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A validated size exclusion chromatography method coupled with fluorescence detection for rapid quantification of bevacizumab in ophthalmic formulations

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Abstract:

Bevacizumab is a full-length human monoclonal antibody used to treat various neovascular diseases such as wet age-related macular degeneration (AMD), diabetic eye disease and other problems of the retina. Monthly intravitreal injections of bevacizumab (Avastin[®]) are effective in the treatment of wet AMD. However, there is a growing demand in the development of sustained release ophthalmic formulations. Therefore, this study aims, for the first time, to develop a rapid, simple, and sensitive method using size exclusion chromatography coupled with fluorescence detection for routine quantification of bevacizumab in ophthalmic formulations and during in vitro release studies. The selected chromatographic conditions included an aqueous mobile phase composed of 35 mM sodium phosphate buffer and 300 mM sodium chloride (pH 6.8), a flow rate of 0.5 mL/min, and the fluorescence detector at an excitation and emission wavelengths of 280 and 340 nm, respectively. The peak area-concentration relationship maintained its linearity over concentration range of 0.1-20 µg/mL ($R^2 = 0.9993$), and the quantitation limit was 100 ng/mL. The method was validated for specificity, accuracy, precision, and robustness. The developed method had a run time of 6 min at temperature 25°C, making it a unique validated method for rapid and cost-effective quantification of bevacizumab.

Keywords:

Protein assay; bevacizumab; HPLC validation; Fluorescence detector

1. Introduction

Bevacizumab is a recombinant humanized monoclonal antibody, which exhibits antiangiogenic activity by inhibiting vascular endothelial growth factor-A (VEGF-A) and hence, used for treatment of cancer (1,2). It has a molecular weight of 149 kDa, and is commercially available as Avastin[®] for intravenous use (3). Avastin[®] was the first anti-VEGF to be approved by Food and Drug Administration (FDA) in 2004 for treatment of metastatic colorectal cancer (3,4). Throughout 2006-2018, FDA subsequently approved Avastin[®] for treatment of lung, brain, kidney, cervical, and ovarian cancers (3,5,6).

Avastin[®] is also used off-label by ophthalmologists as a cheaper therapeutic alternative to the FDA-approved ranibizumab (Lucentis[®]) for treatment of neovascular (wet) age-related macular degeneration (7). Like any other biopharmaceuticals, storage temperature, exposure to

light, interaction with solvents and excipients can cause changes in protein structures, such as unfolding, aggregation, and denaturation. These changes may lead to no, reduced or altered protein bioactivities (8,9). There has been a recognizable growth within the last few years in the research concerned with developing sustained release systems for ocular delivery of bevacizumab (10-13). These systems are engineered for delivering bevacizumab at a rate of few micrograms per day for prolonged periods of time. This enhanced the urge for utilizing robust and cost-effective analytical methods for routine quantification of bevacizumab in the release media at low concentrations in terms of several hundreds of nanograms or few micrograms per millilitre.

There are several recently published methods for assay of bevacizumab using reversed-phase HPLC. The limits of quantitation (LoQs) were 6.5 and 45.0 $\mu\text{g/mL}$ using fluorescence and diode array detectors, respectively (14,15). Todoroki *et al.* (16) and Yamada *et al.* (17) achieved LoQs of 0.120 and 0.032 $\mu\text{g/mL}$, respectively, by employing a column temperature of 75°C, yet this high temperature may have negative consequences on the stability of bevacizumab as well as the stationary phase. Furthermore, the previous methods had relatively long run times (15 – 20 min). Other studies used enzyme-linked immunosorbent assay (ELISA), LC/MS or LC/MS/MS for determination of lower levels of bevacizumab at convenient temperatures (10,18,19).

On the other hand, size exclusion chromatography (SEC) is one powerful tool that is used for separation of biotherapeutics including monoclonal antibodies, and represents a feasible alternative for the more costly and time-consuming LC/MS and ELISA techniques (20,21). SEC separates molecules based on their hydrodynamic volume by selectively excluding them from an inert porous matrix of controlled pore size. On adopting an isocratic elution mode, proteins elute in the order of decreasing their molecular size, hence peaks of aggregates and fragments of protein molecules are separated before and after the protein monomer peak, respectively.

Being a part of sustained ocular drug delivery project, this study exploited SEC to develop a simple, robust, sensitive, and cost-effective method for quantification of bevacizumab in the release media of ocular implants. Up to the authors' knowledge, this is the first published study which couples SEC with fluorescence detector for assay of bevacizumab.

2. Experimental

2.1. Materials

Avastin[®] (aqueous bevacizumab solution, 25 mg/mL) was obtained from Roche Diagnostics Ltd. (Burgess Hill, UK). Sodium chloride, sodium hydroxide, d-(+)-trehalose dihydrate, phosphate buffer saline (PBS) tablets (pH 7.4), 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure[®] 2959), and poly(ethylene glycol) diacrylate 700 were purchased from Sigma-Aldrich (Irvine, UK). Sodium dihydrogen phosphate dihydrate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic anhydrous, and polysorbate 20 were purchased from VWR (Leicestershire, UK). The water utilized was purified using Purelab flex purification system (ELGA LabWater, High Wycombe, UK).

2.2. Chromatographic conditions

Assay of bevacizumab was carried out on an Agilent HPLC infinity II system consisting of quaternary pump, auto-sampler, variable wavelength detector (VWD), and fluorescence detector (Agilent Technologies, Cheadle, UK). Separation was achieved by Phenomenex[®] bioZen[™] SEC-2 column (1.8 μ m, 4.6x150 mm) connected to Phenomenex[®] bioZen[™] SEC-2 4.6 mm SecurityGuard Ultra Cartridge (Phenomenex Ltd, Macclesfield, UK).

Preliminary experiments were performed to select the optimum chromatographic conditions based on bevacizumab peak characteristics. The tried mobile phases were composed of 50 mM phosphate buffer (pH 6.2 – 7.3), with adding 0 – 500 mM sodium chloride, and 0 – 10% v/v acetonitrile. Different column temperatures were used (25, 35, and 45°C). VWD was set at wavelengths 214 and 280 nm (14,22). Fluorescence spectrophotometer (F-2710; Hitachi, Gunma-ken, Japan) was used for preliminary scanning of the fluorescence emission of 10 μ g/mL bevacizumab solution in PBS (pH 7.4, 10 mM) at different excitation wavelengths within the range of 220 – 300 nm, in order to detect the optimum excitation and emission wavelengths of bevacizumab solution in PBS.

The selected mobile phase was composed of 10 mM sodium phosphate dibasic anhydrous, 25 mM sodium dihydrogen phosphate dehydrate, and 300 mM sodium chloride. The solution was adjusted to pH 6.8 using 1 M sodium hydroxide solution (Jenway pH meter 3510; Cole-Parmer, Stone, UK). Bevacizumab samples were analysed in an isocratic mode using an injection volume of 10 μ L, mobile phase flow rate of 0.5 mL/min, and column temperature of 25°C. The run time of analysis was 6 min. The excitation and emission wavelengths were 280 and 340 nm, respectively.

2.3. Preparation of bevacizumab solutions

The samples for calibration curve were prepared using direct dilution method. Firstly, 5 μL of Avastin[®] solution (25 mg/mL) were diluted with PBS (pH 7.4, 10 mM) to prepare bevacizumab stock solution of concentration 20 $\mu\text{g/mL}$. The solution was filtered through 0.22 μm polyethersulfone filter (RephiQuik syringe filter; Reophile Bioscience Ltd, Shanghai, China). Bevacizumab solutions of concentrations 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 $\mu\text{g/mL}$ were prepared by direct dilution of the stock solution with PBS. All stock and standard solutions were prepared directly prior to each analytical run to avoid any instability issues of bevacizumab.

2.4. Method validation

The developed HPLC method was validated in terms of system suitability, robustness, linearity, sensitivity, specificity, accuracy, and precision, according to the guidelines of the international conference on harmonization (ICH Q2(R1)) and FDA recommendations (23,24).

2.4.1. System suitability test

The system suitability tests were carried out by injecting bevacizumab samples (10 $\mu\text{g/mL}$) in triplicates. Theoretical plate numbers and tailing factors were calculated using OpenLab 3.2 software (Agilent Technologies, Cheshire, UK).

2.4.2. Robustness test

Robustness is a measure of the capability of the analytical method to remain unaffected by small deliberate variations in the method parameters (23). The robustness of the developed HPLC method was evaluated by assessing bevacizumab recovery and system suitability parameters on changing each of the following chromatographic conditions independently; flow rate (- 0.1 mL/min), column temperature (+ 2°C), pH of the mobile phase (± 0.5), and fluorescence detection wavelengths (± 5 nm for both excitation and emission wavelengths together). The test was carried out by injecting bevacizumab samples of concentration 10 $\mu\text{g/mL}$ in triplicates.

2.4.3. Specificity

The specificity of the developed method was assessed by injecting a placebo sample to check possible interference from the excipients present in Avastin[®] solution. The prepared placebo

solution contained 0.048 mg/mL of trehalose dihydrate, 0.232 mg/mL of sodium phosphate monobasic monohydrate, 0.048 mg/mL of sodium phosphate dibasic anhydrous and 0.016 mg/mL of polysorbate 20.

2.4.4. Linearity

The linearity was assessed by creating a calibration curve for bevacizumab which consisted of seven standard solutions of concentrations: 0.1, 0.25, 0.5, 1, 2.5, 10 and 20 µg/mL. The solutions set was injected on the same day and repeated on five different days. Peak area was plotted versus concentration, followed by calculation of slope and coefficient of determination (R^2) of the best-fitting straight line using linear regression analysis (Microsoft® Excel 2010). Relative standard deviation (RSD) was calculated for the slope and R^2 .

2.4.5. Sensitivity

Sensitivity of the analytical procedure is a measure of the lowest concentration of the analyte that can be measured reliably. Six replicates of 0.1 µg/mL bevacizumab solution were analyzed to validate the value of LoQ.

2.4.6. Accuracy and precision

The accuracy and precision of the developed HPLC method were evaluated by determining the intra- and inter-day variability for three different concentrations of bevacizumab solutions: 0.5, 10, and 20 µg/mL for low, medium and high quality control (QC) samples, respectively. Intra-day precision was assessed by analyzing QC samples three times on the same day, whereas for inter-day precision, samples were analyzed on five different days. Precision of the developed method was expressed as RSD for peak areas of the replicates. On the other hand, accuracy of the method was expressed as % recovery of bevacizumab.

2.5. Solution stability

Bevacizumab solutions of concentration 10 µg/mL were prepared in PBS (pH 7.4, 10 mM) and stored at three conditions; bench-top ($25 \pm 2^\circ\text{C}$), refrigerator ($5 \pm 1^\circ\text{C}$), and incubator ($37 \pm 0.5^\circ\text{C}$). The solution stability was assessed for 6 months at intervals of 1, 7, 14, 28, 63, 84, 112, 160, and 180 days, by analyzing 10 µL aliquots of the solutions using the developed SEC method. The bevacizumab recovery was calculated at each time point and plotted *vs.* time.

2.6. *In vitro* release study

Bevacizumab-loaded UV-crosslinked ocular implants were fabricated as previously explained by McAvoy *et al.* (25). The *in vitro* release behaviour of bevacizumab from the fabricated implants was investigated, where three replicates of the implants were stored in glass vials containing 5 mL PBS (pH 7.4, 10 mM) at $37 \pm 0.5^\circ\text{C}$. At predetermined time intervals, the release media were collected and replenished with fresh release media. The collected samples were analysed using the developed SEC method for detection of the amount of released bevacizumab.

3. Results and Discussion

3.1. Chromatographic conditions

Bevacizumab solution showed maximum fluorescence emission at 340 nm when excited at 280 nm (Figure 1). Bevacizumab recovery values and tailing factors did not show significant differences ($p > 0.05$) at different column temperatures ($25 - 45^\circ\text{C}$). In contrast, the tailing factors of bevacizumab peak changed from 3.1 to 1.3 on increasing the pH of the mobile phase from 6.2 to 6.8. The selected mobile phase (pH 6.8) yielded maximum signal-to-noise ratio and peak symmetry, and exhibited bevacizumab retention time at 2.96 min. Bevacizumab was not detected using the selected method by UV detector at concentrations $\leq 20 \mu\text{g/mL}$ either at 214 or 280 nm.

3.2. Preparation of bevacizumab samples

PBS (pH 7.4, 10 mM) was used as diluent for samples preparation due to the observed decrease in % recovery of bevacizumab on dilution with water relative to PBS. This supports the findings of Kaja *et al.* who reported the stability of bevacizumab solution on dilution with isotonic saline solution, as well as Hirvonen *et al.* findings that pH 7.4 maintained the stability of bevacizumab being far enough from its isoelectric point (8.8) (26,27). In contrast, Giannos *et al.* reported a 40 – 50% decrease in bevacizumab potency on dilution with PBS (28). The % recovery of bevacizumab showed significant decrease on application of mechanical stress during samples preparation (vortex, vigorous shaking, magnetic stirring, etc), which agrees with the findings of Lahlou *et al.* with cetuximab (29). Therefore, bevacizumab samples preparation involved gentle shaking with excessive caution.

3.3. Method validation

3.3.1. System suitability test

The results of system suitability test of bevacizumab (10 µg/mL) are presented in Table 1. The mean values of tailing factors and number of theoretical plates were found to be within the accepted limits according to ICH Q2(R1) criteria (≤ 2 and ≥ 2000 , respectively), with RSD values $< 2\%$ (23).

3.3.2 Robustness test

The tailing factor and number of theoretical plates of bevacizumab peak remained within the acceptable ranges (≤ 2 and ≥ 2000 , respectively), on undergoing slight variations in the chromatographic conditions (Table 1). This indicates that method accuracy and column efficiency maintained their robustness (30). However, the % recovery of bevacizumab suffered from a significant increase of 11% on reducing the flow rate by 0.1 mL/min, due to increase in the time required for bevacizumab to pass through the detector flow cell, and subsequent peak broadening. In contrast, a significant decrease of 16% was encountered in the % recovery on reducing the fluorescence excitation and emission wavelengths by 5 nm, because of decreased fluorescence intensity of bevacizumab at lower excitation wavelength as shown in Figure 1. Increasing the flow rate was excluded from the tested conditions, because of the limitation of the recommended maximum flow rate for bioZen SEC-2 column (0.5 mL/min) as per the manufacturer's guidelines. Furthermore, column temperature could not be decreased below ambient temperature (25°C).

3.3.3. Specificity

No interfering peaks from Avastin[®] excipients were found at the retention time of bevacizumab (2.96 min), hence the developed analytical method is specific for the analysis of bevacizumab in the pharmaceutical dosage form. The chromatograms of a standard bevacizumab sample (10 µg/mL) and the placebo sample are shown in Figure 2.

3.3.4. Linearity

The bevacizumab peak area versus concentration had an average regression equation of ($y = 1238.4 x - 264.8$) over the concentration range of 0.1 – 20 µg/mL, with R^2 of 0.9993. RSD values for the slope and R^2 over five days were 4.66 and 0.26%, respectively.

3.3.5. Sensitivity

The lowest quantified concentration of bevacizumab was 0.1 µg/mL (Figure 2). The % recovery and RSD for six replicate injections of the lower limit of quantitation (LLOQ) were 107.2% and 2.8%, respectively. Furthermore, the carryover was found to be 17.9%. Therefore, signal-to-noise ratio, accuracy, and precision were within the acceptable ranges for the LLOQ (24). The LoQ of the currently developed method is lower than those of reported reversed-phase HPLC methods (6.5 – 45 µg/mL) (14,15). The recently published methods of Todoroki *et al.* and Yamada *et al.* achieved similar sensitivity levels using C8 columns in reversed-phase HPLC, yet, the currently developed SEC method had drastically lower analysis run time (6 min vs. 15 – 20 min) and analysis temperature (25°C vs. 75°C) (16,17).

3.3.6. Accuracy and precision

The results of accuracy expressed as % recovery, and precision expressed as RSD, are shown in Table 2. The highest exhibited RSD was for the inter-day precision of the low QC sample (1.75%), hence, all the RSD values fulfilled the acceptance criterion ($\leq 2\%$) (14). The recovery values also fulfilled the acceptance criterion falling within the range $100 \pm 3\%$.

3.4. Solution stability

Bevacizumab recovery values were $> 95\%$ on storage for 24 h at different storage conditions. After 6 months, bevacizumab potency decreased to 24, 39, and 51% of its original concentration on storage at 37, 25, and 5°C, respectively. Figure 3 shows the degradation of bevacizumab during 6 months at the three different conditions. The chromatograms showed bevacizumab monomer peak at 2.96 min, eluting separately from its aggregates and fragments which showed peaks at 2.28 and 3.56 min, respectively (Figure 4). The samples stored at 37°C suffered from fragmentation, while those stored at 5°C showed marginal fragmentation with more prominent aggregation.

3.5. *In vitro* release study

The developed SEC method was successfully used for assay of the released bevacizumab from the fabricated ocular implants. The chromatograms of the release samples showed bevacizumab monomer peak at retention time 2.96 min with detected aggregation in samples

collected at 70 days (Figure 5). This is consistent with the aggregation detected in bevacizumab samples stored at 37°C.

4. Summary

The developed chromatographic procedure exploited SEC coupled with fluorescence detector to analyze large number of samples in a short period without the use of organic solvents or high temperature, hence, it is cost and time-effective. The method was validated as per the ICH Q2(R1) guidelines, and showed satisfactory accuracy, precision, robustness, sensitivity and specificity. Therefore, this method is suitable for routine analysis of bevacizumab in pharmaceutical dosage forms and release media of bevacizumab-loaded ocular implants.

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Conflicts of interest

The authors confirm that this article content has no conflict of interest.

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Figures captions:

Figure 1. Fluorescence emission spectra of bevacizumab solution (10 µg/mL) on excitation at 275, 280, and 285 nm

Figure 2. Chromatograms of bevacizumab solutions of 0.1 and 10 µg/mL relative to placebo solution

Figure 3. Stability of bevacizumab solution (10 µg/mL) on storage for 6 months in three different conditions

Figure 4. Chromatograms of bevacizumab solutions (10 µg/mL) stored in (A) refrigerator at $5 \pm 1^\circ\text{C}$ and (B) incubator at $37 \pm 0.5^\circ\text{C}$.

Figure 5. Chromatograms of release samples of bevacizumab from the fabricated ocular implants at different time points.

Table 1 System suitability and robustness tests for bevacizumab standard solutions (10 µg/mL)

Chromatographic conditions	Recovery (%) (± SD, n = 3)	Retention time (min) (± SD, n = 3)	Tailing factor (± SD, n = 3)	No. of theoretical plates (± SD, n = 3)
Optimum chromatographic conditions (Flow rate 0.5 mL/min, column temperature 25°C, pH 6.8, excitation wavelength 280 nm, emission wavelength 340 nm)	100.00	2.96 ± 0.01	1.32 ± 0.01	2048.33 ± 15.42
Flow rate (-0.1 mL/min)	111.03 ± 1.03	3.87 ± 0.02	1.49 ± 0.02	2112.34 ± 23.55
Column temperature (+ 2°C)	99.05 ± 1.08	3.09 ± 0.01	1.42 ± 0.04	2023.57 ± 12.54
pH of the mobile phase (+0.5)	104.43 ± 1.17	2.94 ± 0.01	1.54 ± 0.03	2202.53 ± 14.33
pH of the mobile phase (-0.5)	95.38 ± 2.88	3.03 ± 0.03	1.83 ± 0.05	2190.42 ± 28.00
Excitation and emission wavelength (+5 nm)	86.43 ± 0.42	2.96 ± 0.01	1.52 ± 0.04	2005.03 ± 14.77
Excitation and emission wavelength (-5 nm)	95.95 ± 0.98	2.96 ± 0.01	1.49 ± 0.01	2125.62 ± 32.41
ICH Q2(R1) acceptance criteria [23]	N/A	N/A	< 2	≥ 2000

Table 2 Accuracy (% Recovery) and precision (% RSD) of the developed method for analysis of bevacizumab

Concentration ($\mu\text{g/mL}$)	Recovery (%) ^a	RSD (%)	
		Intra-day ^b	Inter-day ^c
0.5	98.75	0.92	1.75
10	98.22	1.43	0.65
20	99.23	0.28	0.62

^a % recovery is the mean of 3 determinations

^b RSD of 3 determinations on the same day

^c RSD of 5 determinations on 5 different days

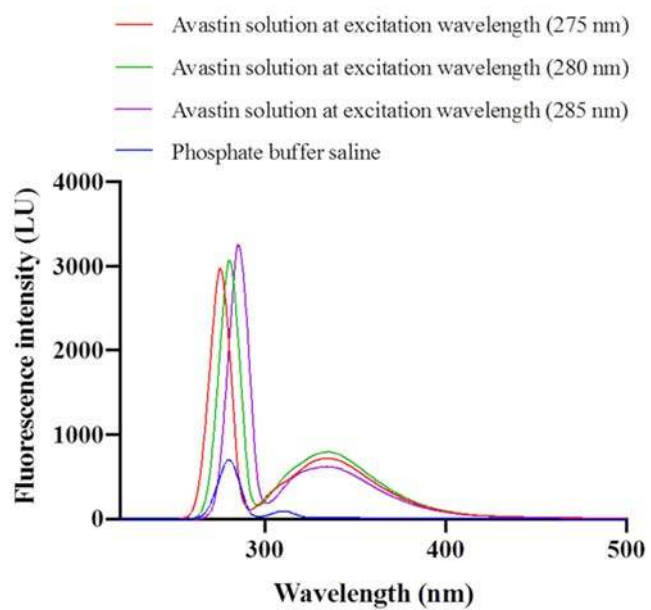


Fig 1

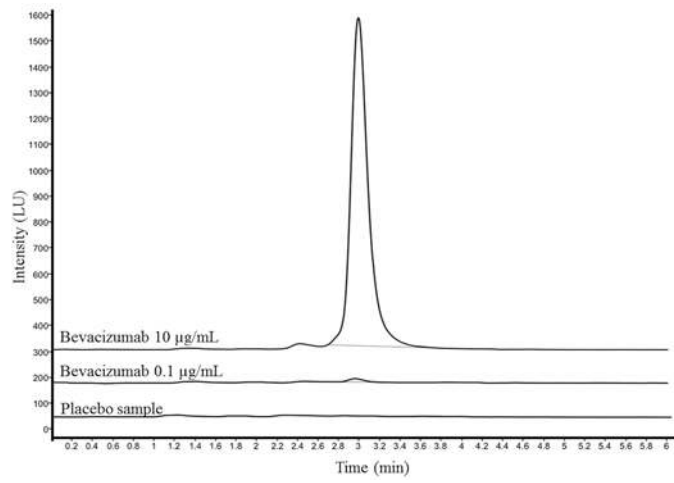


Fig 2

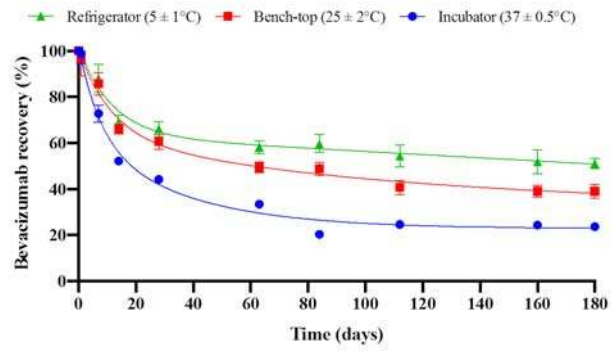


Fig 3

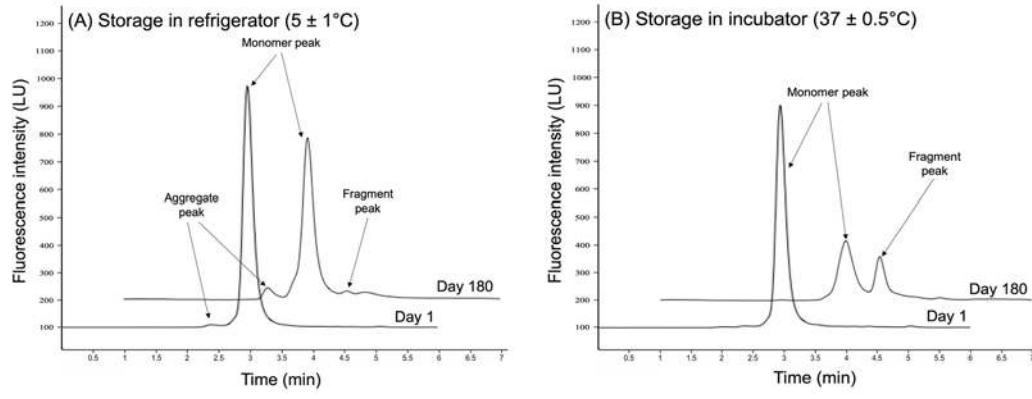


Fig 4

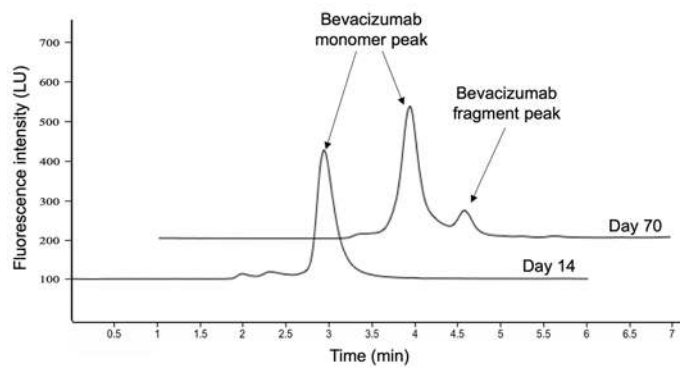


Fig 5