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Preserved bevacizumab (Avastin®) eye drops for application in multidose containers – an in-vitro characterisation

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Abstract

Purpose Monoclonal antibodies have made an immense contribution to the treatment of various human diseases. We aimed at investigating an affordable treatment option for veterinary patients with corneal neovascularization by adding the preservative benzalkonium chloride (BAC) to bevacizumab (Avastin®) for usage in multidose containers. A comprehensive analytical similarity assessment of preserved and unpreserved bevacizumab after dilution and storage was carried out.

Methods Diluted and preserved bevacizumab was analysed at different time points for a 4-week period and compared with unpreserved bevacizumab at the same concentrations at each time point. Native-PAGE, immunoblotting and HP-SEC were used to observe aggregation and degradation. DLS provided information about particle size and dispersity. Bevacizumab quantified by ELISA was conducted to determine its biological activity. Dose response curves and cell migration assays were performed to detect possible toxic effects and determine biological activity and efficacy of the drug using HUVECs.

Results Native-PAGE, immunoblotting and HP-SEC analysis did not show any changes or degradation products in the presence of BAC and after storage compared to unpreserved bevacizumab. The overlapping intensity-based particle size distribution obtained from DLS showed similarity in all tested groups and homogeneity was maintained. ELISA accurately detected bevacizumab at different concentrations. HUVECs incubated with preserved or unpreserved bevacizumab showed a comparable effect on cell migration. No decrease in cell viability was detected.

Conclusion Equivalence tests demonstrated that bevacizumab is stable after dilution, storage and preservation with BAC. Our study shows that preserved bevacizumab applied in mutidose containers can be considered as a cost-effective alternative to the otherwise single-dose treatments.

Keywords Anti-VEGF, Protein aggregation, Cornea, Angiogenesis

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Introduction

Corneal neovascularization (CNV) significantly contributes to ocular disorders, such as superficial pigmentary keratitis, chronic superficial keratitis and consequently rejection of corneal transplantations [1, 2]. Inflammation and other pathological insults disrupt the corneal angiogenic privilege and promote angiogenesis in the physiologically avascular cornea [3]. Although CNV is initially needed after injuries to clear infections, initiate wound healing, and prevent stromal melts, it comes with several disadvantages. Corneal neovascularization leads to tissue scarring, lipid deposition, edema, and potentially sustains inflammation that may impede visual acuity [4, 5]. Furthermore, it's noteworthy that CNV not only affects human patients, leading to a 12% decrease in visual acuity, but it also plays a substantial role in veterinary ophthalmology, where the cornea is involved in approximately 48% of reported lesions in dogs [6, 7]. Of these lesions, 30.8% are inflammatory keratopathies like ulcerative keratitis, pigmentary keratitis and chronic superficial keratitis (CSK) which are also associated with CNV [7]. Moreover, chronic inflammation of the ocular surface also contributes significantly to corneal neoplasm development, as evidenced by the occurrence of corneal vascular neoplasia in canine and feline patients with a history of chronic ocular disease, as well as the emergence of corneal B-cell lymphoma in horses with chronic immune-mediated keratitis [8–11].

In addition, CNV accompanies common causes of corneal blindness such as keratoconjunctivitis sicca in conjunction with pigmentary keratitis [12].

Typical treatment options include topically or systemically administered corticosteroids, cyclosporine and non-steroidal anti-inflammatory drugs, alongside with different surgical approaches [13–15]. However, these drugs show variable success in the clinics and myriad adverse effects. Therefore, proper medication options are still elusive and their development is fundamental to treat CNV and maintain corneal clarity, avascularity and vision [16].

A balance of antiangiogenic and angiogenic factors maintain the avascular state of the cornea [17]. If angiogenic factors predominate, the balance is lost and corneal vascularization is initiated. One key aspect is the action of vascular endothelial growth factor A (VEGF-A) on its receptors (mainly VEGFR-1 and VEGFR-2), as shown in experimental models of CNV [18]. In line with these observations, VEGFR-1 and VEGFR-2 are expressed in canine healthy and diseased eyes and pathological conditions induce their upregulation [19].

Pharmacological inhibition of VEGF-A has revolutionized human patients' care with diseases driven by pathological neovascularization [20–27]. One of the most potent VEGF inhibitors is bevacizumab (Avastin®, Roche). Bevacizumab is a recombinant humanized anti-VEGF immunoglobulin G1 (IgG 1) antibody and was recently administered safe and efficacious to treat dogs with CNV [28]. It was administered via a single-dose vial of 0.5 mL for each treatment time point to address concerns of stability and safety. Of note—despite its efficacy—this treatment regime is not suitable for veterinary practice because of the increased consumption and high costs when the medication is applicated in single dose vials [29]. Approximately two to three mL are needed per month to treat one eye according to the published dosages in single-dose vials [30–32]. Hence, the aim of this study was to overcome this problem by manufacturing bevacizumab eye drops suitable for multidose containers.

The addition of an antimicrobial preservative, most frequently the quaternary ammonium benzalkonium chloride (BAC), is necessary to prevent bacterial contamination of the multidose eye drops [33, 34]. Few studies already used preserved bevacizumab eye drops although the compatibility of the solutions is unknown [1, 30, 31]. We hypothesized that bevacizumab preserved with BAC is a safe and cost-effective alternative formulation to single-dose treatments. The aim of this study was to perform a comprehensive analytical similarity assessment. We investigated the structural, physicochemical and functional similarities of preserved and unpreserved bevacizumab after dilution and storage to assess potential differences, which could impact the clinical performance.

Results

General properties

Avastin® (25 mg/mL) showed a pH value of 6.14. When diluted with sterile 0.9% saline solution the pH value slightly dropped to 6.05. However, the values remained stable when diluted with sterile 0.9% saline solution and preserved with BAC 0.01% (pH 6.05 ± 0.00). No particles were visible macroscopically (Data not shown).

Biochemical properties by native PAGE, SDS PAGE and ELISA

Native PAGE is a quick diagnostic tool for detection of antibody aggregation. It was performed to detect any possible gross degradation or aggregation by separation of proteins and protein complexes in their native state [35]. Only one band was observed, indicating the presence of monomeric mAbs, when 5 µg of preserved or unpreserved bevacizumab were loaded per lane. Similar banding patterns and no indication of aggregation or degeneration was detected in all groups (Fig. 1a). Density obtained of the B-T_0 protein band was similar to preserved (B_{BAC}-T_0), preserved and stored (B_{BAC}-T_4) and diluted and stored (B-T_4) protein bands (Fig. 1b).

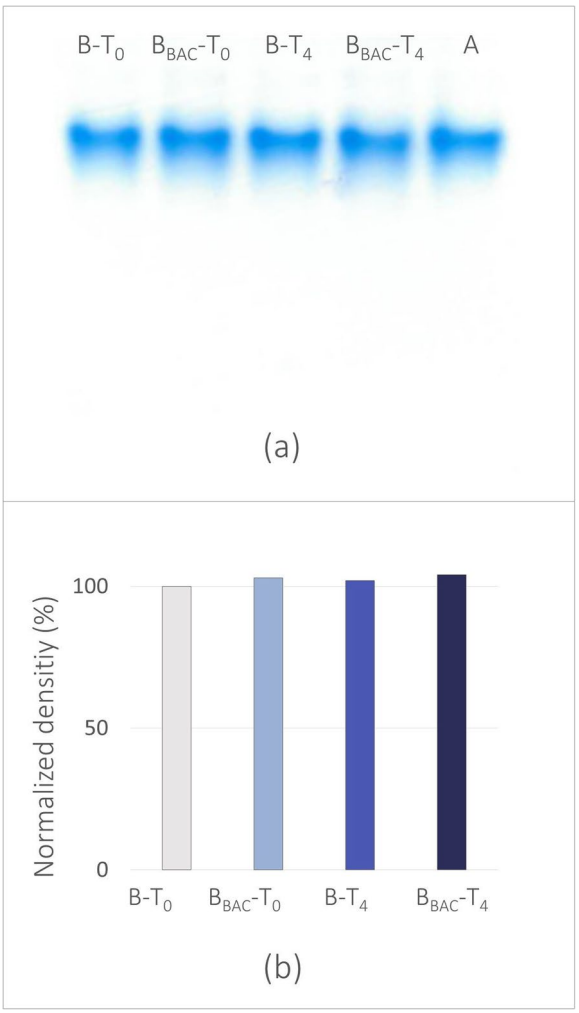

Fig. 1 Native PAGE migration of B-T_0, B_{BAC}-T_0, B-T_4, B_{BAC}-T_4 and A. **a** Representative native PAGE gel shows the electrophoretic pattern of the native proteins stained with Coomassie brilliant blue (5 µg loaded per lane). **b** Densitometry was performed on a representative gel loaded with 5 µg per lane. The density was normalized to B-T_0

Similarly, we did not detect aggregation in Coomassie blue-stained gels at different contrast settings. Our results were reproduced with different amounts (0.1 µg and 1 µg) loaded (Suppl. Figure 1) as well. Native gels are presented in Suppl. Figure 2.

Immunoblotting (SDS PAGE) was used to separate the proteins based on their mass. This allows a qualitative analysis and detection of any possible changes that may affect light or heavy chains of the samples [36–39]. The samples resolved into heavy (~ 50 kD) and light IgG chains (~ 25 kD) in all samples when either 10 ng, 25 ng or 50 ng were loaded per lane (Fig. 2a). Again, we did not observe any signs of aggregation. Densitometric analysis of the heavy (Fig. 2b) and light chain (Fig. 2c) revealed comparable mean intensities of the bands in all groups. Native blots are presented in Suppl. Figure 3.

ELISA quantification was performed to determine the binding activity to VEGF and thus biological activity of bevacizumab after dilution and storage with or without preservative [40]. A standard curve was established using a fresh vial of Avastin® between 0.625 ng/mL and 40 ng/mL. The detected range resulted in a linear relationship (Fig. 3a). The different bevacizumab samples were diluted to a concentration of 5 ng/ml. The calculated concentrations were comparable in all groups (Fig. 3b).

Native PAGE, immunoblotting and ELISA indicated no changes in the biochemical properties of the drug. There is no evidence of degradation or aggregation after dilution and/or storage with or without the preservative BAC. Therefore, we conclude that bevacizumab is still biologically active and accurately detectable.

Protein profile and aggregation by High-performance size-exclusion chromatography (HP-SEC)

HP-SEC is a key tool in demonstrating biosimilarities [41]. This method allows detection of potential aggregation by quantifying the monomer levels and soluble aggregates. Protein elution profiles of the samples are illustrated in Fig. 4. All samples revealed the presence of two protein fractions, with major peaks at ~ 13 mL retention volume, corresponding to the monomeric species. In addition, a relatively minor shoulder (dimer and trimer peak) at the leading edge of the peak was observed within all samples (referred to as higher molecular weight (HMW) species). The amount of HMW was 2.59 ± 0.02%, 2.65 ± 0.22%, 2.73 ± 0.01% and 2.75 ± 0.26% for B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4, respectively (Table 1). Molecular weight obtained from the major peaks centred around 151 kDa (Table 1). Isolated elution profiles of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4 is available in Supplement Fig. 4.

We concluded that dilution, storage and preservation using BAC has no impact on the monodispersity of bevacizumab.

Physicochemical characterization by Dynamic Light Scattering (DLS)

DLS was conducted to analyse particle size distribution and homogeneity of the proteins (Table 2). A Polydispersity Index (PDI) < 0.3 indicates homogeneity, which is an excellent tool to identify aggregates [39]. Particle size was evaluated using the hydrodynamic diameter and was found to be similar in all groups centering around 11.65 nm (Fig. 5a) with a PDI < 0.3 (Fig. 5b). The overlapping intensity-based size distribution of all samples shows similarity in all groups (Fig. 5c). Isolated size

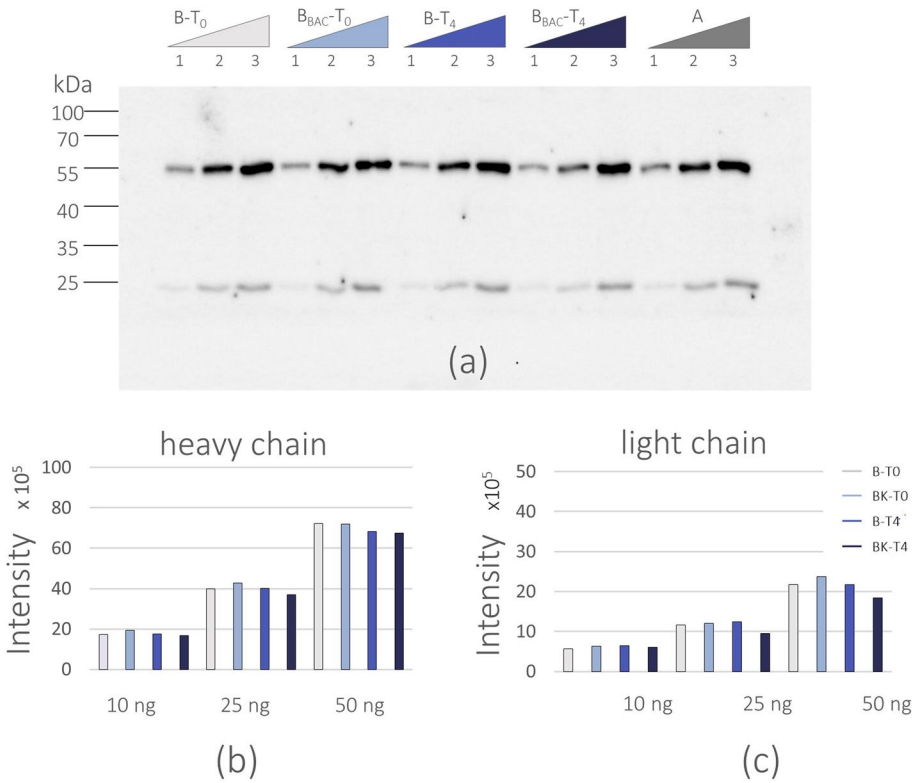

Fig. 2 Immunoblotting of B-T_0, B_{BAC}-T_0, B-T_4, B_{BAC}-T_4 and A. **a** Representative example of bevacizumab detected after immunoblotting on a PVDF membrane. 10 ng (1), 25 ng (2) or 50 ng (3) were loaded per lane. Two distinct bands of 25 and 55 kDa were detected, corresponding to the light and heavy chains of the IgG molecule. (b/c) Densitometry analysis of the heavy (**b**) and light (**c**) chain ($n = 2$)

distribution analysis of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4 is available in Supplement Fig. 5.

When bevacizumab is diluted, stored and/or preserved parameters obtained with DLS remained unchanged compared with control (B-T_0).

Cytotoxicity and efficacy by HUVEC viability and migration assay

To determine the safety and bioactivity of bevacizumab upon dilution and preservation, we studied the effect of the drug on human umbilical vein endothelial cell (HUVEC) viability and migration. HUVECs are used in this experiment since VEGF as a growth factor promotes their proliferation [42]. Therefore, when bevacizumab is present in the medium, there is an interaction between VEGF and bevacizumab, leading to an inhibitory effect on HUVEC growth. To evaluate whether bevacizumab supplemented with BAC has a toxic effect on the viability of HUVECs a dose response curve (CellTiter®-Glo assay) was performed. Comparable viability was detected in all groups (Fig. 6a) after 72 h. We assessed the functionality of preserved bevacizumab by comparing its capacity to reduce cell migration to its unpreserved counterpart (scratch assay). Percentage of scratch filling was calculated using the Adobe® Photoshop® software (Version 23.5.0, Adobe Inc., San Jose, California) (Supplement Fig. 6) modified from the analysis established by Glaß et al. [43] and Jin et al. [44]. Both unpreserved (B) and preserved bevacizumab (B_{BAC}) decreased HUVEC cell migration compared to control (Fig. 6b). After 24 h cells treated with medium alone had already proliferated and initiated the closure of the gap; after 48 h a monolayer was formed (Fig. 6c). In contrast, cells treated with either bevacizumab (B) or bevacizumab diluted with BAC (B_{BAC}) only partially closed the scratch wound leaving a visible gap (Fig. 6c).

These data indicate that dilution and preservation of bevacizumab via BAC do not interfere with its functionality.

Discussion

Monoclonal antibodies have revolutionized the treatment of vascular driven human diseases and beyond [45–48]. It is of utmost importance to favour a widespread use in veterinary health care as well. We aimed at contributing to an affordable treatment option for veterinarian patients with CNV. We have proven here the feasibility to create stable and functional bevacizumab eye drops

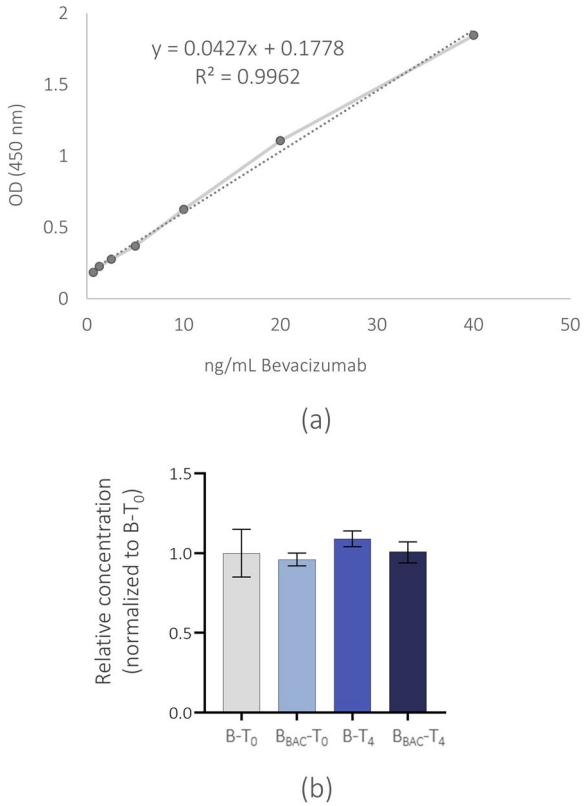

Fig. 3 ELISA of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4. **a** An ELISA standard curve was established to detect 0.625 ng/mL to 40 ng/mL of bevacizumab. The standard curve was generated ($n = 3$) and data points are presented as means. The dotted line represents the best fit determined by linear curve fitting ($r^2 = 0.9962$). **b** Bevacizumab samples were analyzed by ELISA at a concentration of 5 ng/ml ($n = 4$). From the obtained OD values, concentrations were calculated based on the standard curve and were then normalized to B-T_0. Mean relative concentrations with 95% CI compared to B-T_0 are depicted. OD = Optical Density

by adding the preservative BAC. Addition of BAC would allow for longer usage in multidose containers with prevention of bacterial contamination. This, in fact, provides a cost-effective and safe alternate formulation to single-dose treatments that require the usage of new single-dose vials, ending up in the disposal of loads of unused medication.

Alternately, to our approach, preservative-free bevacizumab eye drops could be manufactured using newly developed eye drop bottles containing a silicone membrane filter, and a one-way valve system to prevent retrograde microbial contamination. Rate of microbial contamination is around 21.7 (after two weeks), in standard multidose containers without preservatives. The usage of valve-featured bottles resulted in a slight but statistically insignificant reduction (13% of bacterial growth). Furthermore, the authors observed difficulties in administrating the eye drops using these valve-featured multidose bottles, which negatively affected owners' compliance [49].

Benzalkonium chloride, a nitrogenous cationic surface-acting agent, is the most commonly used preservative in ophthalmic preparations at concentrations ranging from 0.004%—0.02% as seen in ketorolac (ACULAR®, Allergan Inc., California, USA) or latanoprost eyedrops (Xalatan®, Viatris Healthcare GmbH, Delaware, USA) [50]. It is an effective and safe agent, well documented experimentally in clinical trials [34]. Nevertheless, when it comes to BAC usage in veterinary patients, particularly in brachycephalic animals who already suffer from chronic ocular surface conditions due to their anatomical features, BAC could potentially worsen these susceptibilities [51–54]. Median application times of bevacizumab eye drops described in human [30, 31] and veterinary literature [28, 32] are three to four weeks. Adverse effects are most likely to occur after long-term usage like with glaucoma medications in human patients and are not expected in short-term usage [53, 54]. Due to the potential ocular toxicity of BAC, in-vivo studies are essential to evaluate its safety on the ocular surface for cases where extended exposure is required.

Creating and handling therapeutic proteins is challenging because of the mAbs tendency to aggregate [55, 56]. In aggregates, two or more monomeric units of mAb may bind irreversibly to one another, reducing efficacy, enhancing immunogenicity, and causing side effects in patients [57, 58]. The presence of aggregates is considered a critical quality attribute (CQA) for the U.S. Food and Drug Administration (FDA) approval [41]. Therefore, the understanding of how the presence of preservatives may affect the structural stability of bevacizumab is essential for practical applications. Considering the safety, efficacy, immunogenicity (aggregation) and biological activity for mAb quantification we used native PAGE, immunoblot, ELISA, DLS and HP-SEC.

Native PAGE was performed to separate proteins and protein complexes in their native state, immunoblotting to separate the proteins based on their mass and ELISA for analysing the biological activity of mAbs through their specific binding to VEGF. Kaja et al. [59] obtained similar results when investigating preserved bevacizumab with native PAGE, immunoblot and ELISA, although using different storage conditions and concentrations.

To the best of our knowledge, this is the first study investigating preserved bevacizumab eye drops with DLS and HP-SEC. High-performance size-exclusion chromatography was used to quantify levels of monomers and soluble aggregates of preserved and stored bevacizumab eye drops. A population of HMWS was shown in all samples (2.64 ± 0.22%), similar to reported data [60]. Kahook

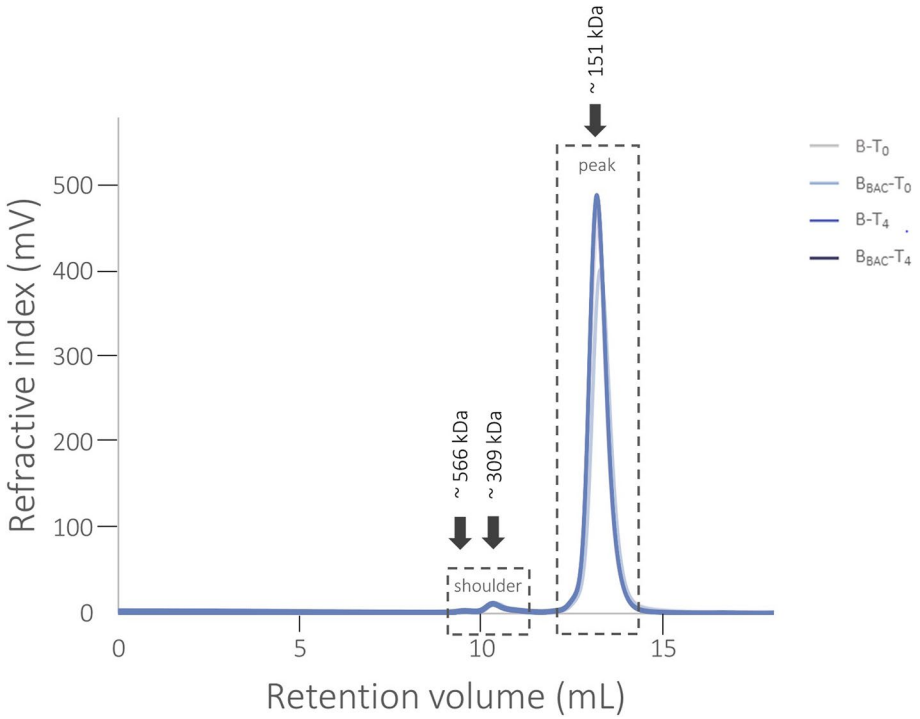

Fig. 4 Overlapped high-performance size-exclusion chromatography (HP-SEC). Chromatograms of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4. Values represent means ± SD (n = 3). All samples showed the presence of two protein fractions, with a major peak at 13.17 ± 0.002 mL, 13.18 ± 0.001 mL, 13.16 ± 0.003 mL and 13.17 ± 0.003 mL retention volume for B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4, respectively. Molecular weight obtained from the major peaks are 152.15 ± 0.19 kDa, 151.68 ± 0.37 kDa, 152.06 ± 0.13 kDa and 152.41 ± 0.55 kDa for B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4, respectively. Mean molecular weight obtained from the minor peaks centred around 309 kDa (dimer formation) and 566 kDa (trimer formation)

Table 1 Relative amounts of monomers and high molecular weight (HMW) species of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4

Sample	Monomers (%)	HMW (%)	MW monomer (kDa)
B-T_0	97.57 ± 0.02	2.59 ± 0.02	152.15 ± 0.19
B_{BAC}-T_0	97.35 ± 0.22	2.65 ± 0.22	151.68 ± 0.37
B-T_4	97.27 ± 0.01	2.73 ± 0.01	152.06 ± 0.13
B_{BAC}-T_4	97.25 ± 0.26	2.75 ± 0.26	152.41 ± 0.55

Values represent means (n = 3) ± SD. Monomers (%) = Main peak area/total peak area × 100; HMW (%) = HMW peak area/total peak area × 100. MW monomer = molecular weight in kDa

HMW high molecular weight species

et al. [61] reported HMWS in diluted bevacizumab and suspected them to arise from intermolecular disulfide crosslinks forming dimers or trimers of bevacizumab IgG monomers. This would be consistent with the measured molecular weight (~ 309 and ~ 566 kDa).

These methods, although highly sensitive, require drug samples to be diluted. Dilution results in a loss of bioactivity (up to 50%) and might induce aggregation [62]. To overcome these difficulties, we further investigated the samples with DLS. It is a well-established method for the determination of aggregates of proteins [63]. In contradistinction to other techniques, no dilution of the samples is required and therefore allows an investigation of the native structure of proteins and aggregates [64]. The hydrodynamic diameter of the drugs with PDI values of ≤ 0.3 remained stable over a four-week period when measured by DLS indicating homogeneity [65]. Similar results were reported by Khalili et al. [36] and Paul et al. [66] for undiluted bevacizumab stored for up to six months. A slight increase of the hydrodynamic diameter of preserved bevacizumab after storage (B_{BAC}-T_4) could be observed (0.12 ± 0.01 nm). It is reported that

Table 2 Physicochemical analysis of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4

Sample	Hydrodynamic diameter (Intesity based, nm) ± SD	PDI ± SD
B-T_0	11.25 ± 0.07	0.05 ± 0.00
B_{BAC}-T_0	11.24 ± 0.07	0.07 ± 0.02
B-T_4	11.6 ± 0.49	0.07 ± 0.01
B_{BAC}-T_4	12.51 ± 0.01	0.12 ± 0.01

Values are mean ± SD

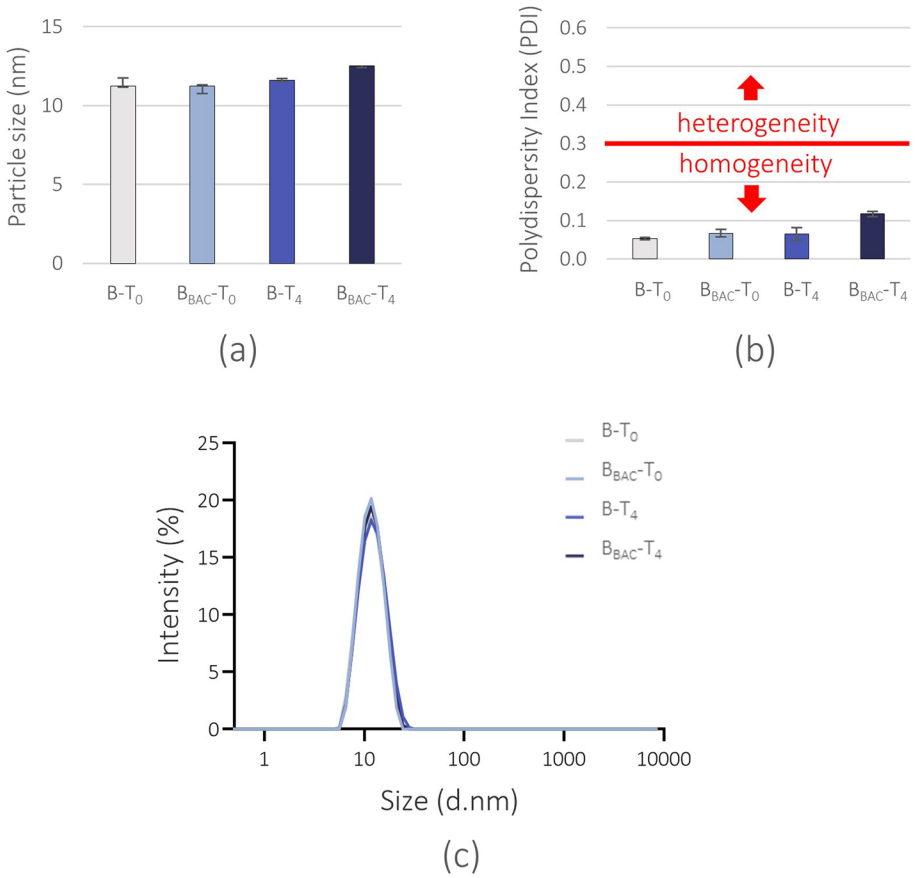

Fig. 5 Dynamic light scattering (DLS) analysis of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4. Values are presented as means ± SD (n = 3). **a** The hydrodynamic diameter was found to be 11.25 ± 0.07 nm, 11.24 ± 0.07 nm, 11.60 ± 0.49 nm and 12.51 ± 0.01 nm with PDI (**b**) of 0.05 ± 0.00 nm, 0.07 ± 0.02 nm, 0.07 ± 0.01 nm and 0.12 ± 0.01 nm at 25 °C for B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4, respectively. Polydispersity Index (PDI) < 0.3 was used to determine homogeneity in particle size distribution. **c** Overlapping intensity-based distribution analysis

pH or medium alterations and formed oligomers can lead to changes in the hydrodynamic radius [64, 67, 68]. Although the main peaks presented a uniform size distribution and no aggregates were detected, minor changes of pH values could be seen in preserved (pH 6.05) and unpreserved (pH 6.14) bevacizumab. This could possibly be an explanation for changes in the hydrodynamic diameter.

Ophthalmic solutions typically require a pH value between 4.5 and 9.0, ideally falling within the physiological range of 7.0–7.7 to prevent local irritation and enhanced lacrimation, which can lead to rapid drug clearance through the lacrimal system [69, 70]. Ophthalmic preparations with lower pH values, such as 6.05, can increase tear turnover, causing swift clearance from the ocular surface [71]. However, benzalkonium chloride acts as a penetration enhancer, possibly counteracting these effects by facilitating drug penetration through the ocular epithelia [72]. Furthermore, some ophthalmic formulations must be prepared within an acidic pH for long-term stability, as seen in the case of pilocarpine with a pH range of 4.5 to 5.5 [73]. Nonetheless, *in-vivo* studies are necessary in order to assess ocular compatibility.

A concentration of 2.5 mg/mL bevacizumab was used. This choice was based on findings from previous veterinary studies, which showed no significant ocular side effects in dogs, neither with nor without ocular surface diseases [28, 32]. A concentration of 0.01% BAC was chosen, as this has previously been described in the literature for the preserving of bevacizumab eye drops in human patients with chronic keratitis [1, 30, 31]. Notably, in these studies BAC and bevacizumab with concentrations as high as 10 mg/mL showed to be safe and without ocular side effects [1, 30, 31].

Alteration of the formulation can be associated with a loss of bioactivity [74]. We therefore studied the effect of clinically relevant doses of preserved and unpreserved bevacizumab eye drops on endothelial cell viability (cytotoxicity) and migration (efficacy). Bevacizumab was able to decrease HUVEC migration when administered

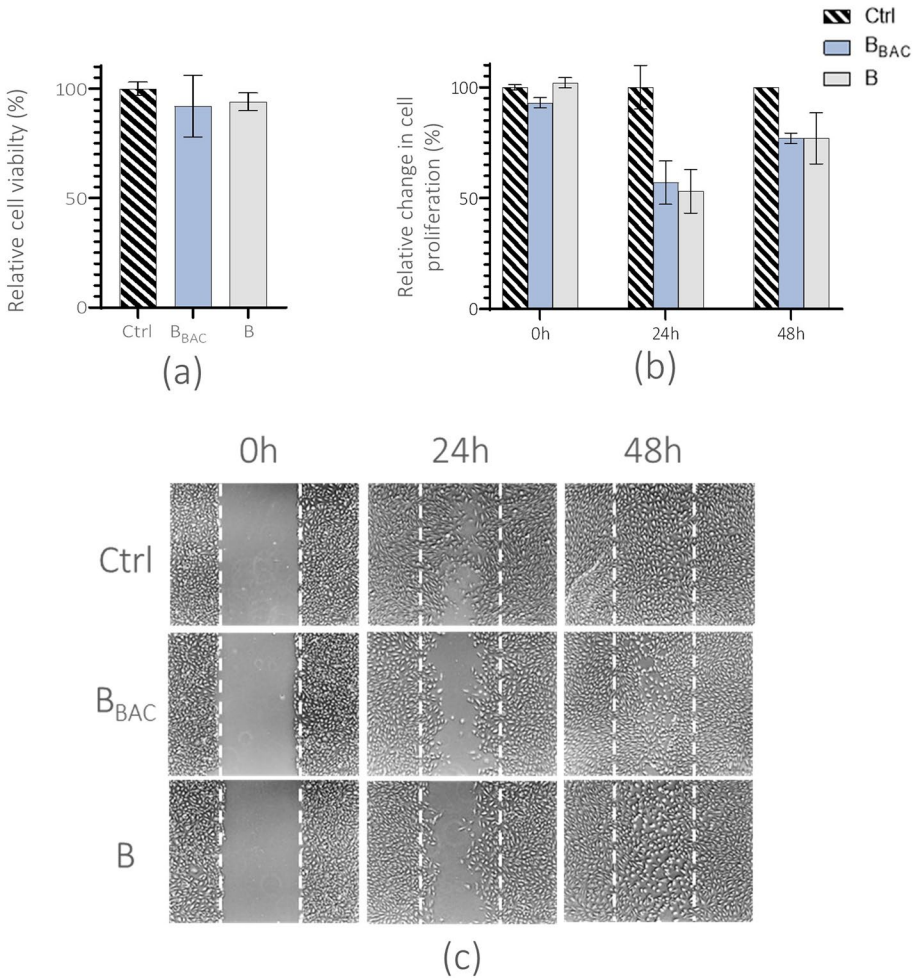

Fig. 6 Cytotoxicity and HUVEC migration assay with (B, B_{BAC}) and without (Ctrl) treatment. Results are shown as means ± SD (n = 3). **a** Quantification of cell viability after treatment measured by the amount of ATP present. Relative cell viability was 92 ± 17% and 94 ± 4% for B_{BAC} and B, respectively. **b** Quantification of cell migration after treatment over a 48 h period. Relative migration rates obtained were 57 ± 17% and 53 ± 17% at 24 h and 77 ± 4% and 77 ± 20% at 48 h for B_{BAC} and B, respectively. **c** Representative images of preserved and unpreserved bevacizumab treated HUVECs after scratch wound. Images were taken over a 48 h period

unpreserved and preserved. Forty-eight hours after treatment a slight recovery and increase in migration of the endothelial cells was detected. This could be due to the relatively short application time (10 min), which was used to mimic the contact time of ophthalmic preparations with the ocular surface *in-vivo*. Further studies are needed to identify appropriate application frequency of preserved bevacizumab eye drops in patients with naturally occurring CNV.

Conclusion

This study shows that bevacizumab is stable in its biochemical properties when diluted and preserved with BAC over a period of four weeks. Equivalence tests demonstrated that both preserved and unpreserved bevacizumab show similar properties. *In-vivo* studies are needed to indicate the use of preserved bevacizumab eye drops in patients with naturally occurring CNV.

Methods

Materials

Drug preparation

Bevacizumab 25 mg/mL (Avastin®) was obtained from Roche (Basel, Switzerland). A 2.5 mg/mL solution of bevacizumab was aseptically prepared in compliance with good manufacturing practice with sterile 0.9% saline solution as a solvent. Four groups were studied (Table 3); the solution was either preserved (B_{BAC}) or unpreserved (B) and subsequently aliquoted to 2 mL in commercially available plastic eye drop containers. Solutions were

Table 3 Sample preparation of B-T_0, B_{BAC}-T_0, B-T_4 and B_{BAC}-T_4

B-T_0	B-T_4	B_{BAC}-T_0	B_{BAC}-T_4
unpreserved (0.25% Bevacizumab) freshly prepared	unpreserved (0.25% Bevacizumab) stored for 4 weeks	preserved (0.25% Bevacizumab + 0.01% BAC) freshly prepared	preserved (0.25% Bevacizumab + 0.01% BAC) stored for 4 weeks

Table 4 Summary of investigations

Safety	Efficacy and/or biological activity	Immunogenicity (Aggregation)
Potentiometric pH measurements	ELISA	Native PAGE
Visual assessment	HUVEC Migration Assay	Immunoblot (SDS PAGE)
HUVEC Viability Assay	HP-SEC	DLS
		HP-SEC

examined at two different time points; either stored for 4 weeks protected from light at 2–4 °C (T_4) or freshly prepared (T_0). Benzalkonium chloride was used as a preservative. The final solution of preserved bevacizumab eye drops contained 0.01% BAC as prescribed in the European Pharmacopeia (EMA/495737/2013) [75]. As a control Avastin® (25 mg/mL, same lot number) diluted to 2.5 mg/mL with sterile 0.9% saline solution was used (B-T_0). A fresh vial of undiluted Avastin® (A) (25 mg/mL, same lot number) was used as a standard.

Methods

Study design

The bevacizumab solutions (B-T_4, B_{BAC}-T_0, B_{BAC}-T_4) were evaluated for their physicochemical stability after dilution and storage with or without BAC as a preservative. Over a four-week period, the solutions were analysed and compared with Avastin® (25 mg/mL, same lot number) diluted to 2.5 mg/mL with sterile 0.9% saline solution (B-T_0) as recommended by the manufacturer (Avastin® prescribing information, 01.2021). Samples were prepared from different vials but the same batch. Different approaches were chosen to investigate selected critical quality attributes (CQAs) concerning safety, efficacy and/or biological activity and immunogenicity (Table 4) [76, 77].

Determination of pH

The pH values of the formulations were determined using a previously calibrated pH-meter (inoLab Level 3, Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany). Measurements were carried out in triplicates.

Visual inspection

Solutions were visually inspected in front of a white and black panel before carrying out further experiments to confirm the absence of particles. Presence of any particles was noted.

Native polyacrylamide gel electrophoresis (Native PAGE)

Native PAGE was carried out as previously described [59]. Briefly, samples were diluted with phosphate buffered saline (PBS) in a total volume of 20 µL. The solution was electrophoresed at concentrations of 5 µg, 1 µg and 0.5 µg on a native 6% poly-acrylamide gel without SDS. Gels were stained with Coomassie Blue (SimplyBlueTM Safe Stain, Invitrogen, Carlsbad, CA) according to manufacturer's protocol, photographed (OM-D E-M10 Mark III, Olympus, Shinjuku, Tokyo, Japan) and/or scanned. Densitometry was performed on a representative gel and data was normalized to B-T_0.

Immunoblotting

Immunoblotting was performed as previously described [59]. Briefly, samples were diluted in PBS to a total volume of 40 µL containing 4×loading dye. Proteins were denatured and resolved by a 10% sodium dodecyl sulphate polyacrylamide gel for 30 min at 70 V and for 90 min at 90 V at concentrations of 10 ng, 25 ng and 50 ng. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Immobilon®-P, Merck, Darmstadt, Germany) by overnight wet transfer at 4 °C. Membranes were blocked in 5% milk for 1 h, and probed with diluted sheep anti-human IgG (GE Healthcare, Piscataway, NJ) at 4 °C overnight. The next day, detection was performed by chemiluminescent visualisation using the ChemiDoc Touch™ Imaging System (BioRad Laboratories Inc., Hercules, California) according to manufacturer's protocol. Protein expression levels were quantified using the Image Lab software (Version 6.1, BioRad Laboratories Inc., Hercules, California). All blots were performed in one experiment.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out as previously described [59]. Briefly, an ELISA was developed using 96-well plates coated with human IgG-specific goat IgG (Abcam, Cambridge, United Kingdom) overnight at 4 °C. Bevacizumab samples were diluted in deionized water to a concentration of 5 ng/mL. After blocking, 100 µL of the diluted samples were added to each well. Wells were then incubated with 30 ng biotinylated recombinant human VEGF-165 (antibodies-online GmbH, Aachen, Germany) for 1 h. Peroxidase-conjugated streptavidin (Extravidin-Peroxidase; Sigma, St. Louis, USA) was diluted 1:2000 in PBS and added to each well. Peroxidase activity was determined by incubation with 100 µL peroxidase substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma). Absorbance at 450 nm was quantified in a microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA). Absorbance at 650 nm was subtracted as a reference. The standard curve was obtained by serial dilutions of Avastin® (25 mg/mL, same lot number) in PBS. The linear range was between 0.625 ng/mL and 40 ng/mL. Experiments were carried out in triplicates.

High-performance size-exclusion chromatography (HP-SEC)

High-performance size-exclusion chromatography analysis was conducted on an OMNISEC multi-detector GPC/SEC (Malvern Panalytical, Worcestershire, UK) equipped with a refractive index, right angle light scattering (RALS), and UV/VIS diode array detector. Briefly, proteins were separated on a Superdex S200 increase 10/300 GL column (Cytiva) maintained at 25 °C, using PBS as an isocratic mobile phase at a flow rate of 0.5 mL/min. The autosample chamber was maintained at 20 °C and detectors were maintained at 25 °C. The injection volume was 60 µL. Protein concentration was measured online by using the refractive index detector. A dn/dc of 0.185 was taken. The instrument was calibrated using commercially available bovine serum albumin solution (BSA) (2 mg/mL) (Thermo Scientific™ Pierce™). Elution profiles of the samples were monitored using an ultraviolet (UV) absorbance detector. A qualitative comparison between samples was conducted using an overlay of these chromatograms. Measurements were carried out in triplicates.

Dynamic Light Scattering (DLS)

The hydrodynamic diameter and polydispersity index of bevacizumab were measured with a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK) according to the manufacturer's protocol. Briefly, samples (~700 µL) were measured using a PMMA semi-micro cuvette (Y197.1, Carl Roth, Germany) at 25.0 °C and with a detecting angle of 173 °C. Data were collected in an automatic mode, typically requiring a measurement duration of 150 s. Data were analysed using the Zetasizer software (Version 8.01.4906, Malvern Instruments Ltd., Malvern, UK). Quality reports of all the measurements were checked to ensure that the obtained data met the quality criteria. Measurements were carried out in triplicates.

Cell culture

Cells were purchased from American Type Culture Collection (ATCC®, USA, cat# PCS-100–010™). Cells were grown in Vascular Basal Medium (ATCC®, USA, cat# PCS-100–030™) supplemented with VEGF Growth kit (ATCC®, USA, cat# PCS-100–041™) containing rhEGF, rhFGF, rhVEGF, rhIGF-1, ascorbic acid, hydrocortisone, heparin, L-glutamine and 2% FBS in a 37 °C and 5% CO^2 humidified incubator. Cells were seeded at 5000 cells/cm^2 in T-75 flasks. After reaching 80—100% confluence the monolayer was washed with PBS (ATCC®, USA, cat# 30–2200) and trypsinized (Trypsin–EDTA for primary cells, ATCC®, USA, cat# PCS-999–003™) before being used in experiments. For experiments, a limited medium was formulated containing only rhVEGF as a growth factor according to Liu et al. [78] to produce a significant drug-specific dose-dependent inhibition. HUVECs up to passage five were used in all experiments.

HUVEC viability

Cells were plated at a density of 1×10^4 cells/well in 96-well plates (Falcon; Becton Dickinson Labware, Plymouth, England). After overnight incubation, cells had formed a monolayer with 80 – 100% confluency and were treated with preserved bevacizumab (B_{BAC}) or unpreserved bevacizumab (B) for 10 min to mimic topical drug treatments [79]. After this time, solutions were removed and normal cell culture conditions were restored for 24 h. At termination of the assay, 100 µl CellTiter-Glo® 2.0 Reagent (Promega, Madison, WI) was added to each well, absorbance at 490 nm was recorded using a microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Waltham, MA, USA) after incubation and shaking (600 rpm) for 30 min following manufacturer's protocol. Results are expressed as a percentage of the measured absorbance of control cells. Experiments were carried out in triplicates.

HUVEC migration

Cells were inoculated onto a 12-well plate (Falcon; Becton Dickinson Labware, Plymouth, England) at a density of 1×10^5 cells/well. After a confluent monolayer had formed overnight, a scratch was introduced with a sterile 1000 µl tip. Cell debris was removed by washing with PBS. Cells were treated with either preserved

bevacizumab (B_{BAC}) or unpreserved bevacizumab (B) for 10 min. After removal of the treatments, medium was supplied. Scratch filling was documented at different time points (0 h, 24 h, 48 h and 72 h). Experiments were carried out in triplicates.

Statistics

The results of the experiments performed in triplicates are expressed as mean ± standard deviation (SD). Native PAGE and SDS-PAGE were performed in duplicates although on different gels. The results are therefore presented in a descriptive manner. A one-sided equivalence test was used to determine whether unpreserved and preserved bevacizumab are equal. Equivalence bounds were specified based on the mean ± 15% of B-T_0. Equivalence between the groups was given, if the 95% CI was completely within the equivalence interval.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04592-4.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.

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Authors' contributions
All authors read and approved the final manuscript. UL contributed to the design of the study, performed analysis, interpreted data and wrote the manuscript. TB contributed to the design of the study, helped to perform analysis, was a major help in interpreting data, helped to write the manuscript and critically revised the manuscript. BN and AHK contributed to the study design, helped to interpret data and critically revised the manuscript. KK, GM, LW and LS performed analysis, helped to interpret the data and critically revised the manuscript. AT performed statistical data analysis, helped to interpret data and critically revised the manuscript.

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Data availability
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Dastjerdi MH, Al-Arfaj KM, Nallasamy N, Hamrah P, Jurkunas UV, Pineda R, Pavan-Langston D, Dana R. Topical bevacizumab in the treatment of corneal neovascularization: results of prespective, open-label, noncomparative study. Arch Ophthalmology. 2009;127(4):381–9.
2. Hayashi T, Zhang W, Hos D, Schrittenlocher S, Le VNH, Siebelmann S, Matthaei M, Bock F, Bachmann B, Cursiefen C. Descemet membrane endothelial keratoplasty in vascularized eyes: outcome and effect on corneal neovascularization. Cornea. 2021;40:685–9.
3. Nicholas MP, Mysore N. Corneal neovascularization. Exp Eye Res. 2021;202: 108363.
4. Inomata T, Mashaghi A, Di Zazzo A, Lee SM, Chiang H, Dana R. Kinetics of angiogenic responses in corneal transplantation. Cornea. 2017;36:491–6.
5. Maddula S, Davis DK, Maddula S, Burrow MK, Ambati BK. Horizons in therapy for corneal angiogenesis. Ophthalmology. 2011;118(3):591–9.
6. Lee P, Wang CC, Adamis AP. Ocular neovascularization: an epidemiologic review. Surv Ophthalmol. 1998;43(3):245–69.
7. Antonia NA, Anoop N, M.K., Anoop, S., Devanand, C.B., Martin, J., Venugopal S.K. Occurrence of ophthalmic disorders in dogs. Indian J Vet Res. 2014;23(2):21–4.
8. Shank AMM, Teixeria LBC, Dubielzig RR. Canine, feline, and equine corneal vascular neoplasia: a retrospective study (2007–2015). Veterinary Ophthalmology. 2019;22(1):76–87.
9. Dreyfus J, Schobert CS, Dubielzig RR. Superficial corneal squamous cell carcinoma occurring in dogs with chronic keratitis. Vet Ophthalmol. 2011;14:161–8.
10. Lisankis AP, Beavis BB, Weigt AK, Nunnery C. A case of malignant transformation of equine immune-mediated keratitis to B-cell lymphoma. Equine Veterinary Education. 2022;00:1–6.
11. Vallone LV, Neaderland MH, Ledbetter EC, Dubielzig RR. Suspected malignant transformation of B lymphocytes in the equine cornea from immune-mediated keratitis. Vet Ophthalmol. 2016;19:172–9.
12. Kaswan R, Pappas C Jr, Wall K, Hirsh SG. Survey of canine tear deficiency in veterinary practice. Adv Exp Med Biol. 1998;438:931–9.
13. Dean E, Meunier V. Feline eosinophilic keratoconjunctivitis: a retrospective study of 45 cases (56 eyes). J Feline Med Surg. 2013;15(8):661–6.
14. Hendrix DVH. Diseases and Surgery of the Canine Anterior Uvea. In: Gelatt KN, Gilger BC, Kern TJ, editors. Veterinary Ophthalmology, vol. two. 5th ed. Ames: Wiley Blackwell; 2013. p. 1160–3.
15. Li X, Liu H, Yu A, Lin D, Bao Z, Wang Y, Li X. Bioinspired self-assembly supramolecular hydrogel for ocular drug delivery. Chin Chem Lett. 2021;32(12):3936–9.
16. Huang J, Wang W, Yu J, Yu X, Zheng Q, Peng F, He Z, Zhao W, Zhang Z, Li X, Wang Q. Combination of dexamethasone and Avastin® by supramolecular hydrogel attenuates the inflammatory corneal neovascularization in rat alkali burn model. Colloids Surf, B. 2017;159:241–50.
17. Shi H, Zhu Y, Xing C, Li S, Bao Z, Lei L, Lin D, Wang Y, Chen H, Xu X. An injectable thermosensitive hydrogel for dual delivery of diclofenac and

Avastin® to effectively suppress inflammatory corneal neovascularization. Int J Pharm. 2022;25(625): 122081.
18. Amano S, Rohan R, Kuroki M, Tolentino M, Adamis AP. Requirement for vascular endothelial growth factor in wound- and inflammation-related corneal neovascularization. Invest Ophthalmol Vis Sci. 1998;39(1):18–22.
19. Binder DR, Herring IP, Zimmerman KL, Phillip Pickett J, Huckle WR. Expression of vascular endothelial growth factor receptor-1 and -2 in normal and diseased canine eyes. Veterinary Ophthalmology. 2012;15:223–30.
20. Andreoli C, Miller JW. Anti-vascular endothelial growth factor therapy for ocular neovascular disease. Curr Opin Ophthalmol. 2007;18:502–8.
21. Arevalo JF, Fromow-Guerra J, Quiroz-Mercado H, Sanchez JG, Wu L, Maia M, Berrocal MH, Solis-Vivanco A, Farah ME. Primary intravitreal bevacizumab (Avastin) for diabetic macular edema. Results from the Pan-American collaborative retina study group at 6-month follow-up. Ophthalmology. 2007;114(4):743–50.
22. Avery RL, Pieramici DJ, Rabena MD, Castellarin AA, Nasir MA, Giust MJ. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Ophthalmology. 2006;113(3):363–72.
23. Beccasio A, Mignini C, Caricato A, Iaccheri B, Di Cara G, Verrotti A, Cagini C. New trends in intravitreal anti-VEGF therapy for ROP. Eur J Ophthalmol. 2022;32(3):1340–51.
24. Heier, J.S., Brown, D.M., Chong, V., Korobelnik, J.F., Kaiser, P.K., Nguyen, Q.D., Kirchhof, B., Ho, A., Ogura, Y., Yancopoulos, G.D., Stahl, N., Vitti, R., Berliner, A.J., Soo, Y., Anderesi, M., Groetzbach, G., Sommerauer, B., Sandbrink, R., Simader, C., Schmidt-Erfurth, U., VIEW 1 and VIEW 2 Study Groups. Intravitreal aflibercept (VEGF trap-eye) in wet age-related macular degeneration. Ophthalmology. 2012;119(12):2537–48.
25. Iliev ME, Domig D, Wolf-Schnurrbursch U, Wolf S, Sarra G-M. Intravitreal bevacizumab (Avastin) in the treatment of neovascular glaucoma. Am J Ophthalmol. 2006;142:1054–6.
26. Krizova D, Vokrojova M, Liehneova K, Studeny P. Treatment of corneal neovascularization using anti-VEGF bevacizumab. Journal of Ophthalmology. 2014;2014:1–8.
27. Wang Y, Du H, Zhao X, Meng L, Chen Y. Observation, laser photocoagulation or anti-VEGF therapy in the management of retinal arterial macroaneurysms. BMC Ophthalmol. 2022;22:417.
28. Muellerleile L-M, Bernkopf M, Wambacher M, Nell B. Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series. Vet Ophthalmol. 2021;2021(24):554–68.
29. Na K-H, Yoo C, Park J-H, Kim YY. Eye Drop Dispenser Type and Medication Possession Ratio in Patients with Glaucoma: Single-Use Containers Versus Multiple-Use Bottles. Am J Ophthalmol. 2018;188:9–18.
30. Cheng SF, Dastjerdi MH, Ferrari G, Okanobo A, Bower KS, Ryan DS, Amparo F, Stevenson W, Hamrah P, Nallasamy N, Dana R. Short-term topical bevacizumab in the treatment of stable corneal neovascularization. Am J Ophthalmol. 2012;154(6):940–8.
31. DeStafeno JJ, Kim T. Topical bevacizumab therapy for corneal neovascularization. Arch Ophthalmol. 2007;125(6):834–6.
32. Muellerleile L-M, Tichy A, Nell B. Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs. Vet Ophthalmol. 2018;2018(00):1–7.
33. Aptel F, Villemont AS, Cunnac P, Sallit R, Rabilloud M, Poli M, Denis P. Comparison of Topical Instillation from Single-dose and Multidose Eye Drop Containers in Glaucoma: A Multicenter Randomized Cross-sectional Trial. J Glaucoma. 2021;30(8):718–24.
34. Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: the good, the bad and the ugly. Prog Retin Eye Res. 2010;29(4):312–34.
35. Sakuma C, Tomioka Y, Li C, Shibata T, Nakagawa M, Kurosawa Y, Arakawa T, Akuta T. Analysis of protein denaturation, aggregation and post-translational modification by agarose native gel electrophoresis. Int J Biol Macromol. 2021;172:589–96.
36. Khalili H, Sharma G, Froome A, Khaw PT, Brocchini S. Storage stability of bevacizumab in polycarbonate and polypropylene syringes. Eye. 2015;29:820.
37. Palmer JM, Amoaku WM, Kamali F. Quality of bevacizumab compounded for intravitreal administration. Eye (Lond). 2013;27(9):1090–7.
38. Roufik S, Paquin P, Britten M. Use of high-performance size exclusion chromatography to characterize protein aggregation in commercial whey protein concentrates. Int Dairy J. 2005;15(3):231–41.
39. Sugimoto M, Toledo V, Cunha M, Carregal VM, Jorge R, Leão P, Fialho SL, Silva-Cunha A. Quality of bevacizumab (Avastin®) repacked in single-use glass vials for intravitreal administration. Arq Bras Oftalmol. 2017;80(2):108–13.
40. Suárez I, Salmerón-García A, Cabeza J, Capitán-Vallvey LF, Navas N. Development and use of specific ELISA methods for quantifying the biological activity of bevacizumab, cetuximab and trastuzumab in stability studies. J Chromatogr B. 2016;1032:155–64.
41. Koza S, Warren B. Modern Size-Exclusion Chromatography Separations of Biosimilar Antibodies at Physiological pH and Ionic Strength. Column. 2022;18(10):16–21.
42. Sadick H, Schäfer E, Weiss C, Rotter N, Müller CE, Birk R, Sadick M, Häussler D. An *in-vitro* study on the effect of bevacizumab on endothelial cell proliferation and VEGF concentration level in patients with hereditary hemorrhagic telangiectasia. Exp Ther Med. 2022;24(3):555.
43. Glaß M, Möller B, Zirkel A, Wächter K, Hüttelmaier S, Posch S. Cell migration analysis: Segmenting scratch assay images with level sets and support vector machines. Pattern Recogn. 2012;45(9):3154–65.
44. Jin W, Shah ET, Penington CJ, McCue SW, Maini PK, Simpson MJ. Logistic Proliferation of Cells in Scratch Assays is Delayed. Bull Math Biol. 2017;79(5):1028–50.
45. Chavda VP, Prajapati R, Lathigara D, Nagar B, Kukadiya J, Redwan EM, Uversky VN, Kher MN, Patel R. Therapeutic monoclonal antibodies for COVID-19 management: an update. Expert Opin Biol Ther. 2022;22(6):763–80.
46. Gallazzi M, Ucciero MAM, Faraci DG, Mahmoud AM, Al Essa W, Gaidano G, Mouhssine S, Crisà E. New Frontiers in Monoclonal Antibodies for the Targeted Therapy of Acute Myeloid Leukemia and Myelodysplastic Syndromes. Int J Mol Sci. 2022;23(14):7542.
47. Sacco S, Amin FM, Ashina M, Bendtsen L, Deligianni CI, Gil-Gouveia R, Katsarava Z, Maassen Van Den Brink A, Martelletti P, Mitsikostas DD, Ornello R, Reuter U, Sanchez-Del-Rio M, Sinclair AJ, Terwindt G, Uluduz D, Versijpt J, Lampl C. European Headache Federation guideline on the use of monoclonal antibodies targeting the calcitonin gene related peptide pathway for migraine prevention - 2022 update. J Headache Pain. 2022;23(1):67.
48. Singh S, Kumar NK, Dwiwedi P, Charan J, Kaur R, Sidhu P, Chugh VK. Monoclonal Antibodies: A Review. Curr Clin Pharmacol. 2018;13(2):85–99.
49. Strauss RA, Genschel U, Allbaugh RA, Sebbag L, Ben-Shlomo G. Evaluation of microbial contamination of canine plasma eyedropper bottles following clinical use in canine patients. Vet Ophthalmol. 2019;22:222–8.
50. Freeman PD, Kahook MY. Preservatives in topical ophthalmic medications: historical and clinical perspectives. Expert Review of Ophthalmology. 2009;4(1):59–64.
51. Goldstein MH, Silva FQ, Blender N, Tran T, Vantipalli S. Ocular benzalkonium chloride exposure: problems an solutions. Eye. 2021;36:1–8.
52. Swan KC. Reactivity of the ocular tissues to wetting agents. Am J Ophthalmol. 1944;27:118.
53. Cohen JS, Gross RL, Cheetham JK, VanDenburgh AM, Bernstein P, Whitcup SM. Two year double-masked comparison of bimatoprost with timolol in patients with glaucoma or ocular hypertension. Surv Ophthalmol. 2004;49(Suppl1):45–52.
54. Fellman RL, Sullivan EK, Ratliff M, Silver LH, Whitson JT, Turner FD, Weiner AL, Davis AA. Comparison of travoprost 0.0015% and 0.004% with timolol 0.5% in patients with elevated intraocular pressure: a 6-month, masked, multicentertrial. Ophthalmology. 2002;109:998–1008.
55. Chopda V, Gyorgypal A, Yang O, Singh R, Ramachandran R, Zhang H, Tsilomelekis G, Chundawat SPS, Ierapetritou MG. Recent advances in integrated process analytical techniques, modeling, and control strategies to enable continuous biomanufacturing of monoclonal antibodies. J Chem Technol Biotechnol. 2022;97:2317–35.
56. Vazquez-Rey M, Lang DA. Aggregates in monoclonal antibody manufacturing processes. Biotechnol Bioeng. 2011;108(7):1494–508.
57. Cordoba-Rodriguez RV. Aggregates in MAbs and Recombinant Therapeutic Proteins: A Regulatory Perspective. Biopharm Int. 2008;21(11):44–53.
58. Das TK, Sreedhara A, Colandene JD, Chou DK, Filipe V, Grapentin C, Searles J, Christian TR, Narhi LO, Jiskoot W. Stress Factors in Protein Drug Product Manufacturing and Their Impact on Product Quality. J Pharm Sci. 2022;111(4):868–86.
59. Kaja S, Hilgenberg JD, Everett E, Olitsky SE, Gossage J, Koulen P. Effects of dilution and prolonged storage with preservatives in a polyethylene container on bevacizumab (Avastin) for topical delivery as a nasal spray

in anti-hereditary hemorrhagic telangiectasia and related therapies. Hum Antibodies. 2011;20:95–101.
60. Seo N, Polozova A, Zhang M, Yates Z, Cao S, Li H, Kuhns S, Maher G, McBride HJ, Liu J. Analytical and functional similarity of Amgen biosimilar ABP 215 to bevacizumab. mAbs. 2018;10(4):678–91.
61. Kahook MY, Liu L, Ruzycki P, Mandava N, Carpenter JF, Petrash JM, Ammar DA. High-molecular-weight aggregates in repackaged bevacizumab. Retina. 2010;30(6):887–92.
62. Giannos SA, Kraft ER, Zhao ZY, Merkley KH, Cai J. Formulation Stabilization and Disaggregation of Bevacizumab, Ranibizumab and Aflibercept in Dilute Solutions. Pharm Res. 2018;35(4):78.
63. Khodabandehloo A, Chen DD. Particle sizing methods for the detection of protein aggregates in biopharmaceuticals. Bioanalysis. 2017;9:313–26.
64. Akbas S, Sahin A, Calis S, Oncel H, Capan Y. Characterization of bevacizumab by dynamic light scattering while maintaining its native structure. Pharmazie. 2018;73(7):369–74.
65. Roggia I, Dalcin AJF, Ourique AF, Da Cruz IBM, Ribeiro EE, Mitjans M, Vinardell MP, Gomes P. Protective effect of guarana-loaded liposomes on hemolytic activity. Colloids Surf, B. 2020;187: 110636.
66. Paul M, Vieillard V, Roumi E, Cauvin A, Despiau MC, Laurent M, Astier A. Long-term stability of bevacizumab repackaged in 1 mL polypropylene syringes for intravitreal administration. Ann Pharm Fr. 2012;70(3):139–54.
67. Kim YC, Chiang B, Wu X, Prausnitz MR. Ocular delivery of macromolecules. J Control Release. 2014;190:172–81.
68. Quigley A, Williams DR. The second virial coefficient as a predictor of protein aggregation propensity: A self-interaction chromatography study. Eur J Pharm Biopharm. 2015;96:282–90.
69. Malmberg GJ, Lupo R. Compounding in veterinary ophthalmology. Veterinary Clinics of North America. Small Animal Practice. 2004;34:825–38.
70. Shell J. Pharmacokinetics of topically applied ophthalmic drugs. Surv Ophthalmol. 1982;26:207–18.
71. Sebbag L, Allbaugh RA, Wehrman RF, Uhl LK, Ben-Shlomo G, Chen T, Mochel JP. Fluorophotometric assessment of tear volume and turnover rate in healthy dogs and cats. J Ocul Pharmacol Ther. 2019;35(9):497.
72. Sasaki H, Yamamura K, Mukai T, Nishida K, Nakamura J, Nakashima M, Ichikawa M. Enhancement of ocular drug penetration. Crit Rev Ther Drug Carrier Syst. 1999;16:85–146.
73. Patil PN. Some factors which affect the ocular drugresponse. Trends Pharmacol Sci. 1984;5:201–4.
74. Jia H, Harikumar P, Atkinson E, Rigsby P, Wadhwa M. The First WHO International Standard for Harmonizing the Biological Activity of Bevacizumab. Biomolecules. 2021;11:1610.
75. European Medicines Agency (EMA). 2014. Questions and Answers on Benzalkonium chloride in the context of the revision of the guideline on Excipients in the label and package leaflet of medicinal products for human. Doc. Ref. (CPMP/463/00) EMA/495737/2013. EMEA Headquarters, London. Available at: https://www.ema.europa.eu/documents/scientific-guideline/questions-answers-benzalkonium-chloride-context-revision-guideline-excipients-label-package-leaflet_en.pdf.
76. Vandekerckhove K, Seidl A, Gutka H, Kumar M, Gratzl G, Keire D, Coffey T, Kuehne H. Rational Selection, Criticality Assessment, and Tiering of Quality Attributes and Test Methods for Analytical Similarity Evaluation of Biosimilars. AAPS J. 2018;20(4):68.
77. Chan C. Critical Quality Attributes Assessment and Testing Strategy for Biotherapeutics Development. American Pharmaceutical Review. 2019;22:29–33.
78. Liu Y, Tian H, Blobe GC, Theuer CP, Hurwitz HI, Nixon AB. Effects of the combination of TRC105 and bevacizumab on endothelial cell biology. Invest New Drugs. 2014;32(5):851–9.
79. De Saint Jean M, Brignole F, Bringuier AF, Bauchet A, Feldmann G, Baudouin C. Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. Invest Ophthalmol Vis Sci. 1999;40(3):619–30.

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