

SIX-MONTH STABILITY OF BEVACIZUMAB (AVASTIN) BINDING TO VASCULAR ENDOTHELIAL GROWTH FACTOR AFTER WITHDRAWAL INTO A SYRINGE AND REFRIGERATION OR FREEZING

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Purpose: To determine the change in anti-vascular endothelial growth factor (VEGF) activity of bevacizumab (Avastin, Genentech, Inc., San Francisco, CA) after refrigeration or freezing.

Methods: Samples of bevacizumab were drawn up from new vials into plastic tuberculin syringes and refrigerated at 4°C for 1 week, 3 weeks, 1 month, 3 months, and 6 months. The vials and syringes were stored at 4°C, and the syringes were capped with a needle. One syringe was frozen at -10°C. The bevacizumab concentration was measured, via its binding to VEGF-165.

Results: The percentage of degradation of bevacizumab in the previously pierced vials stored at 4°C compared with that in the unpierced vial was 9.6% at 3 months and 12.7% at 6 months. The bevacizumab drawn into the syringe and stored at 4°C was degraded by 1.6% at 1 week, 0% at 3 weeks, 8.8% at 3 months, and 15.9% at 6 months. The bevacizumab frozen in a syringe at -10°C was degraded by 12.0% at 6 months.

Conclusion: The anti-VEGF activity of bevacizumab may degrade minimally over time, with storage.

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Bevacizumab (Avastin, Genentech, Inc., San Francisco, CA) is a recombinant humanized monoclonal IgG1 antibody that inhibits human vascular endo-

thelial growth factor (VEGF). The drug is approved by the US Food and Drug Administration for intravenous use in combination with 5-fluorouracil-based chemotherapy for metastatic colorectal cancer. It has been administered intravitreally in VEGF-mediated diseases, such as choroidal neovascularization,^{1,2} central retinal vein occlusion,³ proliferative diabetic retinopathy,⁴ and other retinal diseases.

Bevacizumab is a clear to slightly opalescent, colorless to pale brown solution with a pH of 6.2. It is supplied in 100-mg and 400-mg preservative-free, sin-

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gle-use vials to deliver 4 mL or 16 mL of bevacizumab (25 mg/mL). It is formulated in α,α -trehalose dihydrate, sodium phosphate (monobasic, monohydrate), sodium phosphate (dibasic, anhydrous), polysorbate 20, and water for injection. The most commonly used dose for intravitreal injection is currently 1.25 mg (0.05 mL) in the United States, although up to 2.50 mg (0.1 mL) may be used. Because such a small amount of drug from a large vial is required to treat ocular disease, ophthalmologists have been obtaining the drug from compounding pharmacies that fractionate the vial into smaller amounts, at a lower cost. The stability and anti-VEGF activity of bevacizumab that was drawn up from the vial and refrigerated or frozen for later use is currently not known. The manufacturer's guidelines state that bevacizumab vials must be refrigerated at 2°C to 8°C (36°F to 46°F), should be protected from light, and should be stored in the original carton until time of use. The manufacturer recommends that bevacizumab is used within 8 hours of being opened when diluted for intravenous administration and that it should be neither frozen nor shaken.⁵

The purpose of this study was to test the stability and anti-VEGF activity of bevacizumab that was previously withdrawn from a vial and either refrigerated or frozen up to 6 months.

Methods

Syringes of bevacizumab were drawn up from new vials and refrigerated at 4°C for 1 week, 3 weeks, 1 month, 3 months, and 6 months. The drug was drawn into a 1-mL tuberculin syringe (latex-free syringe, Becton Dickinson & Company, Franklin Lakes, NJ) using a 20-gauge needle (B-D Precision Glide needle, Becton Dickinson & Company). Both the previously pierced vials and their corresponding syringes were stored at 4°C in the tuberculin syringe capped with a 30-gauge needle (B-D Precision Glide needle, Becton Dickinson & Company). In addition, one syringe was frozen at -10°C and capped with a needle. The stored samples as well as a new previously unpierced vial were then analyzed. The new vial was from the same shipment and had been stored under the same conditions recommended by the manufacturer (refrigerated at 4°C). An immunoassay was used to measure the concentration of bevacizumab, via its binding to VEGF-165. All samples were run in duplicate on each individual assay, and each assay was repeated a minimum of two times.

Assay

The 165 amino acid variant of human recombinant VEGF (R&D Systems, Minneapolis, MN), which was

synthesized in Sf21 insect cells using a baculovirus expression system, was immobilized on Microlite 2 (Thermo Labsystems, Franklin, MA) high-binding plates designed for luminescent detection. The VEGF was diluted to a concentration of 1.0 $\mu\text{g/mL}$ in a 50 mmol/L carbonate buffer, pH 9, and then aliquoted onto the Microlite plates at 100 μL per well. After an overnight incubation at 4°C, the plates were washed three times with 1× phosphate-buffered saline (PBS) on an EL50 AutoStrip Washer (Bio-Tek Instruments, Inc., Winooski, VT) and then blocked for 4 hours at 4°C with 1% bovine serum albumin in 1× PBS. After washing three times with 1× PBS on the AutoStrip Washer, the plates were stored dry at 4°C.

Samples to be assayed were diluted so as to be within the linear range of the assay. Samples were diluted in 0.1% bovine serum albumin and 1× PBS, aliquoted onto a VEGF plate at 100 μL per well, and then incubated for 3 hours at room temperature with agitation. For each individual assay, a standard curve was included using bevacizumab of known concentrations ranging from 4 ng/mL to 0.0625 ng/mL. After the initial incubation, the plates were washed three times with 0.05% Tween-20 in 1× PBS on the AutoStrip Washer. The bound bevacizumab was detected with an antihuman IgG antibody (Sigma, St. Louis, MO) labeled with acridinium ester. The labeled antihuman IgG was diluted in 100 mmol/L phosphate buffer (pH 6.0), 150 mmol/L NaCl, 0.4% bovine serum albumin, and 0.05% Tween-20 to the dilution at which a 10- μL sample produced a chemiluminescent signal of 5×10^5 relative light units. The diluted labeled antihuman IgG was aliquoted onto the VEGF plate at 100 μL per well, incubated on the VEGF plate for 1 hour at room temperature with agitation, and then washed 3 times with 1× PBS and 0.05% Tween-20 on the AutoStrip Washer. The amount of chemiluminescence produced in each well was then detected on a Centro LB 960 Luminometer (Berthold Technologies, Oak Ridge, TN). The luminescent signal was initiated by injecting 30 μL of hydrogen peroxide and 30 μL of cetyltrimethylammonium chloride (Nichols Institute Diagnostics, San Clemente, CA) into each well. The luminescent signal was acquired immediately after injection for a total time of 1.0 second.

The concentration of bevacizumab in the stability samples was calculated from the standard curve. The standard curve relates the known concentration of bevacizumab samples with the chemiluminescent signal, in relative light units, of these known samples. The equation of the line from the standard curve is calculated by linear regression. This line equation was

Table 1. Mean Concentration of Bevacizumab, Standard Deviation, and Coefficient of Variation for Each Sample

Sample of Bevacizumab	Mean Bevacizumab Concentration (mg/mL)	Standard Deviation (SD)	Coefficient of Variation (%)	Degradation of Bevacizumab (%)
New vial	25.1	2.5	10	0.0
1-week syringe, 4°C	24.7	2.3	10	1.6
3-week syringe, 4°C	25.5	2.6	10	0.0
1-month syringe, 4°C	23.1	1.9	8	8.0
3-month syringe, 4°C	22.9	2.0	9	8.8
3-month vial, 4°C	22.7	2.6	11	9.6
6-month syringe, 4°C	21.1	1.0	5	15.9
6-month vial, 4°C	21.9	1.2	5	12.7
6-month vial, -10°C	22.1	2.2	10	12.0

The degradation of bevacizumab as compared with that in the new unpierced vial is shown.

then used to calculate the concentration of bevacizumab in the stability samples based on the relative light units produced by these samples. A new vial of bevacizumab was used as the control for each of the assays.

Each sample was included in at least two separate assays, with each assay using a separate VEGF plate and standard curve. The individual assays included each stability sample at several dilutions, with each dilution present in duplicate. There were between 5 and 11 measurements included in the standard deviation (SD) calculations for each sample.

Results

Table 1 shows the concentration of bevacizumab (mg/mL) in each sample, along with the SD and coefficient of variation. The highest concentration of bevacizumab was found in the new, previously unpierced vial. The concentration of bevacizumab decreased progressively in the 1-week, 3-week,

1-month, 3-month, and 6-month samples stored at 4°C and -10°C (Fig. 1). The percentage of degradation of bevacizumab in the previously pierced vials stored at 4°C compared with that in the unpierced vial was 9.6% at 3 months and 12.7% at 6 months. The bevacizumab drawn into the syringe and stored at 4°C was degraded by 1.6% at 1 week, 0 at 3 weeks, 8.8% at 3 months, and 15.9% at 6 months. The bevacizumab frozen in a syringe at -10°C was degraded by 12.0% at 6 months.

The SDs for this assay are listed in Table 1. The SD in our measurements reflects the random error in the laboratory assay. This SD can be attributed to various causes, including differences between VEGF plates and pipetting errors. The measurements for two of the 6-month stability samples have slightly lower SDs; however, this probably is coincidental. There is nothing inherent in the assay that would account for a decrease in SD.

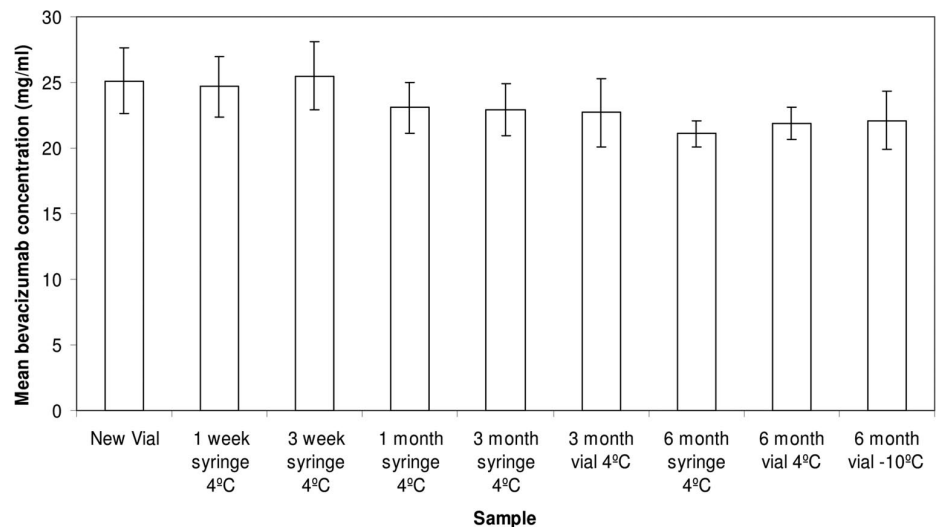


Fig. 1. Bar chart showing the mean concentration of bevacizumab (mg/mL) for each sample. Standard error (SE) bars are included. The bevacizumab progressively degraded as the length of storage increased.

Discussion

Current reports of the use of intravitreal bevacizumab are limited to case series and anecdotal reports, and the optimum dose for each disease has not been established.

The average wholesale price of a 100-mg (4-mL) vial is \$687.50, but most pharmacies obtain it for \$550 for 100 mg. Dividing a \$550 vial into 40 samples of 0.1 mL each (i.e., 0.05 mL for intravitreal injection and 0.05 mL for waste) would provide a dose of bevacizumab at a cost of \$13.75. The question as to how long the withdrawn samples can be stored in a syringe, without affecting the stability of the product, is relevant to our clinical practices. In addition, as clinical trials are designed, it is important to maintain consistency between samples of bevacizumab, to assess its efficacy and establish the optimum dose for each disease. We have shown that there is minimal change in the concentration of bevacizumab in the samples at 3 months and minimal further change from 3 months to 6 months. Given the potential error in accurately dosing 0.05 mL of drug in a clinic setting, a <10% change in drug concentration at 3 months is insignificant. It is interesting to note that at 3 months and 6 months, the bevacizumab in both the vial and stored syringes degrades minimally. Many drugs (e.g., pegaptanib sodium [Macugen[®], (OSI) Eyetech/Pfizer, San Dimas, CA]) are stored under nitrogen to provide an inert atmosphere that may prevent drug degradation. There was no information provided in the package insert for bevacizumab or on the US Food and Drug Administration's Web site regarding whether

bevacizumab vials contain nitrogen. If there is nitrogen in an unpierced bevacizumab vial, this could explain why piercing the vial and injecting air into it may allow the bevacizumab in the vial to minimally degrade, as it would when stored in a syringe. As controls, we did not have unpierced vials that were stored in the same refrigerator for the same length of time, but we did use an unpierced new vial from the same shipment as the control.

We have shown that bevacizumab can be withdrawn into a syringe and stored for 6 months, with a maximum degradation of anti-VEGF activity of 15.9%.

Key words: activity, assay, bevacizumab (Avastin), biologic, choroidal neovascularization, concentration, frozen, immunoassay, refrigerated, stability, syringe, vascular endothelial growth factor, vial.

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