Original Investigation

Evaluation of Compounded Bevacizumab Prepared for Intravitreal Injection

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IMPORTANCE Bevacizumab acquired from compounding pharmacies for intravitreal injection may cause infectious and noninfectious inflammation. In addition to safety issues, the drug itself may have variable efficacy associated with product aliquoting, handling, and distribution.

OBJECTIVE To conduct surveillance cultures, evaluate endotoxin levels, and assess protein concentrations of bevacizumab obtained from compounding pharmacies in the United States.

DESIGN AND SETTING Prospective in vitro study of syringes containing intravitreal preparations of bevacizumab from compounding pharmacies. This study was conducted at a university-based, good manufacturing practice facility and academic ophthalmology practice.

MAIN OUTCOMES AND MEASURES Microbial culture growth, endotoxin levels, and quantity and binding affinity of protein in each sample.

RESULTS There were no microbial contaminants or endotoxin detected in any of the samples. Of the 21 compounded samples of bevacizumab obtained from 11 pharmacies, 17 (81%) had lower protein concentrations (mean [SD], 22.2 [4.9] mg/mL; range, 19.2-24.5 mg/mL) compared with bevacizumab acquired directly from Genentech (25 mg/mL; $P < .05$). In 3 of 10 compounding pharmacies where more than 1 sample was available, there were statistically significant differences in the protein concentration between samples from the same compounding pharmacy.

CONCLUSIONS AND RELEVANCE Test results from intravitreal preparations of bevacizumab acquired from compounding pharmacies were negative for microbial contaminants and endotoxin. However, there were significant variations in protein concentration that appear in general to be lower than bevacizumab acquired directly from Genentech. The clinical implications of these variable protein levels remain uncertain.

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In an era of increasing health care costs and quality-control mandates, compounding pharmacies have been under intense scrutiny. The health care system, including ophthalmologists, relies on compounded drug formulations particularly intravitreal bevacizumab (Avastin; Genentech/Roche) for the treatment of a variety of choroidal and retinal vascular diseases. Clinical trials, such as the Comparison of AMD Treatments Trials[1] and the Inhibition of Vascular Endothelial Growth Factor (VEGF) in Age-related choroidal Neovascularization trial,[2] support the effectiveness of intravitreal bevacizumab for the treatment of age-related macular degeneration. While both bevacizumab and ranibizumab (Lucentis; Genentech/Roche) have been found to have similar safety and efficacy,[1,2] substantial cost savings may be derived from using bevacizumab, which is reimbursed approximately $50 from Medicare in comparison with nearly $2000 for ranibizumab.[3] Indeed, the researchers of the Inhibition of Vascular Endothelial Growth Factor (VEGF) in Age-related choroidal Neovascularization trial estimated that the National Health Service in the United Kingdom could potentially save an estimated £84.5 million (US $140.9 million) per year by switching from ranibizumab to bevacizumab and administering medication on an as-needed basis.

Compounding pharmacies prepare intravitreal bevacizumab for clinical use but the quality, consistency, and safety

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of compounding pharmacies have been questioned. Although the complication rate due to improper formulation is low, several incidents nationwide, involving not only intravitreal drugs but also other compounded medications including steroids for spinal injection, have brought increased public scrutiny on compounding pharmacies. In July 2013, the US Senate Committee on Health, Education, Labor, and Pensions introduced a bill (S. 959) that tightens regulations on compounding pharmacies through increased oversight from the Food and Drug Administration. Furthermore, on November 27, 2013, President Obama signed the Drug Quality and Security Act, legislation that contains provisions relating to the oversight of the compounding of human drugs.[4]

Although compounding pharmacies are required to adhere to US Pharmacopoeia Chapter 797 requirements to ensure quality control, the exact magnitude and scope of microbial contamination or potential variations in drug levels remain unknown. In a cost-conscious health care environment, the demand for high-quality safe drugs from compounding pharmacies has never been higher. Inspired to take a data-driven approach to surveying the compounding pharmacy landscape, the current study evaluated bevacizumab prepared for intravitreal injection from 11 locations throughout the United States using in vitro methods including microbiological surveillance cultures, endotoxin levels, and protein quantification.

Methods

Drug Acquisition

Three syringes of bevacizumab were acquired from each of 11 different compounding pharmacies in the United States in 1.25-mg/0.05 mL or 2.5-mg/0.10 mL syringes. Syringes were capped with either a 30-gauge needle or rubber stopper on receipt from the compounding pharmacy. Two samples from each pharmacy were used for protein testing. The remaining syringe was used to conduct surveillance cultures and endotoxin testing. All samples arrived within a 10-day interval at Weill Cornell Medical College and were analyzed by our laboratory within 30 days of receipt. All samples were analyzed before their indicated expiry date (which ranged from 30 to 90 days, depending on the specific compounding pharmacy). The identity of all samples was blinded for each assay and coded by number from 1 to 22.

Testing for microbial contamination was conducted at the Clinical Microbiology Laboratories of New York-Presbyterian Hospital, New York. All other analyses were carried out at the clinical manufacturing facility of the Belfer Gene Therapy Core Facility, Department of Genetic Medicine, Weill Cornell Medical College, New York, New York. This study was exempt from institutional review board approval at Weill Cornell Medical College.

Polyacrylamide Gel Electrophoresis

Bevacizumab samples were separated on 4% to 12% Tris-glycine gel (Invitrogen) using 1X Tris-glycine sodium dodecyl sulfate/sulfate running buffer (Invitrogen). One microgram of each sample was prepared in 1X phosphate-buffered saline (PBS) with 10X NuPage (Invitrogen) reducing agent and 2X Tris-glycine loading buffer. Samples were heated to 75°C for 5 minutes before loading into the gel. Gels were run for 100 minutes at 125V and then washed with distilled water at room temperature on a rocker. This was repeated twice, changing the distilled water every 5 minutes. Gels were stained with Coomassie blue (Bio-Safe) for 1 hour and destained for 2 hours.

Western Analysis

One microgram of each bevacizumab sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as just described. Gels were transferred via iBlot 7-minute Blot (Invitrogen) onto polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat milk for 1 hour on a rocker at room temperature and then stained with antihuman kappa light-chain horseradish peroxidase (HRP) conjugated at 1:5000 (A7164, Sigma-Aldrich) and antihuman IgG HRP conjugated at 1:1000 (sc-2453, Santa Cruz Biotechnology) in 5% nonfat milk overnight at 4°C. Membranes were washed with 1X PBS-Tween 4 times. Membranes were probed with Amersham ECL detection reagents (GE Healthcare) for 1 minute before a 2-second exposure.

Protein Quantification via Bicinchoninic Acid Assay

Total protein content was determined by bicinchoninic acid method (Micro BCA Protein Assay Kit; Pierce Biotechnology). Bevacizumab samples were diluted 1:400 in 1X PBS into a 96-well enzyme-linked immunosorbent assay (ELISA) plate in quadruplicates. Serial dilutions of bevacizumab (Genentech, 125-16 mg/mL) served as the standard curve. The plate was incubated at 37°C for 30 minutes and then cooled to room temperature. The absorbance at 595 nm was read in a Benchmark microplate reader (Bio-Rad). Bevacizumab protein concentration is reported as the average of quadruplicate assays against the Genentech-acquired bevacizumab standard curve.

Enzyme-Linked Immunosorbent Assay

Ninety-six-well microplates were coated with recombinant human VEGF 165 (0.02 µg/well; R&D Systems) and carbonate buffer (100 µL/well; Sigma-Aldrich) and stored overnight at 4°C. Microplates were washed 3 times with 1X PBS and then blocked with 200 µL/well of 5% bovine serum albumin (BSA) for 1 hour at 23°C. Microplates were then washed 3 times with 1X PBS-Tween. Bevacizumab samples were diluted 1:100 000 in 1% BSA for the initial well followed by 2-fold dilutions in 1% BSA for a final volume of 100 µL/well. Following 1 hour incubation at room temperature and 3 washes with 1X PBS-Tween, 100 µL/well with antihuman kappa light chain HRP conjugated at 1:5000 dilution in 1% BSA was incubated at 23°C for 1 hour. Following 3 washes, 100 µL of HRP substrate was added to each well and incubated in the dark for 15 minutes at 23°C. The reaction was stopped by the addition of 200 µL/well of 2% oxalic acid. The absorbance at 415 nm was measured with a Benchmark microplate reader (Bio-Rad). Bevacizumab content was reported as the average of duplicate assays.

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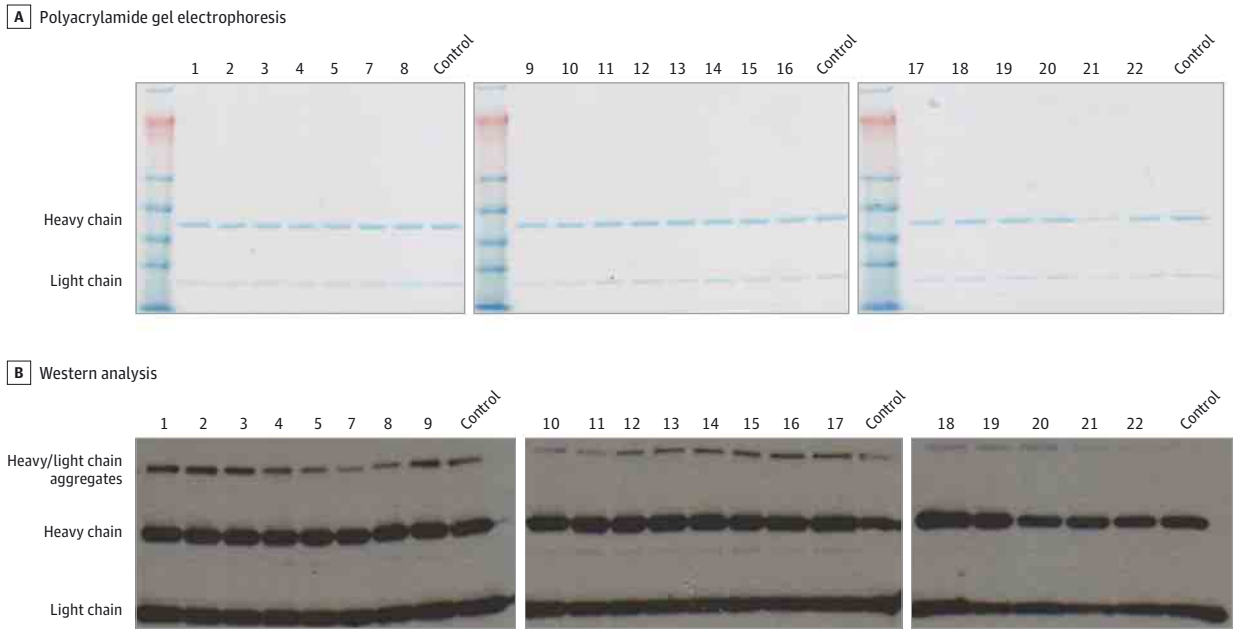
Figure 1. Polyacrylamide Gel Electrophoresis and Western Analysis of Compounded Intravitreal Bevacizumab



A, Polyacrylamide gel electrophoresis of bevacizumab samples stained with Coomassie blue alongside Genentech bevacizumab control. B, Western analysis of bevacizumab samples stained alongside control representing bevacizumab acquired directly from Genentech. Secondary antibodies include antihuman kappa light chain horseradish peroxidase conjugated and anti-human IgG horseradish peroxidase conjugated.

Cultures

All specimens were cultured on Trypticase Soy Agar II with 5% Sheep Blood (Becton Dickinson) incubated at 35°C with 5% carbon dioxide and examined at 24 and 48 hours by a single reviewer for visual signs of growth.

Endotoxin Assay

Endotoxin analysis was performed at 1:100 dilutions on an Endoscan V (Charles River).

Dynamic Light Scattering

Samples of 5 µL were analyzed in a quartz cuvette on the Wyatt Nanostar (Wyatt Technology) to assess for protein aggregation. The intensity of scattered light from macromolecular particles undergoing Brownian motion provides a measure of the distribution of monomers and aggregates in solution. The results are provided as the relative intensity vs radius.

Statistical Analysis

SPSS version 21 (IBM Corp) was used for statistical analysis. To compare the average protein concentration of each compounding pharmacy sample with the control bevacizumab from Genentech, a 1-sample, 2-tailed *t* test was performed using a control protein concentration of 25 mg/mL. To compare the protein concentrations of different samples received from the same compounding pharmacies, a 2-sample, 2-tailed *t* test was performed assuming either equal variance or unequal variance as determined by a Levene test of homogeneity of variance.

Results

With the exception of 1 syringe (sample 6), all of the samples were analyzed. The syringe containing sample 6 was provided with the plunger drawn up to 0.05 mL but was found to contain no medication on receipt from the compounding pharmacy. In all the samples tested, no microbial contamination was noted on culture, and endotoxin was less than 0.05 EU/dose.

To confirm the purity of the compounding pharmacy samples, we assessed them by polyacrylamide gel electrophoresis and stained with Coomassie blue (**Figure 1**A). Bands representing the heavy chain and light chain of the bevacizumab protein were visible at the expected molecular weight for each sample and matched the results for the control bevacizumab from Genentech. There were no obvious signs of protein contamination or degradation.

To confirm the identity of each sample as an immunoglobulin, the samples were subjected to Western analysis using secondary antibodies for human kappa light chain and antihuman IgG (Figure 1B). Each sample revealed bands representing the heavy and light chains of the bevacizumab antibody, and all the samples contained a band representing heavy/light chain aggregates to varying degrees. This result was consistent with that found in the bevacizumab control.

The total protein concentration was quantified using the bicinchoninic acid assay. Samples from the compound-

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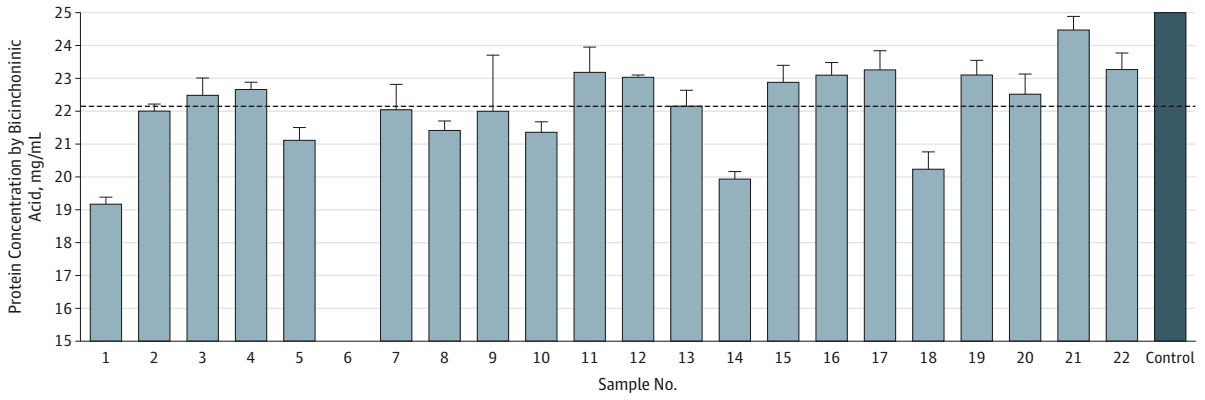
Figure 2. Protein Quantification of Compounded Intravitreal Bevacizumab by Bicinchoninic Acid Assay



Protein quantification by bicinchoninic acid assay alongside Genentech bevacizumab control. The black dotted line represents the mean (SD) concentration of compounding pharmacy samples (22.2 [4.9] mg/mL; range, 19.2-24.5 mg/mL). Sample 6 did not contain drug and is excluded from the mean concentration. The error bars represent standard error of the mean for each quadruplicate sample.

Table. Statistical Analysis of Compounded Intravitreal Bevacizumab as Quantified by a Bicinchoninic Acid Assay[a]

Sample	Average Protein Concentration, mg/mL	*P* Value	
		Comparison With Control	Comparison With Other Sample From Same Pharmacy
1	19.2	<.001[b]	<.001[b]
2	22.0	.001[b]	
3	22.5	.02[b]	.77
4	22.6	.003[b]	
5	21.1	.002[b]	NA
6	NA	NA	
7	22.0	.03[b]	.497
8	21.4	.001[b]	
9	22.0	.18	.74
10	21.3	.002[b]	
11	23.2	.10	.86
12	23.0	<.001[b]	
13	22.1	.01[b]	.007[b]
14	19.9	<.001[b]	
15	22.9	.03[b]	.71
16	23.1	.02[b]	
17	23.3	.06	.008[b]
18	20.2	.003[b]	
19	23.1	.02[b]	.46
20	22.5	.03[b]	
21	24.5	.28	.10
22	23.3	.04[b]	

Abbreviation: NA, not available.

[a] Comparison of protein concentrations as measured by the bicinchoninic acid assay. In the comparison of the average protein concentration from each compounding pharmacy and the control bevacizumab from Genentech, a 1-sample, 2-tailed *t* test was performed assuming a control protein concentration of 25 mg/mL. To compare the protein concentrations of different samples received from the same compounding pharmacies, a 2-sample, 2-tailed *t* test was performed assuming either equal variance or unequal variance as determined by a Levene test of homogeneity of variance.

[b] $P < .05$.

ing pharmacies had a mean (SD) concentration of 22.2 (4.9) mg/mL (range, 19.2 -24.5 mg/mL) as shown in **Figure 2**. All samples were lower in protein concentration, with 17 of 21 significantly reduced in comparison with the bevacizumab control from Genentech ($P < .05$; **Table**). To measure variation within each compounding pharmacy, we also compared the protein concentrations of the paired samples received from the same pharmacy. Three of 10 compounding pharmacies had statically significant differences in the protein concentrations between the 2 samples provided ($P < .05$; Table).

As a measure of active protein, the binding affinity to VEGF by ELISA as a quantitative surrogate was tested. In comparison with a standard curve generated with the bevacizumab ac-

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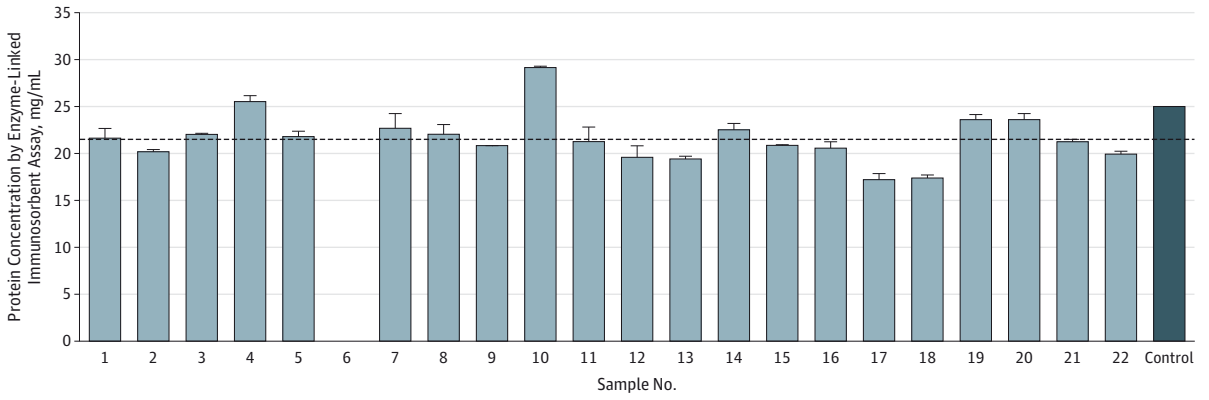
Figure 3. Protein Quantification of Compounded Intravitreal Bevacizumab by Enzyme-Linked Immunosorbent Assay



Protein quantification by enzyme-linked immunosorbent assay alongside Genentech bevacizumab control. The black dotted line represents the mean (SD) concentration of compounding pharmacy samples (21.6 [5.2] mg/mL; range, 17.2-29.1 mg/mL). Sample 6 did not contain drug and is excluded from the mean concentration. The error bars represent standard error of the mean for each quadruplicate sample.

quired from Genentech, compounded samples revealed a mean (SD) protein concentration of 21.6 (5.2) mg/mL (range, 17.2-29.1 mg/mL), with 19 of 21 samples having protein concentrations lower than the control (**Figure 3**).

Evaluation of the distribution of macromolecular size in the bevacizumab samples from each source by dynamic light scattering demonstrated that all sources, except that acquired directly from Genentech, had substantial evidence of aggregation (**Figure 4**). The relative intensity of scattered light corresponding to the hydrodynamic radius for the compounded bevacizumab (representative sample shown in Figure 4A) revealed a broader range of dynamic light scattering peak intensity for larger size aggregates, while the Genentech sample was narrowly distributed (Figure 4B).

Discussion

The medical community previously brought attention to deficiencies in the bevacizumab compounding process for intravitreal injection and their association with infectious endophthalmitis, as highlighted by several outbreaks nationwide.[5] Endophthalmitis clusters have been traced to unsafe practices at multiple compounding pharmacies throughout the United States including in Nashville, Tennessee; Minneapolis, Minnesota; Los Angeles, California; Augusta, Georgia[6]; and Miami, Florida.[5] One incident resulted in 12 cases of endophthalmitis and a Food and Drug Administration warning concerning drugs compounded by a pharmacy in Hollywood, Florida.[5,7] There have also been reports of endophthalmitis outbreaks outside the United States including in Japan,[8,9] South Korea,[10] and Germany.[11] Problems with the preparation of bevacizumab have not only been related to bacterial contamination and infection, but also sterile endophthalmitis caused by excess silicone oil residue and particulates.[12,13] In response to these events, retinal specialists have endorsed implementation and strict adherence to the US Pharmacopoeia Chapter 797 requirements as a means of reducing the chance of future outbreaks.[5] Other investigators[14] have called for a data-driven approach to analyzing the potential risks associated with drug delivery by compounding pharmacies.

Proponents of compounding pharmacies reference a seminal study examining the incidence of endophthalmitis in compounded bevacizumab and direct-from-manufacturer ranibizumab that found no significant difference between the 2 drugs[15] and the low rate of bevacizumab-associated endophthalmitis in the Comparison of AMD Treatments Trials.[1] Indeed, to our knowledge, no case series subsequent to this study have detected an increased risk for endophthalmitis with bevacizumab in comparison with ranibizumab. Yet, the bevacizumab used in many of these series was from compounding pharmacies that may not have been representative of the standard national product.

Over the past 2 years since the most recent review on the compounding controversy,[5] there have been several reports of compounding-related issues. Eight patients were diagnosed as having fungal endophthalmitis after receiving an intravitreal injection of combined bevacizumab-triamcinolone produced by a compounding pharmacy in New York.[16] In another incident, an injection of counterfeit bevacizumab in Shanghai, China, led to acute intraocular inflammation caused by endotoxin.[17]

With the spotlight on endophthalmitis and sterile compounding practices, it was reassuring to find that in our analysis of syringes from 11 compounding pharmacies, there were no microbial contaminants noted in any of the samples when cultured by our clinical microbiology laboratory. Moreover, none of the samples had significantly elevated endotoxin levels. Although there were no signs of bacterial contamination in this survey, given the low rate of infectious endophthalmitis after intravitreal bevacizumab, our relatively small sampling is likely underpowered to detect biologically viable contaminants, even if they were present at any of these pharmacies.

Unsterile practices have not been the only concern surrounding compounding pharmacies. Other literature has fo-

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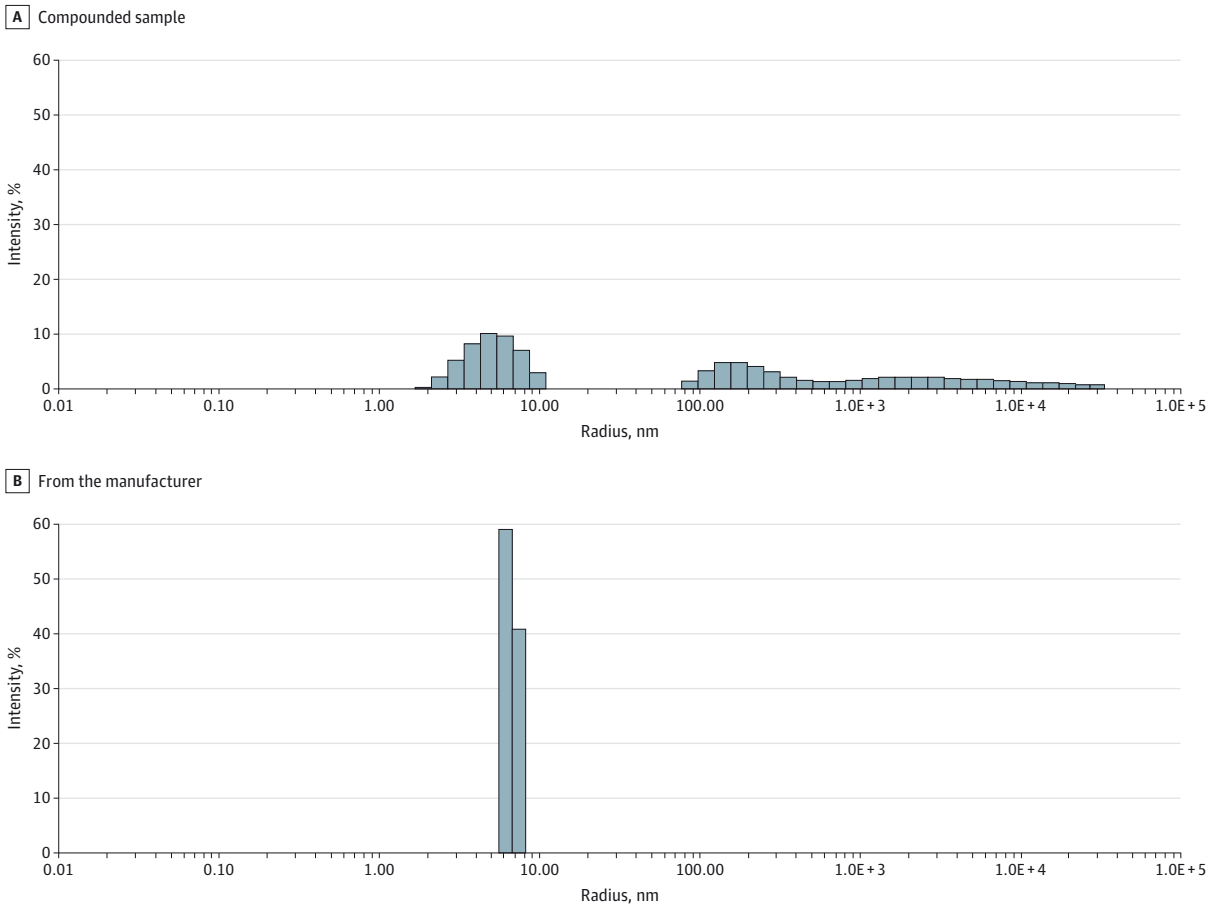
Figure 4. Distribution of Particle Size of Compounded and Direct-From-Manufacturer Bevacizumab



Dynamic scattered light data were evaluated by autocorrelation to plot the intensity vs hydrodynamic radius. Depicted is the relative intensity for a representative compounded bevacizumab sample (A) and the direct-from-manufacturer bevacizumab (B).

cused on variations in the quantity, quality, and stability of compounded drugs. A study analyzed the quality and stability of repackaged bevacizumab for intravitreal injection from 5 licensed compounding pharmacies in the United Kingdom and compared it with bevacizumab in its original glass vial.[18] A significant difference in the distribution of particle density was found between bevacizumab batches from the 5 suppliers, which increased as the samples were stored. Protein concentration, IgG content, and molecular weight were comparable between all the batches acquired from the compounders and the bevacizumab acquired directly from the manufacturer. However, particle size distribution was found to fall outside of guidelines set forth by the US Pharmacopeia for injectable ophthalmic solutions.

A separate study used an ELISA, size exclusion chromatography, polyacrylamide gel electrophoresis, and microflow imaging to analyze repackaged bevacizumab from 3 different compounding pharmacies in the United States and compared it with bevacizumab obtained directly from Genentech.[19] Analysis of the drugs revealed that 2 of the compounding pharmacies' batches had significantly less functional IgG in solution. Those pharmacies with the lowest levels of IgG also had 10-fold the number of micron-sized particulate matter on microflow imaging and this large particulate matter was hypothesized to lead to obstruction of aqueous outflow and pressure elevations after intravitreal injection.

In this study, we detected significant variation in the concentration and ELISA titer of the drugs we acquired. Furthermore, we found that compounded formulations had significantly lower drug concentrations than drug acquired directly from the manufacturer. We also detected a greater degree of protein aggregation in the compounded samples in comparison with the Genentech sample, which exhibited a profile consistent with previous findings.[20]

Although no protein or microbial contaminants were noted in any of the samples, this variability in drug concentration may have important clinical implications. It may also have ethical implications for ophthalmologists and patients who deserve medications that adhere to a minimum quality control as set forth for other Food and Drug Administration–approved drugs.

Filtration or packaging methods during compounding may be responsible for the variation in protein concentration. It is hypothesized that the use of a polymer stop-cock device to ex-

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tract bevacizumab from the original vial may result in loss of protein owing to drug adherence to the polymer as the 0.05- to 0.01-mL aliquots are prepared. Compounding pharmacies may not be using low protein binding 0.20- or 0.22-µm sterilization filters, leading to a reduction of active ingredient concentration. The amount of active drug lost during these types of processes is unknown.[5]

Additionally, certain syringe types may be made from materials that have an affinity for the bevacizumab protein, lowering the concentration of drug in each prepared dose. It has previously been shown that repacking bevacizumab in plastic syringes can result in protein aggregation and contamination with silicone oil microdroplets.[21] Aggregation in compounded protein products has been linked to steps in dispensing and storing in syringes, shipping, and delivery.[22,23] Proteins may also adhere to glass syringes where silicone oil is used to lubricate the surface, rubber from stoppers, and stainless steel from filling pumps.[19] These factors may contribute both to the general trend of lower drug levels in compounded samples and the substantial variability among samples.

The ELISA data in our study also demonstrated decreased titers for binding to VEGF, which may not only be due to decreased protein amounts, but also functional degradation. Compounded drug may be stored in suboptimal conditions, affecting protein quality and variability from sample to sample. The propensity for bevacizumab's anti-VEGF activity to degrade over time, even with storage in freezing or 4°C conditions, has been shown.[24] Despite the mechanism, the unreliability of protein concentrations and drug activity levels in these samples may have clinical implications.

The limitations of this study include the relatively small number of compounding pharmacies from which samples were obtained, which may limit the power to robustly detect microbial contamination. Likewise, given the limited volume of sample from each compounding pharmacy, we were unable to test for silicone oil contamination. Most importantly, our study provided in vitro analysis of compounded bevacizumab for intravitreal injection; however, it was not designed to investigate the real-life clinical implications of this variability for the treatment of vitreoretinal disorders. Nevertheless, among currently published studies, to our knowledge, this study contains one of the larger conglomerates of samples from different compounding pharmacies to date.

Conclusions

This study demonstrated significant variability in the protein concentration of bevacizumab prepared for intravitreal injection from compounding pharmacies in the United States. There was no evidence of endotoxin or microbial contamination in any of the samples tested. The clinical implications of the variability with regard to efficacy of compounded product destined for intravitreal administration in the treatment of vitreoretinal diseases remain uncertain and would be of interest in future investigations. However, the in vitro variability deserves attention as patients, governments, and physicians select from a variety of anti-VEGF agents available to treat retinal disease.

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Study concept and design: Yannuzzi, Klufas, Kaminsky, Crystal, D'Amico, Kiss.
Acquisition, analysis, or interpretation of data: Yannuzzi, Klufas, Quach, Beatty, Kaminsky, Crystal, Kiss.
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Critical revision of the manuscript for important intellectual content: Yannuzzi, Klufas, Beatty, Kaminsky, Crystal, D'Amico, Kiss.
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OPHTHALMIC IMAGES

Conjunctival Rhinosporidiosis

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A, Clinical photograph showing a pedunculated mass attached to the palpebral conjunctiva with white dots studding the surface. B, Histology shows sporangia (arrowhead) visible in the subepithelial stroma with surrounding inflammatory infiltrate (asterisk) (hematoxylin-eosin; original magnification ×200).

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