# Stability of Bevacizumab Divided in Multiple Doses for Intravitreal Injection

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***Objective:*** *To investigate the stability of bevacizumab in multiple doses divided from a single-use vial for intravitreal injection after storage at 4°C for up to six months and under drug transfer condition in tropical climate.*
***Material and Method:*** *Five syringes (0.1 mL, 2.5 mg) of bevacizumab were withdrawn each from five new bevacizumab single-use vials (4 mL, 100 mg) under sterile technique. The concentration of bevacizumab in each syringe was measured at two dilutions ($2x10^6$ and $4x10^6$ fold) using enzyme-linked immunosorbent assay at baseline and after storage at 4°C for 1-, 3-, and 6-month. Each assay was performed at least twice. To simulate the drug transfer condition, bevacizumab was placed in a brown plastic bag and put in another transfer plastic bag with an ice cube for 30 minutes prior to the assay at 1-, 3-, and 6-month.*
***Results:*** *The concentrations of bevacizumab (mean ± standard deviation) at baseline, 1-, 3-, and 6-month were 26.24±1.95, 25.43±3.80, 27.87±2.81, and 24.25±2.00 mg/mL, respectively. The lowest lower limit of 95% confidence interval for the mean concentration was 23.32 mg/mL at 6-month storage, which was 89% of the mean baseline concentration and considered to be non-inferior to the baseline concentration.*
***Conclusion:*** *Bevacizumab in a single-use vial could be divided into multiple small doses for intravitreal injection with sufficient stability when refrigerated at 4°C for up to six months and under the drug transfer condition in tropical climate.*

***Keywords:*** *Bevacizumab, Intravitreal injection, Stability*

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Bevacizumab (Avastin®) is a recombinant humanized monoclonal antibody that binds to all isoforms of vascular endothelial growth factor (VEGF) [1] resulting in inhibition of angiogenesis. This anti-angiogenic effect of bevacizumab has been initially employed for the treatment of metastatic colorectal cancer[2], for which it is approved by the United States Food and Drug Administration. VEGF is also well known to mediate the pathogenesis of many ocular diseases such as diabetic retinopathy and age-related macular degeneration. As a result, despite being an off-label use, bevacizumab had been administered intravitreally in those VEGF-mediated ocular diseases worldwide with encouraging results[3-6].

The treatment of colorectal cancer requires about 5 mg/kg of bevacizumab per dose intravenously[2] but intravitreal injection of bevacizumab for ocular diseases requires only 1.25 to 2.5 mg of the drug per one injection[5,7]. For the purpose of cost-saving, a single-use vial of Avastin®, which contains 100 mg of bevacizumab in 4 mL solution (25 mg/mL), could be divided and would be adequate for more than 30 intravitreal injections. Nevertheless, the stability of the bevacizumab after dividing from a single-use vial for intravitreal injection is of major concern. The United States study by Bakri et al, 2006 reported that bevacizumab divided into plastic syringes degraded minimally (15.9%) over six months of storage at 4°C without drug transfer conditions[8].

In tropical countries including Thailand, despite widespread use of divided bevacizumab for intravitreal injection, there were no reports regarding the stability of bevacizumab after it was divided from a fresh vial. The stability could be lost during the drug dividing process, drug storage, and drug transfer to patients because of the tropical climate.

The purpose of the present study was to evaluate the stability of bevacizumab divided in multiple doses from a single-use vial, after storage at 4°C for up to six months and under a simulated condition of drug transfer in our clinical settings at Siriraj Hospital.

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## Material and Method

### *Samples*

The study protocol was approved by Siriraj Institutional Review Board (SIRB), Mahidol University in November 2010.

Bevacizumab (Avastin®, Roche, Switzerland) was prepared under aseptic techniques at the Cytotoxic Dispensary Unit, Pharmacy Department, Faculty of Medicine Siriraj Hospital, as routinely used for patients at Siriraj Hospital between November 2010 and June 2011. Sterility test by microbial culture was performed at random as a part of routine safety check of the hospital. From the manufacturer, bevacizumab was a clear to slightly opalescent, colorless to pale brown, sterile liquid. Under a laminar flow cabinet, 0.1 mL of bevacizumab was drawn from a 4-mL vial (25 mg/mL) into a 1-mL sterile plastic syringe (Terumo, Japan) and capped with a sterile syringe cap. The capped syringe was then sealed in a sterile plastic package (Stericlin®, Vereinigte Papierwarenfabriken GmbH, Germany) and ready for intravitreal injection.

Five syringes (sample 1-5) containing 0.1 mL of bevacizumab were prepared for the study, each of which was drawn from a separate vial of bevacizumab. The bevacizumab in each of the syringes was divided and assayed immediately for the baseline concentration. The remaining bevacizumab in each syringe was then stored in multiple single-use aliquots (in 0.5-mL polypropylene microtubes) at 4°C for further stability assays after storage at 1-, 3-, and 6-month.

Prior to each stability assay at one, three, and six months, all the aliquots of bevacizumab were placed in a brown plastic bag (to protect from light) and put in another plastic bag with an ice cube for 30 minutes (an average time required for the transfer of the drug from the pharmacy to the patient). This was done in order to simulate the condition during drug transfer (from the pharmacist to the patient) in our real clinical setting, which might affect the stability of bevacizumab.

### *Determination of bevacizumab concentration*

Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of bevacizumab. The human VEGF-165 isoform (Cell Signalling Technology, Inc., MA, USA) was coated on the plates (Costar® Stripwell™ plates, Corning Incorporated, NY, USA). The VEGF-165 was diluted to a concentration of 1 ug/mL in 50 mmol/L carbonate buffer, pH 9, and aliquoted onto the plates at 100 ul/well. The plates were incubated at 4°C overnight and washed three times with 200 ul/well of phosphate-buffered saline (PBS). The plates were then blocked with 3% bovine serum albumin in PBS 200 ul/well at 37°C for two hours. After washing three times again with 200 ul/well of PBS, the resulting VEGF plates were ready for bevacizumab assays.

The bevacizumab samples were diluted with 0.1% bovine serum albumin in PBS at $2x10^6$-fold and $4x10^6$-fold dilutions in order to be within the linear range of the assay. The assay at each dilution was performed at least twice. The diluted samples were aliquoted onto the VEGF plates at 100 uL/well and incubated at 37°C for 90 minutes. After the incubation, the plates were washed three times with 0.05% Tween-20 in PBS at 200 ul/well. The bound bevacizumab was then detected with peroxidase - o-phynylenediamine substrate system. In detail, the goat anti-human IgG peroxidase conjugate (Sigma-Aldrich, Inc., MO, USA) was diluted at 1:2,000 dilution with PBS and then aliquoted onto the plates at 100 ul/well. After incubation at 37°C for 60 minutes, the plates were washed 3 times with 0.05% Tween-20 in PBS at 200 ul/well. The o-phynylenediamine substrate (Sigma-Aldrich, Inc., MO, USA) was then aliquoted onto the plates at 100 ul/well and incubated at room temperature in the dark for 15 minutes for development of a yellow color. Finally, 2M $H_2SO_4$ was added at 100 uL to each well to stop the color reaction and the optical density was measured at 450 nm using Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek® Instruments, Inc.).

For each plate assay, a standard curve, relating the bevacizumab concentration and optical density, was constructed using a known bevacizumab concentration (ranging from 3.125 to 12.5 ng/mL) diluted from a newly opened vial. One plate assay was performed at each time period, resulting in four plate assays (at baseline, 1-, 3-, and 6-month) during the whole study and four new Avastin® vials were required to construct the standard curves. All of the four vials were from the same lot number to ensure the inter-assay precision. By linear regression, each standard curve was used to calculate the concentration of bevacizumab from the optical density readings in each plate.

### *Statistical analysis*

According to the experimental design, at least 20 samples were assayed at each time point (five bevacizumab vials, each vial in two dilutions ($2x10^6$ and $4x10^6$ fold), each dilution assayed at least twice). The concentrations of bevacizumab of all samples at each time point were analyzed for mean, standard

deviation, coefficient of variation (CV) and 95% confidence interval (CI) for the mean. Each individual sample was stored and measured separately and was considered independent. A standard approach for testing pharmaceutically equivalence employed 90% CI to entirely fall within 80 to 125% of the reference experimental population[9]. Since the present study was a non-inferiority trial, we used 95% CI and applied 80% of the baseline drug concentration as a reference limit for declaring non-inferiority. Therefore, the concentration of bevacizumab after storage was considered 'non-inferior' to the baseline concentration if the lower bound of the 95% CI was above 80% of the mean concentration at baseline. The statistical calculation was performed using SPSS for Windows version 11.5 (SPSS, Inc., Chicago, IL, USA).

## Results

Table 1 showed the concentrations of bevacizumab divided from single-use vials and stored at 4°C for one, three, and six months and under the simulated drug transfer condition. The concentration at each time point was the average of at least 20 measurements from five bevacizumab vials, except for month 3, in which two measurements were invalid and were excluded from the analyses. The CV ranged from 7.43% to 14.96%, indicating acceptable precision of our assays. Furthermore, the CV 7.43% at baseline reflected the homogeneity of the drug concentrations from five different vials at the start of the experiment. The lowest lower limit of 95% CI was 23.32 mg/mL at 6-month storage, which was 89% of the mean baseline concentration. In other words, the degradation of the drug was no more than 11% with 95% confidence. According to our predetermined level of 80%, the remaining concentrations of divided bevacizumab after storage at 4°C for up to six months were non-inferior to the baseline concentration. Fig. 1 depicted a trend of minimal declination of divided bevacizumab with storage time, although variations of the concentrations were observed.

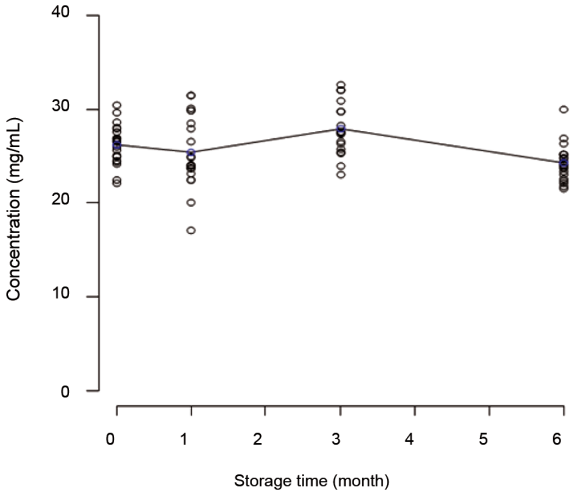


**Fig. 1** Concentrations of divided bevacizumab at baseline (month 0) and after storage at 4°C for 1, 3, and 6 months with the simulated drug transfer condition. The line connects the mean concentration at each time point.

## Discussion

The present study demonstrated that bevacizumab divided in multiple doses from a single-use vial minimally degraded when refrigerated at 4°C for six months. For intravitreal injection, this magnitude of degradation was unlikely to affect the clinical efficacy, given the imprecision of the volume of the drug drawn into the syringe and injected into the vitreous.

In spite of being in a tropical climate, the drug transfer condition as simulated in the present study did not significantly affect the drug stability. The present study was the first to consider the drug transfer condition.

Two previous studies investigated stability of divided bevacizumab at 4°C[8,10]. The study by Bakri et al, 2006[8] reported 15.9% degradation at 6-month while the study by Chen et al, 2009[10] reported 8.4% degradation at 6-month. The authors found that the

**Table 1.** Concentrations of divided bevacizumab at different storage time at 4°C with the simulated drug transfer condition

| Time (month) | n | Concentration (mg/mL) | | | |
|---|---|---|---|---|---|
| | | Mean | SD | 95% CI of mean | Coefficient of variation (%) |
| 0 (baseline) | 25 | 26.24 | 1.95 | 25.44-27.05 | 7.43 |
| 1 | 21 | 25.43 | 3.80 | 23.70-27.16 | 14.96 |
| 3 | 18 | 27.87 | 2.81 | 26.47-29.27 | 10.10 |
| 6 | 20 | 24.25 | 2.00 | 23.32-25.19 | 8.23 |

degradation was no more than 11% (with 95% confidence), which was close to that of the previous studies. Some discrepancies, however, could be due to differences among the studies. The study by Bakri et al, 2006[8] was done outside the tropical climate and the number of bevacizumab vials involved in their study was not mentioned. The study by Chen et al, 2009[10] employed only three vials of bevacizumab for measurement at each time point (compared to five vials in our study). The design of Chen et al[10]'s study was also different from the present study in that they measured bevacizumab stored in previously pierced vials while our study measured bevacizumab withdrawn into syringes and stored separately, as this was our actual clinical situation. Neither of the previous studies investigated the effect of drug transfer conditions.

It was observed that the mean concentration of bevacizumab at 3-month storage was 6.2% higher than the baseline concentration (Fig. 1). The standard deviations at 1-month (3.8 mg/mL) and 3-month (2.8 mg/mL) were wider than at baseline (1.9 mg/mL) and 6-month (2.0 mg/mL). These variations could be explained by the nature of quantitative bioassays regarding such factors as difference between plates, pipetting errors and different lots of reagents in different assays. It might also be caused by inhomogeneous temperature during the transfer condition. Furthermore, according to the Avastin®'s manufacturer, the drug was not to be shaken. Shaking could have occurred during mixing in the assays or unintentionally during drug transfer. This could also be the source of variations of the measured concentrations.

The exact temperature during the drug transfer was not controlled since we intended to reflect the real situation in our clinical setting. This drug transfer condition was simple and could be used in most hospitals. However, we speculated that the transfer condition could be improved so as to keep the optimal temperature for the entire drug syringe and to minimize shaking. This might be accomplished by designing a special container rather than using an ice cube in a plastic bag.

The multiple divided doses of bevacizumab in our hospital were prepared under sterile techniques. Routine sterility tests by microbial culture at random were negative. This was similar to a previous study reporting no microbial contamination for six months of storage at 4°C in three repeatedly withdrawn vials[10]. Nevertheless, sterility should always be of high concern for any intraocular injections.

The results from ELISA were expressed in the form of concentration of bevacizumab. However, ELISA actually measured the antigen-antibody reaction and represented the in vitro bioactivity of the drug. VEGF-165 is the major pathogenic isoform of VEGF in VEGF-mediated ocular diseases[11]. Although bevacizumab can bind to all isoforms of VEGF[1], the present study measured only the activity of bevacizumab in binding to VEGF-165. It was reasonable that the stability of the activity of bevacizumab against VEGF-165 could imply the stability of the whole bevacizumab molecule, and thus, the activity against the other VEGF isoforms.

**Conclusion**

The present study suggested that bevacizumab in single-use vial could be divided into multiple small doses for intravitreal injection, with sufficient stability, when refrigerated at 4°C for up to 6 months. The simulated drug transfer condition in tropical climate did not significantly affect the drug stability. Further in vivo studies would be useful to translate this stability to clinical efficacy and safety. This would reduce the cost of treatment and aid the patients losing vision from VEGF-mediated ocular diseases.

**What is already known on this topic?**

Originally, bevacizumab is used intravenously for the treatment of colorectal cancer. Currently, however, it is accepted worldwide for intravitreal injection to successfully treat many ocular diseases. The dose for intravitreal injection is very small compared with the intravenous dose. The cost of the drug is rather expensive. To reduce the cost of treatment, the drug can be divided from the original vial, manufactured for intravenous injection, into multiple doses for intravitreal injection. Since bevacizumab is a monoclonal antibody, factors such as time and temperature during drug division, storage, and transfer condition can affect its stability. There are few data on the stability of the drug after division but without consideration of drug transfer conditions. This is particularly important in tropical countries including Thailand.

**What this study adds?**

Despite being in a tropical climate, bevacizumab in single-use vial could be divided into multiple small doses for intravitreal injection, with sufficient stability, when refrigerated at 4°C for up to six months. The simulated drug transfer condition in

tropical climate (placing the divided drug in a brown plastic and putting in another plastic bag with an ice cube for 30 minutes) did not significantly affect the drug stability. This could be applied to clinical practice to reduce the cost of treatment for patients and the country.

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**Potential conflicts of interest**

None.

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## เสถียรภาพของยา bevacizumab ที่แบ่งเก็บเป็นหน่วยย่อยจากขวดยาที่เปิดใช้ครั้งเดียวเพื่อใช้ในการฉีดเข้าวุ้นตา

นพศักดิ์ ผาสุขกิจวัฒนา, จุฑาไล ตันฑเทอดธรรม, ดรุณพร เลิศพงศ์ภาคภูมิ

**วัตถุประสงค์:** เพื่อทราบเสถียรภาพของยา bevacizumab ที่แบ่งเก็บเป็นหน่วยย่อยจากขวดยาที่เปิดใช้ครั้งเดียว เพื่อใช้ในการฉีดเข้าน้ำวุ้นตา หลังจากเก็บยาในตู้เย็น 4°C นานถึง 6 เดือน และภายใต้สภาวะจำลองของการส่งยาไปยังผู้ป่วยในโรงพยาบาลศิริราช

**วัสดุและวิธีการ:** ทำการแบ่งยา bevacizumab ขวดใหม่ 5 ขวด (ขวดละ 4 มิลลิลิตร, 100 มิลลิกรัม) ใส่กระบอกฉีดยา ขวดละ กระบอก (กระบอกละ 0.1 มิลลิลิตร, 2.5 มิลลิกรัม) ภายใต้สภาวะปลอดเชื้อ ปริมาณยาในแต่ละกระบอกฉีดยาจะถูกวัดโดยใช้วิธี enzyme-linked immunosorbent assay โดยทำการเจือจางตัวอย่างก่อนการวัดเป็นสองความเข้มข้น คือ เจือจาง $2x10^6$ และเจือจาง $4x10^6$ เท่า โดยวัดค่าที่เวลาเริ่มต้น และหลังจากผ่านการเก็บรักษาในตู้เย็นที่อุณหภูมิ 4°C นาน 1, 3 และ 6 เดือน ตามลำดับ และเพื่อเป็นการจำลองสภาวะของการส่งยาจากห้องยาไปยังผู้ป่วยในโรงพยาบาลศิริราช ก่อนทำการวัดปริมาณที่ 1, 3 และ 6 เดือน จะนำยามาใส่ในซองยาพลาสติกกันแสง แล้วใส่ในถุงพลาสติกอีกใบหนึ่งที่มีน้ำแข็งอยู่ 1 ก้อน ทิ้งไว้เป็นเวลานาน 30 นาทีก่อน แล้วจึงทำการวัดปริมาณต่อไป

**ผลการศึกษา:** ปริมาณยา bevacizumab (ค่าเฉลี่ย ± ค่าเบี่ยงเบนมาตรฐาน) ที่เวลาเริ่มต้น, 1 เดือน, 3 เดือน และ 6 เดือน มีค่า 26.24±1.95, 25.43±3.80, 27.87±2.81 และ 24.25±2.00 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ ค่าขอบล่างของช่วงความเชื่อมั่นที่ร้อยละ 95 มีค่าต่ำที่สุดที่เวลา 6 เดือน (23.32 มิลลิกรัมต่อมิลลิลิตร) และค่านี้คิดเป็นร้อยละ 89 ของค่าเฉลี่ยปริมาณยาที่เวลาเริ่มต้น ซึ่งแปลผลได้ว่าปริมาณยาหลังจากเก็บนานถึง 6 เดือน และภายใต้สภาวะของการส่งยา ไม่ด้อยไปกว่าปริมาณยาที่เวลาเริ่มต้น

**สรุป:** ผลการศึกษาชี้ให้เห็นว่า ยา bevacizumab จากขวดที่ใช้ครั้งเดียว สามารถแบ่งเก็บเป็นหน่วยย่อย ๆ เพื่อใช้ในการฉีดเข้าน้ำวุ้นตาได้ โดยยายังคงมีเสถียรภาพที่ดีหลังจากผ่านการเก็บรักษาในตู้เย็น 4°C ได้นานถึง 6 เดือน และยังคงความเสถียรภายใต้สภาวะของการส่งยาไปยังผู้ป่วยในประเทศภูมิอากาศร้อนชื้นด้วย