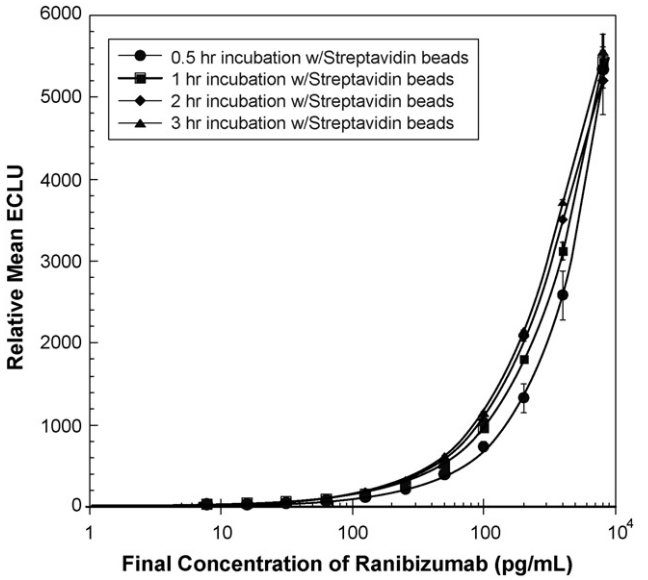

Fig. 2. Effect of streptavidin-coated-bead incubation time on ranibizumab standard curve during assay development. Values are represented by relative mean electrochemiluminescence units (ECLU). Error bars represent ±1 standard deviation of the mean from duplicate determinations.

ranibizumab in each serum sample was derived from the standard curve. This method was validated and shown to have a lower limit of quantitation of 20 ng/mL in neat human serum (data not shown).

3. Results

3.1. ECLA assay optimization and qualification

Several parameters were evaluated during assay development, including sample incubation time, streptavidin-coated-bead incubation time and concentration, and reagent concentration, as well as sample dilution. In the first set of experiments, biotin-labeled rabbit anti-ranibizumab (1:100 dilution) and BV-TAG-conjugated rhVEGF (0.5 μg/mL) were incubated with serially diluted ranibizumab (range 62.5–4000 pg/mL) in 1% NHS for 2, 3, 4, 22, or 66 h at room temperature. ECL signal improved with increased incubation time up to 22 h incubation (Fig. 1).

Due to limited success with the above-described reagents, a different assay format was subsequently assessed using streptavidin-coated beads, biotin-labeled rhVEGF, and BV-TAG-rabbit anti-ranibizumab. Initially, incubation times with the streptavidin-coated beads varied from 0.5 to 3 h following an overnight incubation of sample with biotin-labeled rhVEGF (0.25 μg/mL), and BV-TAG-rabbit anti-ranibizumab (1 μg/mL). In these experiments, ranibizumab standard curves were prepared by serial dilution in 10% NHS, with ranibizumab concentrations ranging from 7.8 to 8000 pg/mL. The ECL signal increased slightly with bead incubation time to a maximum at 2 h incubation (Fig. 2).

The biotin-rhVEGF and BV-TAG-anti-ranibizumab concentrations were then optimized. For these experiments, ranibizumab standard curves that ranged in concentration from 5 to 500 pg/mL in 10% NHS were used. The combination of 0.25 μg/mL biotin-rhVEGF with 1 μg/mL BV-TAG-anti-ranibizumab resulted in the most robust ECL signal (Fig. 3). Thus, these concentrations were used for further assay optimization.

The minimum sample dilution also affected the strength of the ECL signal. Biotin-rabbit anti-ranibizumab and BV-TAG-rhVEGF were incubated overnight with serially diluted ranibizumab in assay diluent containing varying proportions of NHS (1–50% serum). ECL signal decreased as serum concentrations increased (Fig. 4); however, the estimated assay sensitivity based on 2× the background ECLU was better at higher serum concentrations (Fig. 4). Therefore, 50% serum was used for further assay optimization.

Finally, the effect of various concentrations of streptavidin-coated beads on ECL signal was tested. Biotin-rhVEGF and BV-TAG-anti-ranibizumab were incubated with serially diluted ranibizumab (ranging from 3.9 to 16,000 pg/mL) in 50% NHS for 18 h at room temperature. Varying concentrations of streptavidin-coated beads (50, 100, 250, 500, 1000, or 2000 μg/mL) were then added to the mixture, which was then incubated for 2 h, followed by ECL measurement. Two concentrations of streptavidin-coated

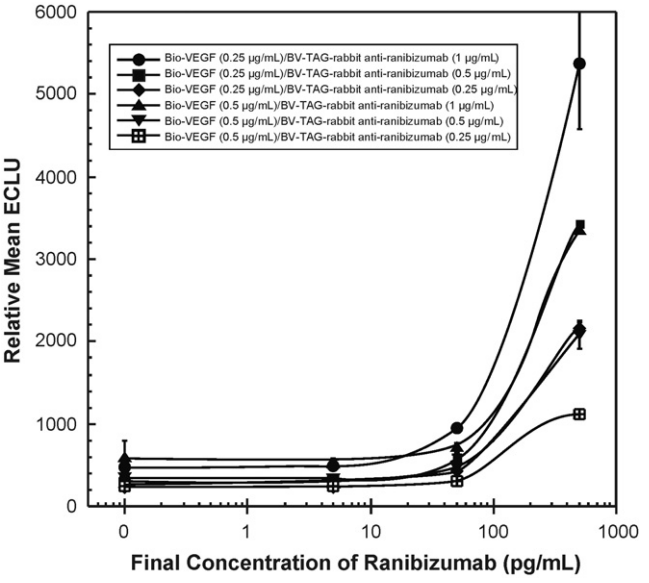

Fig. 3. Effect of biotin-VEGF and BV-TAG-rabbit anti-ranibizumab concentrations on ECLA during assay development. Values are represented by relative mean electrochemiluminescence units (ECLU). Error bars represent ±1 standard deviation of the mean from duplicate determinations.

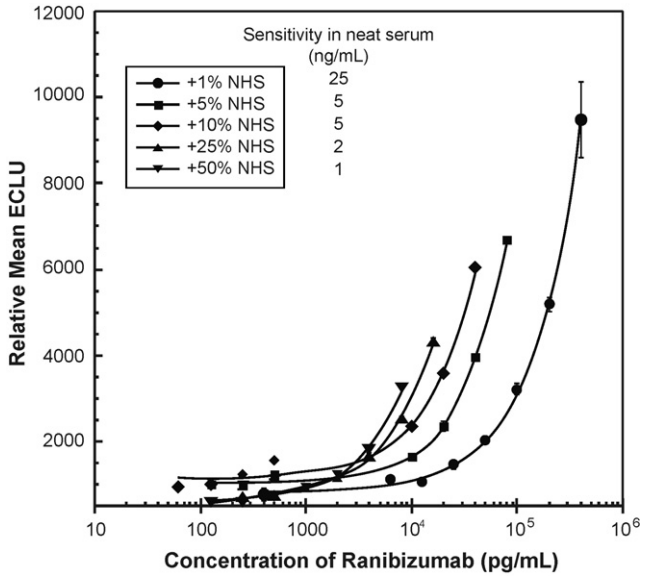

Fig. 4. Effect of normal human serum concentration on ranibizumab standard curve during assay development. Values are represented by relative mean electrochemiluminescence units (ECLU). Error bars represent ±1 standard deviation of the mean from duplicate determinations.

beads yielded the strongest ECL signal in these experiments: 250 and 500 μg/mL. Higher and lower concentrations of streptavidin-coated beads reduced the signal significantly (Fig. 5), indicating that there is an optimal bead-to-sample ratio, above or below which assay sensitivity diminishes. At high bead concentrations, this effect is most likely due to the surface area on the tricorder not being sufficiently wide to allow an even dispersion of all the beads in a single monolayer, resulting in the accumulation of multiple layers of beads on top of those covering the tricorder surface and increasing the percentage of beads with no analyte-containing complexes attached.

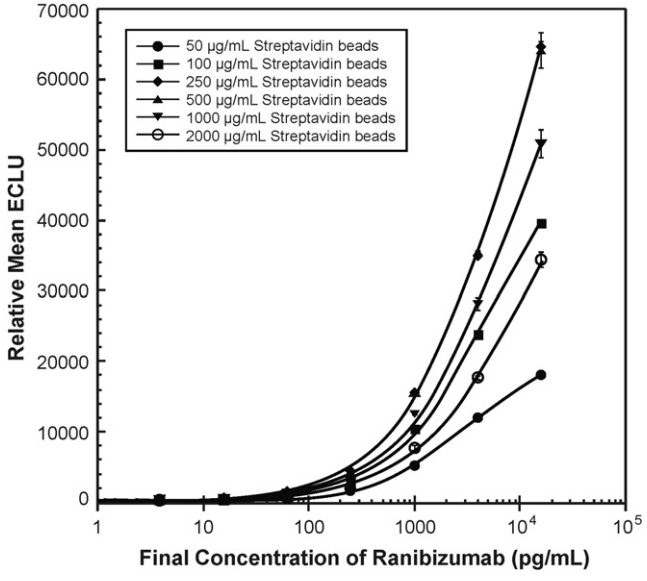

Fig. 5. Effect of streptavidin-coated-bead concentration on ranibizumab standard curve during assay development. Values are represented by relative mean electrochemiluminescence units (ECLU). Error bars represent ±1 standard deviation of the mean from duplicate determinations. Sensitivities, estimated based on 2× background ECLU, were 62, 41, 31, 36, 41, and 50 pg/mL for streptavidin-coated-bead concentrations of 50, 100, 250, 500, 1000, and 2000 μg/mL, respectively.

Table 1
Assay accuracy in pooled human serum.

Nominal concentration[a] (pg/mL)	Mean concentration (pg/mL)	SD	%CV	Mean recovery (%)	Range of recovery[b] (%)
200	180	13	7	90	82–100
400	399	34	9	100	89–114
4000	3959	272	7	99	83–106

CV: coefficient of variation; SD: standard deviation.
[a] Known concentrations of ranibizumab were added to pooled human serum at three different concentrations and the samples were analyzed in 10 assays in duplicate.
[b] Accuracy is calculated as $[\text{ranibizumab}]_{\text{observed}}/[\text{ranibizumab}]_{\text{added}}$.

These assay optimization experiments allowed the establishment of a basic standard procedure, which was then further evaluated for accuracy and specificity.

3.2. Accuracy

Assay accuracy was assessed during assay qualification. The recovery of three concentrations of ranibizumab (200, 400, and 4000 pg/mL) added to pooled normal human serum was determined from 10 assays run in duplicate on the same day (Table 1). Overall, the mean recovery rates ranged from 82–114%.

3.3. Specificity

Specificity of the ECLA for ranibizumab was confirmed by measuring the reactivity of ranibizumab, six other recombinant humanized monoclonal antibodies (full length or Fabs), and three generic purified human IgG samples [full length, $F(ab')_2$, or Fab]. Pooled normal human serum was prepared by combining serially diluted ranibizumab (range 31–32,000 pg/mL or 0.64–662 pM) and each of the nonspecific antibodies at concentrations ranging from 10 pg/mL (0.067 pM) to 10 μg/mL (66,667 pM) and analyzed in the PK ECLA. None of these full-length or Fab IgGs cross-reacted in the assay (data not shown), demonstrating the high specificity of this assay for ranibizumab.

The assay subsequently underwent a thorough characterization of several additional performance attributes, after which it was considered validated for testing of clinical serum samples from ranibizumab-treated AMD patients. The following sections highlight the parameters tested during assay validation.

3.4. Assay linearity

Linearity of dilution was tested to determine if the calculated concentration of analyte (after correction for sample dilution) was similar for a serial dilution of a sample. This was tested in sera from five normal individuals. Ranibizumab was added to each of the 5 individual serum samples. Subsequently, each sample was diluted to the minimum dilution in assay diluent, followed by three 1/2 serial dilutions in standard/sample diluent (50% NHS). Linearity was evaluated by comparing the percent difference of the

Table 2
Lower limit of quantitation.

Ranibizumab nominal concentration (pg/mL)	300	400	500
Mean measured concentration (pg/mL)	284	400	510
% recovery	94	100	102
Inter-assay variability (%CV)	18	14	15
Intra-assay variability (%CV)	12	12	10

CV: coefficient of variation.

Table 3
Intra-assay and inter-assay precision in human serum.

Matrix control	Ranibizumab nominal concentration (pg/mL)	Mean measured (pg/mL)	Number of replicates	Intra–assay (%CV)	Inter–assay (%CV)
Low	704	689	64	9	20
Mid	1600	1600	64	9	15
High	16,000	15,800	64	8	18

CV: coefficient of variation.

Table 4
Recovery of ranibizumab in normal[a] individual serum samples.

Ranibizumab nominal concentration[b] (pg/mL)	Mean concentration (pg/mL)	Mean recovery[c] (%)	Range of recovery (%)
704	835	119	79–133
1600	1700	107	79–125
16,000	16,000	100	62–120

CV: coefficient of variation.
[a] Patients aged ≥50 years.
[b] Known concentrations of ranibizumab were added to 10 different individual serum samples and assayed in duplicate.
[c] Recovery is calculated as $100 \times [\text{ranibizumab}]_{\text{observed}}/[\text{ranibizumab}]_{\text{added}}$.

dilution-corrected concentration value of each sample serial dilution relative to preceding dilution. The linearity was considered acceptable, as no trend or shift was observed (data not shown).

3.5. Sensitivity

The lower limit of quantitation (LLOQ) for this assay was determined using samples prepared with ranibizumab at 300, 400, and 500 pg/mL in NHS. The LLOQ was determined to be 300 pg/mL (Table 2). Thus, ECLA is approximately 67-fold more sensitive than ELISA, which has a LLOQ of 20 ng/mL in human serum (data not shown).

3.6. Assay precision

Matrix control samples were prepared by adding three concentrations of ranibizumab (704, 1600, and 16,000 pg/mL; low, mid, and high spikes, respectively) to normal pooled human serum; these matrix controls were used for generating precision data. Precision (the degree of agreement among individual test results when the analytical method is repeated for multiple analyses), intra-assay precision (the variability within an assay), and inter-assay precision (the variability between assays) parameters were calculated by using a one-way analysis of variance. Intra-assay precision was consistently less than 10%, and inter-assay precision was consistently less than 20% (Table 3).

3.7. Recovery

Serum samples collected from 10 normal donors (50 years or older) were prepared by adding ranibizumab at 704, 1600, and 16,000 pg/mL and consequently used to assess assay recovery. Overall, the mean percent recovery ranged from 100% to 119% (Table 4).

3.8. Interference

Assay interference was assessed using different concentrations of a variety of potential interfering molecules. rhVEGF was found to interfere at 200,000 pg/mL (5222 pM) (Table 5), and biotin at 50,000 pg/mL (89,767 pM) (data not shown), which are well above the normal physiological concentrations of those two molecules (range 0.3–8 pM for VEGF-A [25,26]) and 200–2.86×10^5 pM for biotin [27]. These findings indicate that the assay can accurately quantify ranibizumab bound to VEGF-A, suggesting that the two molecules are likely to dissociate during the 16–20-h sample incubation step. Verteporfin, hemolysis, lipids, and bilirubin did not interfere in the assay performance (data not shown).

3.9. Analyte stability: freeze–thaw and 2–8 °C

The stability of ranibizumab in normal human serum was evaluated following 6 days of storage at 2–8 °C, as well as by subjecting it to one, three and six freeze–thaw cycles (stressed controls). Matrix controls (low, mid, and high) were used to evaluate stability. The results from the stressed controls were compared with freshly thawed controls (unstressed). The percent difference between stressed and unstressed samples was less than 12% after 6 days of storage at 2–8 °C (Table 6). The percent recovery of multiple frozen–thawed samples relative to single frozen–thawed samples varied from 96–109% (Table 7).

3.10. Robustness

The assay's robustness is evidenced by the fact that qualification and validation experiments were performed in different laboratories by different individuals. The results demonstrated comparable assay performance between laboratories and different individuals. Other experiments were performed during assay validation that provided further evidence of the assay's robustness (data not shown).

Table 5
Interference of rhVEGF on the quantitation of ranibizumab.

rhVEGF (pg/mL)	Ranibizumab nominal concentration (125 pg/mL)	Ranibizumab recovery (%)	Ranibizumab nominal concentration (500 pg/mL)	Ranibizumab recovery (%)	Ranibizumab nominal concentration (2000 pg/mL)	Ranibizumab recovery (%)
0	122	100	508	100	1992	100
200 (5 pM)	124	102	512	101	2118	106
2000 (52 pM)	122	100	528	104	2130	107
20,000 (522 pM)	102	84	440	87	1758	88
200,000 (5222 pM)	38	31	184	36	728	36

rhVEGF: recombinant human vascular endothelial growth factor.
Recovery: (measured concentration of ranibizumab with interfering substance/sample concentration without interfering substance) × 100.

Table 6
Stability of ranibizumab in human serum for 6 days at 2–8 °C.

Matrix control	Ranibizumab (pg/mL)		Difference (%)
	Unstressed[a]	Stressed[b]	
Low	701	614	−12
Mid	1540	1540	0
High	15,100	15,700	4

[a] Unstressed: freshly thawed controls.
[b] Stressed: following 6 days of storage at 2–8 °C.

Table 7
Freeze–thaw stability of ranibizumab in human serum.

Matrix control	1 F/T (pg/mL)	3 F/T (pg/mL)	Recovery 3 F/T vs 1 F/T (%)	6 F/T (pg/mL)	Recovery 6 F/T vs 1 F/T (%)
Low	688	673	98	683	99
Mid	1630	1750	107	1720	105
High	15,600	15,000	96	16,900	109

F/T: freeze–thaw cycle.

4. Discussion

Accurate and robust measurement of drug concentrations in the serum compartment is an essential component of preclinical and clinical studies. This information enables proper interpretation of safety and efficacy data. An understanding of the PK of recombinant protein therapeutics is important not only for predicting therapeutic outcome but also for explaining any safety signals that may be seen in a study. In this study, we report the development, qualification, and validation of a novel and highly sensitive ECL-based assay for quantitation of serum levels of ranibizumab following intravitreal injection in patients with neovascular AMD. This solution-phase ECLA was accurate and robust. The assay did not cross-react with other recombinant antibodies, and it showed good recovery when testing serum samples collected from individual patients and simulated samples with ranibizumab. It also exhibited high intra- and inter-assay precision.

High-quality reagents are crucial for the development of an immunoassay that is capable of detecting sub-nanogram levels of a recombinant therapeutic drug in a serum matrix after intravitreal administration. The challenge of producing such reagents is further amplified when the analyte of interest is a Fab molecule because of its relatively small size and the limited number of antigenic domains on Fab molecules. In the ranibizumab ECLA described here, a biotinylated version of ranibizumab's soluble ligand, rhVEGF, and a BV-TAG-conjugated anti-ranibizumab polyclonal antibody were successfully employed for development and validation of a novel immunoassay.

An ECL-based PK assay offers several advantages over conventional ELISA for clinical PK analysis, including high serum tolerance, as evidenced by the ranibizumab recovery data shown here. Most importantly, the ECLA reliably quantified ranibizumab at concentrations as low as 300 pg/mL, whereas the minimum quantifiable concentration for the ranibizumab ELISA was 20 ng/mL. Measurement of low-level serum concentrations of ranibizumab was needed to monitor systemic exposure to ranibizumab in AMD patients following intravitreal administration. This information was used to assess whether exposure remains below the ranibizumab concentrations thought to be necessary to inhibit the activity of pharmacologically relevant levels of VEGF-A by 50%, as measured by an in vitro cellular proliferation assay [13]. The ranibizumab PK ECLA was used in phase I and I/II clinical trials to measure serum drug concentrations following intravitreal injection in patients with neovascular AMD. In the majority of patients with measurable drug levels, circulating concentrations of ranibizumab ranged between 300 and 2500 pg/mL [28]. These observations confirmed the importance of the capability to quantify serum ranibizumab in treated patients at concentrations below 20 ng/mL. Based on the serum PK data, ranibizumab serum clearance was calculated and the average vitreous elimination half-life was estimated at approximately nine days [28].

In summary, the ECLA described here shows greatly improved sensitivity compared with an analogous ELISA-based method. This PK ECLA has a reporting range of 300–24,000 pg/mL, and it accurately and reliably quantifies pharmacologically relevant ranibizumab concentrations in human serum.

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