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Effects of dilution and prolonged storage with preservative in a polyethylene container on Bevacizumab (Avastin™) for topical delivery as a nasal spray in anti-hereditary hemorrhagic telangiectasia and related therapies

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Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular dysplasia and severe, recurrent epistaxis is a common clinical phenotype associated with HHT. An intranasal treatment regime of diluted Avastin™ (Bevacizumab; recombinant humanized anti-vascular epithelial growth factor immunoglobulin G1) using a pulsatile nasal irrigator has proven efficacious in clinical practice. However, concerns regarding the stability of Avastin™ following dilution and prolonged storage in standard containers used for drug delivery such as polyethylene bottles have so far prevented a more widespread clinical use. Compatibility with the preservative benzalkonium chloride was also unknown. We performed a detailed biochemical and electrochemical analysis of Avastin™ and did not detect any evidence of degeneration or aggregation following dilution and prolonged, refrigerated storage. Our data provide important insight into the stability of Avastin™ and allow the consideration of novel Avastin™ formulations, including its use in a metered-dose nasal spray for the treatment of HHT and other applications.

Keywords

Hereditary hemorrhagic telangiectasia; bevacizumab; Avastin™; storage; dilution

1. Introduction

Hereditary hemorrhagic telangiectasia (HHT) is a severe autosomal dominant vascular dysplasia leading to abnormal blood vessel formation in mucous membranes and skin as well as internal organs including the brain [1]. These vascular malformations result in mucocutaneous telangiectases, severe epistaxis and gastrointestinal hemorrhage [1]. In addition, arteriovenous shunts in lung, liver and brain are common in patients with HHT [1].

HHT is caused by mutations in the genes encoding endoglin or activin receptor-like kinase 1 (ALK-1). Haploinsufficiency is considered the pathophysiological origin of dysregulated angiogenesis, manifesting in the clinical phenotype of HHT [1]. Vascular epithelial growth factor (VEGF) is a critical modulator of angiogenesis and believed to induce mitotic activity in endothelial cells, leading to capillary sprouting [2, 3]. Elevated levels of VEGF were measured in plasma of HHT patients [4], however, the magnitude of the increase did not correlate with the severity of epistaxis or other HHT phenotypes in these patients [4]

Avastin™, a recombinant humanized anti-VEGF immunoglobulin G1 (IgG 1) antibody, has been reported to improve HHT symptoms, especially internal bleedings and anemia [5-7].

Treatment options for patients experiencing severe epistaxis include estrogens, anti-fibrinolytic agents, cauterization, septal dermoplasty, or closure of the nasal airway (Young's procedure). However, these treatment modalities are invasive and/or often associated with unwanted side effects such as thrombosis and septal perforation, limiting their clinical use [1].

Recently, intranasally-applied Avastin™ was successfully used to treat epistaxis in HHT patients and has been shown to be both safe and efficacious [8]. Specifically, Avastin™ was diluted to 10 mg/mL (1%) and 0.1 mL (1 mg) was delivered twice daily via a pulsatile, metered-dose nasal spray after nasal irrigation with hypertonic saline [8-10].

In order to address concerns of the long-term stability of Avastin™ when stored in a standard high-density polyethylene (HDPE) bottle and in a diluted form suitable for administration via metered-dose nasal spray, we performed a detailed biochemical analysis of diluted Avastin™ prior to and after refrigerated storage.

2. Materials and Methods

2.1. Avastin™

Avastin™ (Genentech Inc., South San Francisco, CA) was obtained in single 4 mL vials at a concentration of 25 mg/mL. Avastin™ was diluted to 10 mg/mL with 0.9% sterile normal saline. The final solution, containing 0.013% benzalkonium chloride (BAC) as a preservative, was subsequently aliquoted (0.2ml) into plastic vials. Samples were either frozen immediately in -60 °C (time-point T₀) or stored at 4 °C for two weeks and then frozen in -60 °C (time-point T₁₄). A fresh vial of undiluted Avastin™ (25 mg/mL, same lot number) was used as a standard for ELISA.

2.2. Native polyacrylamide gel electrophoresis

For native polyacrylamide gel electrophoresis (PAGE), 5 µg, 1 µg and 0.5 µg Avastin™ diluted in deionized water were electrophoresed on a native 3-8% gradient TRIS-acetate PAGE gel in TRIS-glycine buffer (both from Invitrogen, Carlsbad, CA) for 2:30 hr at 210 V. Gels were then stained with Coomassie Blue (SimplyBlue™ Safe Stain, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and imaged for 25 ms using a G Box Chemi XT imaging system (Synoptics, Cambridge, UK).

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

For sodium dodecyl sulfate (SDS)-PAGE, Avastin™ was diluted in deionized water to 10 ng, 25 ng, 50 ng and 100 ng in a total volume of 10 µL. Samples were denatured by addition of 2 µL 6 × SDS sample buffer (final concentrations: SDS 10%, glycerol 10%, β-mercaptoethanol 1%, bromophenol blue 0.004%, tris(hydroxymethyl)aminomethane (TRIS)-HCl 0.5 M, pH 6.8) and boiled for 5 min in a heating block. Samples were loaded on

4-12% bis-TRIS gels (Invitrogen, Carlsbad, CA) and proteins separated electrophoretically in 3-(N-morpholino) propanesulfonic acid running buffer (Invitrogen, Carlsbad, CA) for 45 min at 20 mA. Proteins were transferred onto a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI) in transfer buffer containing 25 mM TRIS pH 8.6, 192 mM glycine, 0.1% SDS, 20% methanol for 1 hr at 900 mA. The membrane was blocked with 5% milk, 0.2% Tween-20 in phosphate-buffered saline (PBS) for 1 hr, and probed with 1:10,000 diluted sheep anti-human IgG (GE Healthcare, Piscataway, NJ) at 4 °C overnight. The next day, the membrane was washed three times with 0.2% Tween-20 in PBS. Avastin™ was detected with the Lumina Forte Enhanced Chemoluminescence reagent (Millipore, Billerica, MA). Membranes were imaged using a G Box Chemi XT imaging system (Synoptics, Cambridge, UK).

2.4. Enzyme-linked immune-sorbent assay (ELISA)

An Avastin™ ELISA was developed using 96-well plates coated with human IgG-specific goat IgG (BD Biocoat; BD Biosciences, San Jose, California, USA) for one hour at room temperature. Avastin™ samples were diluted to 0.25 mg/mL in deionized water and 100 µL (25 ng) were added to each well. Wells were then incubated with 30 ng biotinylated recombinant human VEGF165 (Fluorokine Biotinylated Human VEGF; R&D Systems, Minneapolis, MN) diluted in 100 µL PBS for one hour. Peroxidase-conjugated streptavidin (Extravidin-Peroxidase; Sigma, St. Louis, MO) was diluted 1:2,000 in PBS and added to each well. Peroxidase activity was determined by incubation with 100 µL peroxidase substrate solution (SigmaFast OPD; Sigma). Absorbance at 450 nm was quantified in a microplate reader (FlexStation3; Molecular Devices, Sunnyvale, CA) after 10 min. Absorbance at 650 nm was subtracted as a reference. The standard curve was obtained by serial dilutions of Avastin™ in PBS. The linear range was between 2.5 to 50 ng Avastin™.

2.5. Isoelectric focusing

Avastin™ was diluted in deionized water and two different amounts (5 µg and 1 µg) were run on vertical isoelectric focusing (IEF) gels pH 3-10 (Invitrogen, Carlsbad, CA) for 2:30 hr at increasing voltages from 100 to 500 V, according to the manufacturer's instructions. Gels were then stained with Coomassie Blue (SimplyBlue™ Safe Stain, Invitrogen) and imaged for 15 ms using a G Box Chemi XT imaging system (Synoptics, Cambridge, UK). Bands were excised, denatured in 1 × SDS sample buffer (for composition, see 2.3.) for 10 min and then resolved on 2D TRIS acetate gels under denaturing conditions, as described above (see Section 2.3.). Gels were then stained with Coomassie Blue (SimplyBlue™ Safe Stain, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and imaged for 25 ms using a G Box Chemi XT imaging system (Synoptics, Cambridge, UK).

2.6. Densitometry and statistical analysis

Densitometry analysis was performed using Image J software (National Institute of Health, Bethesda, MD). At least three separate experiments were performed for each experimental approach. Data were analyzed for statistically significant differences using Student's *t*-test. Prism 5.01 software (GraphPad Software Inc., La Jolla, CA) was used for plotting and statistical analysis of the data.

3. Results and Discussion

3.1. Avastin™ does not degrade or aggregate after storage

In order to detect any possible gross degradation or aggregation of diluted Avastin™ after a period of 2 weeks storage at 4 °C, we performed native PAGE. Native PAGE is an excellent tool to assess protein stability [11]. Three different amounts of Avastin™ (0.5, 1 and 5 µg)

were loaded for the each time-point. Measured density was corrected for background and normalized to the density of the T₀/5 µg. We detected a linear relationship between density and Avastin™ amounts loaded on the gel (Fig. 1A). Density of the Avastin™ protein band did not differ between diluted (T₀) and diluted, stored (T₁₄) time-points (Fig. 1B). *P* values obtained from comparing three separate experiments were 0.834, 0.959 and 0.951 for 5 µg, 1 µg and 0.5 µg Avastin™ loaded per lane, respectively. No smaller products or smearing indicative of protein degradation, or larger products indicative of protein aggregation were detectable on the gel (Fig. 1A), not even after altering the exposure time and/or contrast settings (data not shown).

Whilst native PAGE is an excellent fast screening tool, low levels of degeneration that may affect either or both light and heavy chains of the Avastin™ antibody molecule may not be detected by this technique. Therefore, we next electrophoresed Avastin™ under reducing conditions on SDS-PAGE gels. 10 ng, 25 ng, 50 ng and 100 ng for each condition were resolved on TRIS/glycine gels and transferred onto nitrocellulose membranes. Using Enhanced Chemiluminescence, we could detect and quantify one higher molecular weight band of approx. 50 kDa and one lower molecular weight band of approx. 25 kDa (Fig. 2A), corresponding to the heavy and light chains of the Avastin™ IgG1 molecule [12]. Densitometry analysis revealed no significant differences between the two groups. *P* values obtained from three separate experiments were 0.830, 0.974, 0.928 and 0.990 for the high molecular weight band for 10 ng, 25 ng, 50 ng and 100 ng Avastin™, respectively (Fig. 2B), and 0.978, 0.852, 0.540 and 0.246 for the low molecular weight band for 10 ng, 25 ng, 50 ng and 100 ng Avastin™, respectively (Fig. 2C). We identified a linear relationship between the amount of Avastin™ loaded per lane and the background corrected mean density for loading amounts between 10 ng and 50 ng. Loading a 100 ng was close to saturating the signal and, therefore, likely outside the linear range of detection (Fig. 2).

Overall, these data indicate the absence of protein degradation as a result of the prolonged storage of diluted Avastin™).

3.2. ELISA can accurately detect diluted Avastin™ after prolonged storage

ELISA is the preferred technique to determine the Avastin™ concentration in tissue or bodily fluids after Avastin™ administration [13, 14]. It was thus important to test, whether an accurate determination of Avastin™ could be made after dilution in BAC and prolonged storage at 4 °C. We established a standard curve using off-the-shelf Avastin™ (25 mg/mL), using between 2.5 ng and 50 ng (n=3; Fig. 3A). This detection range resulted in a linear relationship and falls within a similar range to those reported previously [13, 14]. We then tested T₀ and T₁₄ Avastin™ samples at a calculated 25 ng concentration. The mean concentrations obtained from ELISA were 23.0 ± 1.7 ng and 22.4 ± 2.4 ng for T₀ and T₁₄, respectively, and statistically not significantly different (n=4, *P*=0.835; Fig. 3B). Our Avastin™ ELISA showed minimal inter-experimental variability, as highlighted by the small standard error obtained for the standard curve and the goodness of fit for the linear curve fitting (*r*²=0.993). The slightly larger variation for the samples can be explained by an additional dilution step required for ELISA analysis. Given the similar coefficients of variation between the two groups (15% for T₀ and 19% for T₁₄), there is no evidence of degeneration of Avastin™ following dilution and storage.

3.3. No change of electrochemical properties

In order to exclude any subtle changes on the electrochemical properties of Avastin™ that may have been masked by under native and/or SDS-PAGE conditions, we also performed IEF analysis on all samples. The isoelectric point of Avastin™ was at approximately pH 8.3 (Fig. 4A), in accordance with previous reports [15]. Densities obtained from loading 5 µg

Avastin™ were similar for T₀ and T₁₄ (n=3, $P=0.966$; Fig. 4B). Subsequent separation using SDS-PAGE revealed the same banding pattern of bands of 50 kDa and 25 kDa in size, characteristic of IgG proteins (Fig. 4B), no statistically significant changes in density were found (n=3, $P=0.598$ for the high molecular weight band and $P=0.118$ for the low molecular weight band; Fig. 4B). Our data clearly show unaltered electrochemical properties of Avastin™ after dilution and storage.

3.4. Conclusions

We investigated the possible effects of dilution in BAC and prolonged storage at 4 °C on the biochemical and electrochemical properties of Avastin™. Specifically, we used native and reducing PAGE to detect degeneration or aggregation of Avastin™, ELISA, and IEF, to determine the electrochemical properties. All our assays yielded similar results for our two experimental conditions T₀ (Avastin™ diluted to 10 mg/mL in BAC and frozen) and T₁₄ (Avastin™ diluted to 10 mg/mL in BAC, stored for 2 weeks at 4°C, and then frozen), indicating that dilution and storage did not affect the biochemical and electrochemical properties of Avastin™.

To our knowledge, this is also the first detailed report on the biochemical and electrochemical properties of Avastin™. One recent study addressed the effect of storage of Avastin™ in syringes and the contamination by silicone oil microdroplets, however, did not investigate degradation and/or aggregation [16].

Our data provide important insight into the stability of Avastin™ in HDPE bottles, used among other for nasal sprays, and in the presence of the preservative BAC. BAC is a frequently used preservative in drugs and 0.013%, as used in our study, is a typical concentration for nasal as well as otic and ophthalmic use [17].

Our data allow for the consideration of novel Avastin™ formulations that may require lower concentrations, addition of a preservative and/or the prolonged refrigerated storage of the drug, including its use in a metered-dose nasal spray for the treatment of HHT.

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Abbreviations

ALK-1	activin receptor-like kinase 1
BAC	benzalkonium chloride
HDPE	high-density polyethylene
HHT	hereditary hemorrhagic telangiectasia
IEF	isoelectric focusing
IgG	immunoglobulin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
VEGF	vascular endothelial growth factor

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Figure 1. Avastin™ migration on native PAGE

(A) Representative native PAGE gel of Avastin™ (5, 1 and 0.5 µg loaded per lane, as indicated), either frozen immediately as 10 mg/mL dilution (T₀) or stored for 2 weeks at 4 °C as 10 mg/mL dilution and then frozen (T₁₄). The banding pattern was similar for both conditions, and no high molecular weight or low molecular weight bands indicative of aggregation or degeneration, respectively, were detected. (B) Densitometry was performed on three different gels with separately diluted samples, and data was normalized to the density of the T₀/5 µg condition. Densities were similar between T₀ and T₁₄ and statistically not significantly different. Data is presented as mean ± s.e.m. (n=3).

Figure 2. SDS-PAGE of Avastin™

(A) Representative example of Avastin™ detected on nitrocellulose membrane using ECL. Two distinct bands of approximately 25 and 55 kDa were detected, corresponding to the light and heavy chains of the IgG molecule. (B/C) Densitometry analysis of the bands did not reveal any statistically significant differences between the two experimental groups, T₀ and T₁₄. Data is shown as mean ± s.e.m. (n=3).

Figure 3. Avastin™ ELISA

(A) An ELISA standard curve was established to detect between 2.5 ng and 50 ng of ELISA, using fresh off-the-shelf Avastin™ as standard. The standard curve was generated from three separate experiments and data points are presented as mean $\pm$ s.e.m. The dotted line represent the best fit determined by linear curve fitting ($r^2=0.993$). (B) No statistically significant difference between fresh and stored Avastin™ was detected by ELISA (n=4, $P=0.835$).

Figure 4. Electrochemical properties of Avastin™

(A) The isoelectric point of Avastin was approximately at pH 8.3 as detected by our vertical IEF gel. A representative image is shown. (B) Densitometry was performed on the 5 μ g band and data was normalized to the density of the T₀/5 μ g condition. Data was not statistically significantly different ($n=3$, $P=0.966$). Data is presented as mean $\pm$ s.e.m. (C) Subsequent separation on SDS-PAGE revealed the characteristic IgG pattern of bands of approximately 25 kDa and 55 kDa in size. No banding pattern indicative of aggregation or degeneration was found. (D) Normalized density (compared to T₀/5 μ g condition) was similar for both the high molecular weight band (HMW; $n=3$, $P=0.598$) and the low molecular weight band (LMW; $n=3$, $P=0.118$).