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Stability study over time of clinical solutions of ziv-aflibercept prepared in infusion bags using a proper combination of physicochemical and functional strategies

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ABSTRACT

A range of biopharmaceutical products are used to target Vascular Endothelial Growth Factor (VEGF), including Eylea® (aflibercept, AFL) and Zaltrap® (ziv-aflibercept, ziv-AFL). The first is indicated for ophthalmological diseases such as neovascular (wet) age-related macular degeneration, while the second is used in the treatment of metastatic colorectal cancer. The stability of AFL in prefilled syringes has been widely studied; however, no research has yet been done on the stability of ziv-AFL in polyolefin infusion bags. Therefore, the purpose of the present research is to evaluate the stability of ziv-AFL (Zaltrap®) clinical solutions prepared under aseptic conditions in polyolefin infusion bags at two different concentrations, i.e. 4.0 and 0.6 mg/mL, and stored refrigerated in darkness at 2–8 °C for 14 days. With that aim, the ziv-AFL clinical solutions were assessed by analysing changes in its physicochemical and functional properties. The distribution of the particulates was studied over a range of 0.001–10 μm by Dynamic Light Scattering (DLS); oligomers were analysed by Size-Exclusion High-Performance Chromatography with Diode Array Detection (SE/HLPC-DAD); the secondary structure of the protein was studied by far UV Circular Dichroism (CD) and the tertiary structure by Intrinsic Tryptophan Fluorescence (IT-F) and Intrinsic Protein Fluorescence (IP-F); charge variants were assessed by Strong Cation Exchange Ultra-High-Performance Chromatography with UV detection (SCX/UHPLC-UV); functionality was evaluated by ELISA by measuring the biological activity as manifested in the extension of the immunological reaction of the ziv-AFL with its antigen (VEGF). Neither aggregation nor oligomerization were detected by the techniques mentioned above. Secondary and tertiary structures remained unchanged over the 14-day period, as did charge variants. The functionality observed initially was maintained along time. Therefore, it could be proposed that the ziv-AFL clinical solutions studied showed great physicochemical and functional stability over a period of two weeks, regardless of the concentration, i.e. 4 or 0.6 mg/mL.

1. Introduction

Ziv-aflibercept (Ziv-AFL) is a Vascular Endothelial Growth Factor (VEGF)-trap [1] that consists of the VEGF receptor extracellular binding domains 1 and 2 fused to the Fc portion of a human IgG1. Ziv-AFL acts as a soluble decoy receptor that binds to circulating VEGF A, VEGF B and Placental Growth Factor (PlGF). When it traps these ligands, it prevents the endogenous ligands from activating their respective receptors and thus blocks signaling. It is a recombinant Fc-fusion protein produced in a Chinese Hamster Ovary (CHO) k-1 mammalian expression system by recombinant DNA technology. In combination with irinotecan/5-fluorouracil/folinic acid (FOLFIRI) chemotherapy, it is indicated in adults with metastatic colorectal cancer (MCRC) that is resistant to or has progressed after an oxaliplatin-containing regimen [2]. There are other pharmaceutical products on the market that target VEGF in the treatment of different common chorioretinal vascular diseases, such as neovascular age-related macular degeneration (nAMD) [3], diabetic macular edema (DME) and retinal vein occlusion (RVO) [4]. These include aflibercept (AFL) (Eylea®; Regeneron, Tarrytown, NY and

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Bayer Healthcare, Leverkusen, Germany), ranibizumab (Lucentis®; Genentech, South San Francisco, CA and Roche, Basel, Switzerland) and bevacizumab (Avastin®; Genentech, South San Francisco, CA and Roche, Basel, Switzerland).

The prefix "ziv" is used to differentiate the AFL in Zaltrap® from the AFL in Eylea®; both medicines share the same active substance but have different formulations, and therefore different indications. Although AFL is manufactured by Regeneron Pharmaceuticals Inc, different pharmaceutical companies are responsible for batch release: Sanofi Aventis for Zaltrap® and Bayer AG for Eylea®.

The manufacturer of Zaltrap® indicates that this drug product is chemically and physically stable for 24 h at 2–8 °C and for 8 h at 25 °C once diluted in infusion bags and assuming that dilution has been performed under controlled and validated aseptic conditions [2]. Despite these indications, it recommends, due to microbiological issues, that the solutions prepared for infusion should be used immediately. Any decision to extend this recommended period must be taken by the user and it will be their sole responsibility [2]. Manufacturers usually establish short maximum stability periods for biotechnological and biological medicines due to their fragile nature and possible risks to patient safety [5]. Stability studies on biopharmaceuticals that reproduce hospital storage conditions are essential [6–8] as they guarantee the reuse of the daily surplus in hospital pharmacies including the pharmaceutical preparations not administered due to medical treatment interruption. As an example, A. Kannan et al. [9] studied the in-use interfacial stability of monoclonal antibody (mAb) formulations diluted in saline i.v. bags to evaluate the mAb aggregation propensity in saline medium 0.9 % NaCl in comparison with low ionic strength formulation buffer; results indicated higher tendency to aggregate in the saline medium. Since the approval of AFL and ziv-AFL [10,11], several stability studies have been carried out, the majority on AFL rather than on ziv-AFL. These studies assessed their stability in compounded syringes and most sought to evaluate the continuing functionality of the drug over time.

In 2015, A.M. Mansour et al. [12] proposed that ziv-AFL maintained its functionality for 4 weeks after being compounded in polycarbonate syringes and stored refrigerated. Similarly, in 2017, M. Schicht et al. [13] evaluated the functionality of AFL compounded in syringes over 120 h stored at 4 °C by means of cell proliferation assays and VEGF-ELISA, evaluating inhibition of cell proliferation and binding to VEGF respectively. In contrast to the specifications included in the summary of product characteristics for Eylea® [14], they demonstrated that AFL maintained its biological function over the five-day study period. For their part, S. Cao et al. [15] reported the functional stability of AFL when placed in plastic syringes and stored refrigerated for up to 4 weeks by determining half maximal inhibitory concentration (IC50) performing competitive binding assays against PlGF.

In 2018, J. De Lima Farah et al. [16] demonstrated the stability of AFL and ziv-AFL when compounded in tuberculin-type 1-mL plastic syringes and stored at 4 °C and -8 °C for 28 days. This was done by evaluating the mass recovered from the syringes and checking functional properties in ELISA experiments. M.S. Sivertsen et al. [17] published a stability study in which AFL was compounded in prefilled plastic syringes and stored refrigerated over 4 weeks. The results obtained from a set of suitable analytical techniques such as dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography (SEC), antihuman Fc ELISA, FcRn binding ELISA, surface plasmon resonance (SPR) and differential scanning fluorimetry (DSF) confirmed the quality, stability and antigen/Fc binding properties of the protein over the 4-week period. These authors also developed a novel method for compounding and storage of three anti-VEGF drugs (including AFL) in prefilled silicon oil-free syringes [18]. Applying the same analytical techniques as in the previous study [17], they demonstrated that AFL maintained its physical stability and functionality over a period of 7 days when stored at 4 °C in dark conditions.

Recently, D.P. Han et al. [19] compounded AFL in polycarbonate syringes and stored them at 4 °C and -20 °C for 14 days. They demonstrated the physical and functional stability of AFL at these conditions using ELISA and DLS techniques. M.R. Moreno et al. [20] undertook a more complete study, evaluating AFL stability over 30 days under physiological conditions and when formulated in a drug delivery system (DDS) based on microparticles. Stability was checked using SEC, circular dichroism (CD), fluorescence spectroscopy (FS), SDS-PAGE and ELISA. These authors proposed the physicochemical and functional stability of AFL at 37 °C and at different pH values ranging from 4.5–8.5 over 30 days. They also demonstrated the stability of AFL after simulating a water/oil (w/o) emulsification process, a first step towards protein entrapment, using different organic solvents such as triacetin.

Although all these studies assure that AFL and ziv-AFL are stable when stored in prefilled syringes under the right conditions, no research has yet been conducted into the stability of ziv-AFL diluted in NaCl (0.9 %) and stored in polyolefin bags. Up until now, interest has focused on assessing the stability of AFL in prefilled syringes due to the high cost of this treatment. However, ziv-AFL is also very expensive compared to traditional treatments and its stability in real hospital preparations should be evaluated so as to provide scientific evidence on which to base the complex decisions that must be taken regarding the reuse of this product.

In this research, a comprehensive set of previously validated physicochemical and functional methods [21,22] were applied to assess the stability over time (two weeks) of clinical solutions of ziv-AFL. The main critical attributes (CQA) of proteins such as particulates, oligomers, aggregates, secondary and tertiary structures, charge variants and binding to VEGF were investigated. These attributes need to be strictly monitored during process development and manufacturing in order to ensure product quality [23] and must also be controlled in real conditions of use in stability studies.

2. Materials and methods

2.1. Materials and reagents

4 mL fresh vials containing 100 mg of ziv-AFL (Zaltrap®, Sanofi Aventis) were kindly supplied by the San Cecilio University Hospital (Granada, Spain).

Nunc maxisorp microplates were supplied by Labclinic S.A. (Barcelona, Spain). Anti-human IgG (whole molecule)-peroxidase antibody from rabbit, O-Phenylenediamine Dihydrochloride -as "the sigmafast opd"- (OPD), Vascular Endothelial Growth Factor (VEGF) produced in *E.coli*, sodium phosphate monobasic monohydrate were supplied by Sigma Aldrich (Madrid, Spain). Sodium carbonate, sodium hydrogen carbonate, sodium chloride, potassium chloride, disodium phosphate monohydrate and potassium phosphate monobasic were purchased from Panreac (Barcelona, Spain).Tween 20 was supplied by Fluka Chemika (Madrid, Spain). Skimmed milk powder "La Asturiana" (Oviedo, Spain) was acquired in the local market. Size exclusion column standard calibration (Advance BioSEC 300 Å protein standard) was supplied by Agilent technologies (California, USA). Reverse-osmosis-quality water purified (≥18 M/cm) with a Milli-RO plus Milli-Q station from Millipore Corp. (Madrid, Spain) was used throughout the study. Reverse-osmosis-quality water was purified with a Milli-Qstation from Merck Millipore (Darmstadt, Germany).

2.2. Stability study

The stability of ziv-AFL was assessed using an *ad hoc* work procedure that was mainly based on the ICH Q6B international guideline [24]. To this end, we assessed a range of physicochemical and biological attributes in the ziv-AFL solutions. The physicochemical attributes were tested using a combined set of analytical techniques, and functionality was assessed using a biochemical approach. Table 1 in Supplementary Data indicates the methodology used in this research.

2.2.1. Ziv-AFL samples

Two different ziv-AFL concentrations were evaluated to cover the range commonly used in real clinical practice: 4 mg/mL (intermediate concentration) and 0.6 mg/mL (low concentration).

The medicinal product Zaltrap® contains 25 mg/mL of ziv-AFL in a concentrated solution for dilution for infusion [2]. Two doses of the medicine are currently available: a) vials containing 100 mg of the active substance in 4 mL of water for injection and b) vials containing 200 mg in 8 mL. In this study, the lower dose, i.e. vials containing 100 mg of ziv-AFL (batch number: 7F008C/ expiration date: 10–2020) was used to prepare the diluted solutions for infusion following the instructions set out in the technical report [2]. In this way, several vials of ziv-AFL 25 mg/mL were used to prepare 50 mL of 4.0 and 0.6 mg/mL ziv-AFL diluted solutions in NaCl 0.9 % under validated aseptic conditions using a laminar flow cabinet (Aura Vertical S.D.4, Bio Air Instruments, Italy). This procedure was conducted according to the Hospital Pharmacy routine protocol: appropriate syringes were used to withdraw from the vials the volume of medicine needed to reach the desired concentrations (i.e. 4.0 mg/mL and 0.6 mg/mL) in the 50 mL NaCl 0.9 % polyolefin infusion bags (Baxter, Spain, batch number: 19E05E3S/ expiration date: 07–2020), from which the whole contents had previously been extracted with 50 mL BD Plastipak syringes (batch number: 1707229/ expiration date: 06-2022). The necessary volumes of medicine and solution were then added to the bags to prepare the final concentrations. Due to the limits on obtaining samples of the medicine, we prepared two independent replicates for each storage container as accepted for this situation [8]. Therefore, two bags of each concentration were prepared.

2.2.2. Methodology applied

The stability-indicating nature of all the methods used in this study was validated by accelerated degradation studies performed in a recently published work about ziv-AFL [21,22], in which the following stresses were applied: 12 h light irradiation, exposure to high temperature (60 °C and 70 °C), subjection to several freeze/thaw cycles, pH variation of the drug product, dilution in a hypertonic 1.5 M NaCl solution and 6 M guanidine HCl denaturing solution.

2.2.3. Stability studies over time

4 and 0.6 mg/mL ziv-AFL clinical solutions, stored in polyolefin bags, were analysed for a period of 14 days in order to assess their stability over time when stored refrigerated (2−8 °C) in darkness. In all cases, Day 0, the day when the clinical solutions were prepared, was established as the control or reference day, and the results from all subsequent experiences were compared to this day.

The stability of the two clinical solutions, i.e. 4.0 and 0.6 mg/mL, was studied by using the analytical techniques included in Table 1 (Supplementary Data), over a period of 14 days. The solutions were analysed on: Day 0 (preparation of the clinical solutions), Day 1, Day 2, Day 7or 8, Day 10 or 11 and Day 14. All the techniques were used at these intervals, except for the fluorescence spectroscopic technique, for which the analyses had to be stopped on Day 10, due to technical problems.

2.3. Analytical methods

2.3.1. Visual inspections

Visual inspections were carried out prior to experimentation in order to detect the formation of large aggregates, turbidity, suspended particles, colour changes and gas formation. To this end, samples were visually inspected using the naked eye.

2.3.2. Far UV circular dichroism (CD)

Ziv-AFL secondary structures were studied using a JASCO J-815 spectropolarimeter (JASCO, Tokyo, Japan). The method was optimized at a concentration of 0.25 mg/mL of ziv-AFL. Each spectrum was registered with a scanning speed of 20 nm/min, every 0.2 nm and a total of 5 accumulations were averaged between 200 and 250 nm. A 1 mm quartz cuvette was used throughout the stability study. Temperature was controlled at a constant 20 °C. Raw spectra were smoothed using the Means-Movement method with a convolution width of 25. All the spectra were visually inspected in order to detect changes in the secondary structures of ziv-AFL. The content of the secondary structure was estimated using the Dichroweb website [25].

2.3.3. Intrinsic tryptophan fluorescence spectroscopy (IT-FS)

For Emission, spectra measurements were carried out on a Cary Eclipse spectrofluorometer (Agilent, U.S.A) and recorded from 300 to 400 nm with the excitation wavelength set to 298 nm. The temperature of the samples was kept at 20 °C using a thermostatically controlled cell holder. A total of 5 spectral accumulations were averaged per spectrum. The excitation and emission slits were set to 5 nm for the 4 mg/mL bag samples and to 10 nm for the 0.6 mg/mL bag samples. The blank of the appropriate solvent without the antibody was measured and then subtracted from each sample spectrum. Each spectrum was converted mathematically to a number (Spectral Centre of Mass) from 300 to 400 nm, with the following equation:

$$S.C.M. = \frac{\sum(\lambda_i f_i)}{\sum f_i}$$

Where λi is the wavelength and fi the corresponding intensity

2.3.4. Intrinsic protein fluorescence spectroscopy (IP-FS)

Emission spectra measurements were carried out on a Cary Eclipse spectrofluorometer (Agilent, U.S.A) from 300 to 400 nm, with the excitation wavelength set to 280 nm. The temperature of the samples was kept at 20 °C using a thermostatically controlled cell holder. A total of 5 spectral accumulations were averaged per spectrum. Excitation and emission slits were set to 5 nm. The blank of the appropriate solvent without the antibody was measured and then subtracted from each sample spectrum. Each spectrum was converted into a number (Spectral Centre of Mass, C.M.) from 300 to 400 nm using Equation 1.

2.3.5. Dynamic light scattering

DLS records were obtained on a Zetasizer Nano-ZS90 Malvern (UK). Particle size distribution was determined on 1 mL of sample volume using 10 mm spectrophotometry disposable cuvettes. Each sample record was a result of 100 accumulated readings taken at a thermostatically controlled temperature of 20 °C and with a time acquisition of 5 s per reading. The average hydrodynamic diameter (HD), polydispersity index (PDI), Z-average and size distribution by volume and intensity were compared and discussed for all the bag samples over the 14-day study period.

2.3.6. Size-exclusion high-performance chromatography with diode array detection (SE/HLPC-DAD)

The analysis was performed by liquid chromatography using an Agilent 1100 chromatograph equipped with a quaternary pump, degasser, autosampler, column oven and photodiode array detector (Agilent Technologies, USA).

Samples were evaluated in a SEC-5 column (300Bio, Agilent Technologies, USA) in which temperature was set at 25 °C. The flow rate was 0.35 mL/min and the mobile phase was composed of 150 mM of phosphate buffer pH 7.0. An isocratic elution mode was applied for 17 min. The UV–vis spectra were recorded between 190 nm and 400 nm with a data point every 0.5 nm. Chromatograms were registered at 214 nm with a reference band at 390 nm. This method was previously reported [21].

The column was calibrated in order to establish the relationship between the molecular weight and the retention time of the Fc-protein. The calibration kit (Agilent, USA) was composed of 5 proteins: thyroglobulin (670 kDa), γ-globulin (150 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and angiotensin II (1 kDa).

2.3.7. Strong cation exchange ultra-high-Performance chromatography (SCX/UHPLC-UV) charge variant analysis

This analysis was conducted using a proper analytical platform (Thermo Scientific, Waltham, MA, USA). The chromatographic separation was carried out using a Dionex UltiMate 3000 chromatograph (Thermo Scientific, Waltham, MA, USA), equipped with two ternary pumps, a degasser, an autosampler, a thermostatted column compartment, and a multiple-wavelength detector (MWD-3000Vis-UV detector). The method was previously reported [22] and developed to detect changes in the ziv-AFL charge variant profile.

Chromatographic separation was carried out in a MabPac SCX-10 RS 2.1 mm × 50 mm column with 5 μm particles (Thermo Fisher Scientific, Waltham, MA, USA). The column temperature was 30 °C and the flow rate was 0.4 mL/min. The eluent system was composed of 10 mM of Na_2HPO_4 at pH 5.0 (mobile phase A) and 10 mM of Na_2HPO_4 and 300 mM of NaCl at pH 10.0 (mobile phase B). The column was equilibrated with 10 % of mobile phase B for 9 min. A linear gradient was applied when the percentage of mobile phase B was increased from 10 % to 40 % in 15 min. The percentage of mobile phase B was then increased again to 100 % in 1 min and was maintained at this level for 4 min. The initial conditions were restored in 1 min. The UV chromatograms were registered at 214 nm [22].

2.3.8. ELISA

An indirect, non-competitive ELISA based on the specific interaction of ziv-AFL with VEGF was applied to check for ziv-AFL biological activity, and was then used to study the diluted ziv-AFL clinical solutions stored in the bags over a period of 14 days. This method was adapted from our previous works [21,26]. In brief, 96-well Maxisorp immune plates were sensitized with VEGF by incubation overnight (18 h) at 4 °C adding 100 μL/well of 0.25 μg/mL VEGF diluted in 0.1 M carbonate buffer solution (pH 9.6). The plates were washed (VASHER RT-2600C microplate washer, Comecta, Abrera, Barcelona) four times with PBS-Tween® 20 (pH 7.4 containing 0.3 % (v/v) Tween® 20). They were then treated with 100 μl of the blocking buffer (PBS pH 7.4 containing skimmed milk 2 % (w/v)) per well for 2 h at 37 °C to eliminate nonspecific absorptions. Each plate was washed again and filled with 100 μL of ziv-AFL control samples and samples from the bags appropriately diluted in 0.1 M carbonate buffer pH 9.6 at several concentrations ranging from 1 ng/mL to 100 μg/mL. After incubating for 45 min at 37 °C, the plates were washed four times with PBS and incubated again with 100 μl/well of a solution of 10 μg/mL of peroxidase-labelled rabbit anti-human IgG prepared in 0.1 M carbonate buffer solution pH 9.6 for 30 min at 37 °C. After washing four times with PBS, 100 μL of the substrate solution (OPD) was added to each well and incubated for 20 min in darkness at room temperature (25 °C). The reaction was stopped by adding 50 μL of 1 M sulfuric acid solution. The absorbance was recorded at 450 nm and 620 nm, and the analytical signal was the difference between the two absorbance values (TECAN SUNRISE™ microplate absorbance reader for 96-well plates connected to a computer loaded with XFluor4 software, Tecan, Austria, GMB). On each test day, the absorbance of the ziv-AFL bag samples was compared to that of freshly prepared ziv-AFL samples.

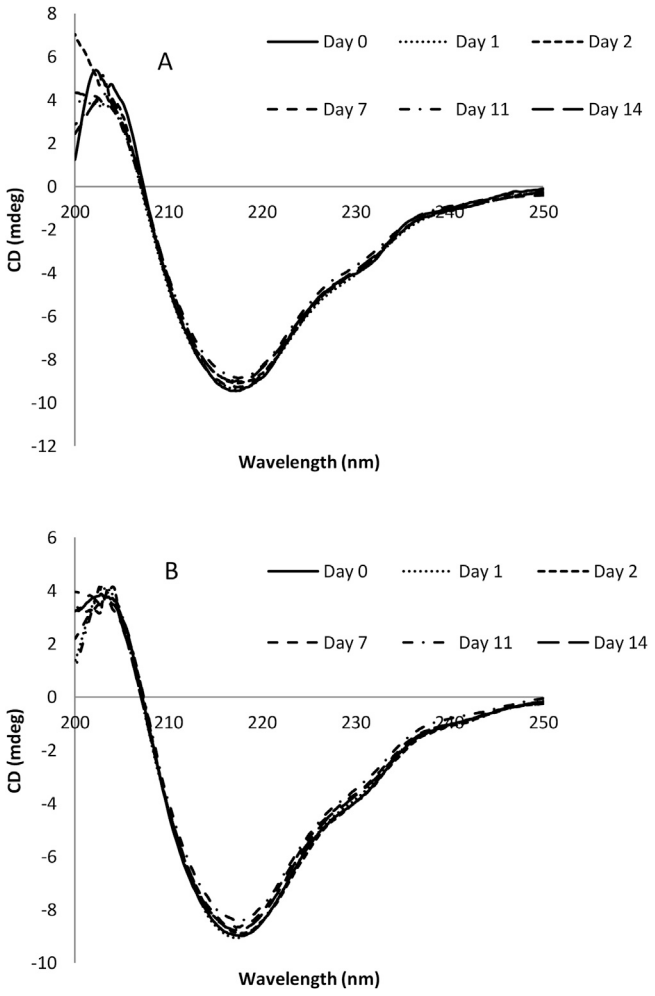

Fig. 1. Representative Circular Dichroism (CD) spectra of ziv-AFL (Zaltrap®) solutions stored in polyolefin infusion bags over the 14-day stability study period: A) 4 mg/mL bag; B) 0.6 mg/mL bag.

3. Results

3.1. Visual inspections

All the solutions remained clear without turbidity throughout the stability study, indicating that no visual aggregates had formed. The solutions also remained colourless throughout the study period.

3.2. Circular dichroism spectroscopy (CD)

The two bag replicate samples for each concentration studied showed practically identical spectral features. Therefore, Fig. 1 shows the ziv-AFL representative spectra (from one of the two

Table 1
CD spectral features of ziv-AFL representative bags samples over the stability study period: 4 and 0.6 mg/mL.

4 mg/mL	*Spectral minima*	*Wavelength (CD = 0)*	*Minima Ellipticity*	0.6 mg/mL	*Spectral minima*	*Wavelength (CD = 0)*	*Minima Ellipticity*
Day 0	217.4	207.4	−9.3	Day 0	217.8	207.2	−9.0
Day 1	217.4	207.0	−9.4	Day 1	217.4	207.1	−9.1
Day 2	217.2	207.1	−9.1	Day 2	217.6	207.3	−9.0
Day 7	217.4	207.0	−9.3	Day 7	217.2	207.1	−8.7
Day 11	217.4	207.2	−8.9	Day 11	217.6	207.3	−8.5
Day 14	217.0	207.2	−9.0	Day 14	217.0	207.0	−8.8

CD (Circular Dichroism).

Table 2
Representative fluorescence spectral centre of mass (C.M) values of the ziv-AFL bags samples over the stability study.

C.M. (at λexc 298 nm, IT-FS)						
C.M.	Day 0	Day 1	Day 2	Day 7	Day 10	Day 14
4 mg/mL Bag	346	346	346	346	346	–
0.6 mg/mL Bag	345.8	345.7	345.8	345.8	345.8	–
C.M. (at λexc 280 nm, IP-FS)						
C.M.	Day 0	Day 1	Day 2	Day 7	Day 10	Day 14
4 mg/mL Bag	343.9	343.8	343.9	343.9	343.9	–
0.6 mg/mL Bag	343.9	343.9	343.9	344.0	343.9	–

C.M (Centre of Mass); λexc (excitation wavelength); IT-FS (Intrinsic Tryptophan Fluorescence Spectra); IP-FS (Intrinsic Protein Fluorescence Spectra).

replicates) for the bag samples from Day 0 to Day 14 for 4.0 and 0.6 mg/mL bag samples. These representative spectra are described in more detail in Table 1 via their spectral features. As can be seen in Fig. 1, the ziv-AFL CD spectra are identical for the 4.0 and 0.6 mg/mL bag samples. The spectral shape remains unchanged over the 14 days. A more detailed analysis of these spectral features of ziv-AFL is shown in Table 1, including the CD spectrum minimum and the wavelength at ellipticity 0. Ziv-AFL has a minimum at 217−218 nm and a wavelength (ellipticity = 0) at 207–207.5 nm. These values were corroborated to remain unchanged in all the bag replicate samples throughout the study, so indicating that no changes had taken place in the secondary structure of the ziv-AFL in any of the bag samples studied. An estimation of the secondary structure content can be found in the Supplementary Data (Tables 2 and 3). These results indicate that secondary structure of ziv-AFL remained unchanged over the 14-day test period for both concentrations assessed.

3.3. Intrinsic tryptophan fluorescence spectroscopy (IT-FS)

The tertiary structure of ziv-AFL in the solutions at 4.0 and 0.6 mg/mL was analysed by IT-FS. Representative results of the fluorescence C.M. from one of the bag replicates for each concentration studied are shown in Table 2 since the standard deviation between the two replicates were close to 0 value. These results in Table 2 indicate that this CQA remained unchanged throughout the test period (10 days). The representative C.M. value of the IT-F spectra for the 4 mg/mL ziv-AFL bag samples was 346 nm at Day 0; this value remained constant over the 10-day test period for two replicates. For the two bag samples of 0.6 mg/mL ziv-AFL, this value was 345.8 at Day 0 and also remained unchanged over the 10 days of study.

3.4. Intrinsic protein fluorescence (IP-FS)

The fact that the tertiary structure of the ziv-AFL in the clinical solutions remained unchanged was also corroborated similarly by IP-FS. The representative C.M. of the spectra from the 4 mg/mL and the 0.6 mg/mL ziv-AFL solution bag samples was 343.9 at Day 0; value that remained over the 10-day study period for all the samples (Table 2).

3.5. Dynamic light scattering (DLS)

Representative results for particulates of up to 10 μm in the solutions over time are shown in Fig. 2 (volume size distribution graphs) and in Table 3 (Z-average and PDI values). For Z-average and HD results, statistical comparison was performed on the results from the two bag replicates for each concentration at a confidence level of 95 %, corroborating there was not statistical differences between the two values compared. Therefore, the results show next are from one of these two bag replicates as representative.

In Fig. 2 there are two size distribution graphs (A and B) for the 4 and 0.6 mg/mL ziv-AFL bag samples; in both cases, a single particulate population was detected and was attributed to protein monomers with an average HD of 11 ± 3.4 nm and 11.3 ± 3.1 nm respectively. These values were maintained over the 14 days with no significant differences as can be corroborated in graphs A and B of Fig. 2.

As regards the size distribution by intensity shown in Supplementary Data (Fig. 2); for 4 mg/mL bag samples, the only peak that contributed to the intensity was the protein monomer population. The only exception was on Day 0, in which a peak was detected at 2.5 μm, although this contributed just 0.1 % of total volume, and was therefore considered an artefact, and was not observed in the other bag replicate. For 0.6 mg/mL bag samples, intensity was caused mainly by the peak associated with the protein monomer population, with other peaks contributing just 0.1 %, and therefore being considered as artefacts, and similarly occurs in the other bag replicate.

The Z-average values for the ziv-AFL 4 mg/mL solutions (Table 3) indicated that no aggregation process was taking place as the values remained similar throughout the 14-day period in all the samples. Results for the most diluted solution at 0.6 mg/mL were discarded as the concentration was too low to enable us to interpret this parameter. As regards the PDI value (Table 3), this ranged between 0.1−0.2 for ziv-AFL 4 mg/mL bag samples and between 0.1−0.5 for 0.6 mg/mL bag samples. These results indicated that there was no significant dispersion in the size of the population, as it was always < 0.7 [27] for all samples. For 0.6 mg/mL samples the higher PDI value obtained in some of the samples (up to 0.5) was also attributed to this higher dilution of the ziv-AFL sample, which could

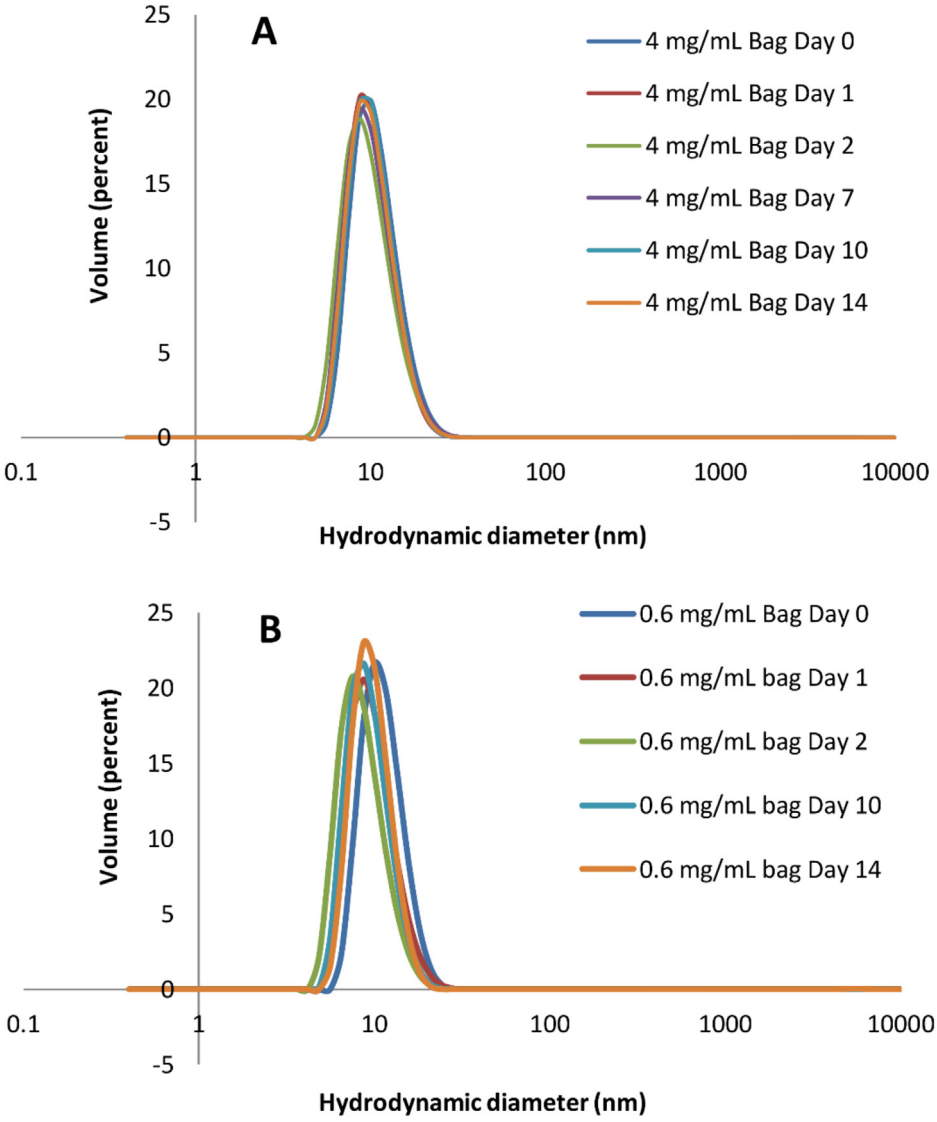

Fig. 2. Representative size distribution by volume of 4 mg/mL A) and 0.6 mg/mL B) ziv-AFL (Zaltrap®) solutions stored in polyolefin bags over 14 days.

Table 3
Z-average and polydispersity index (PDI) values for the representative ziv-AFL bags samples over the stability study.

	Z-average	PDI	
Day	4 mg/mL bag	4 mg/mL bag	0.6 mg/mL bag
0	14 ± 5	0.25	0.32
1	11 ± 6	0.10	0.46
2	12 ± 5	0.15	0.28
7	12 ± 5	0.15	0.28
10	12 ± 5	0.18	0.16
14	12 ± 5	0.18	0.22

PDI (Polydispersity Index).

make the results not interpretable, not only for the Z-average but also for the PDI value.

3.6. Size-Exclusion chromatography (SE/HLPC-DAD)

The chromatographic profile for the oligomers was checked using (SE)HPLC-DAD, so as to detect possible incipient aggregation in the ziv-AFL bags (4 and 0.6 mg/mL). Representative control chromatograms (those registered at Day 0) are shown in Fig. 3. Both concentrations tested (considering the two bags replicates, and in this case two replicates of each bag) were characterised by two peaks: one small peak eluting at 6.7 ± 0.2 min and another, the main peak, eluting at 7.57 ± 0.1 min. The first peak was attributed to the presence of the natural dimer (representing 2% of the total area for ziv-AFL in the chromatogram) and the second peak was attributed to the monomer in accordance with previous works [21] in which this issue was investigated in depth.

Fig. 3 shows the UV-SEC chromatograms corresponding to representative replicates of ziv-AFL in the stability study, showing the results at Day 0 (control), 7 and 14. These chromatographic profiles remained unchanged over the study period, in terms of both the elution time and the area under the peak, as can be seen in Fig. 3 for the two concentrations of ziv-AFL studied. The value of the area of the peaks corresponding to the dimer and the monomer remained constant over the period of study, as did the proportion between them. No new aggregations or fragmentations were detected on any of the test days. The analysis of all chromatographic profiles clearly indicates high stability and a high degree of similarity in the solutions over the study period.

The concentration of the ziv-AFL bags solutions was estimated using the chromatographic area under the peak and this was continued throughout the study period. The concentration estimated

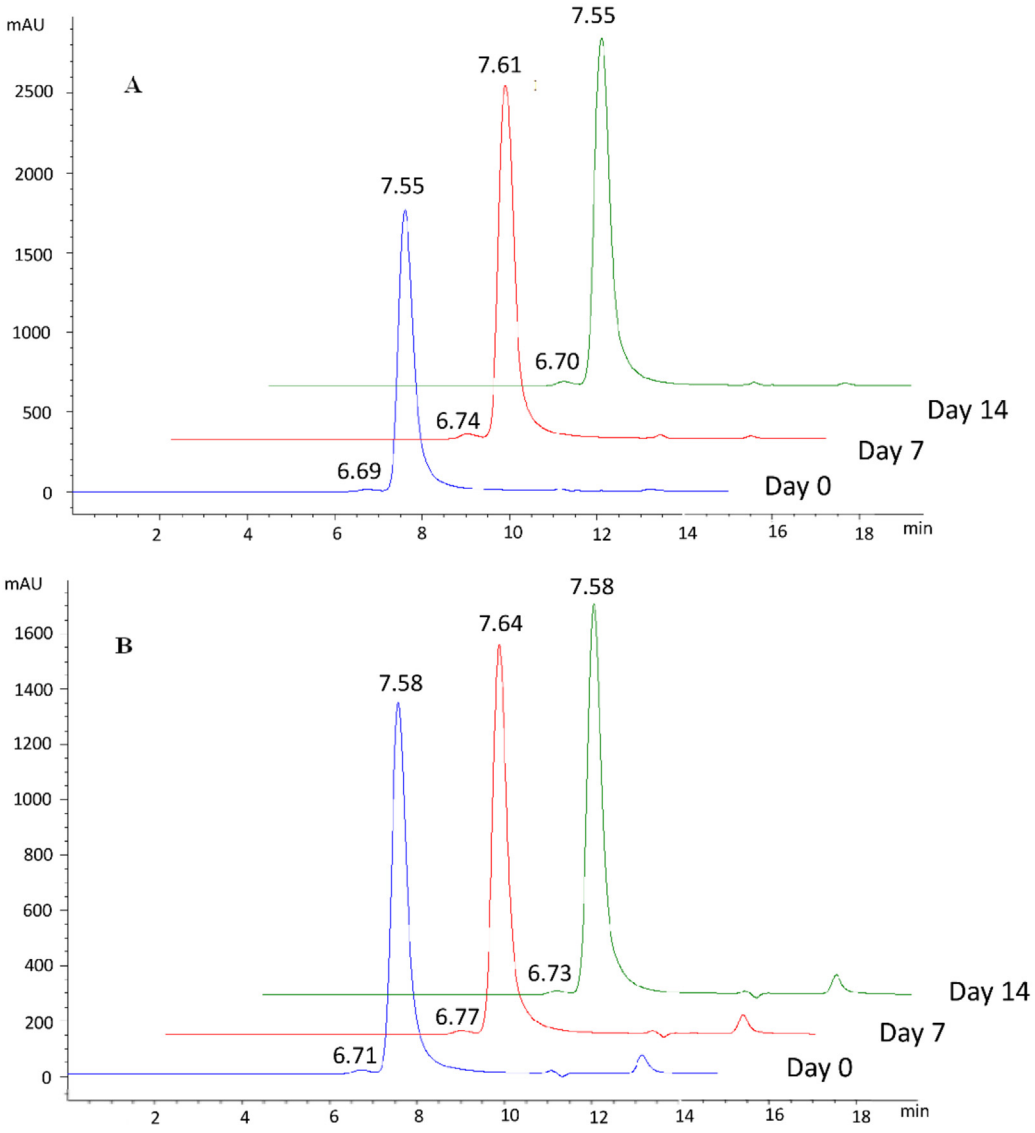

Fig. 3. Representative size exclusion high-performance chromatography with diode array detection (SE-HLPC-DAD) chromatograms of Ziv-AFL (Zaltrap®) solutions stored in polyolefin bags over the 14-day study period: A) 4 mg/mL bag. B) 0.6 mg/mL bag.

was always at around 4 ± 0.27 mg/mL and 0.6 ± 0.20 mg/mL. No significant changes were observed, indicating that the concentrations of ziv-AFL remained constant over the 14-day study period, with no adsorption on the wall of the container or degradation.

3.7. *Strong cation exchange ultra-high-Performance chromatography ((SCX)UHPLC/UV)*

The variant profile is one of the most frequently analysed characteristics in protein stability studies and is related with the presence or absence of different posttranslational modifications. In this paper, the charge variant profile was assessed using the (SCX)UHPLC/UV method, which had previously demonstrated its ability to detect changes when ziv-AFL samples were submitted to different physical stresses [22].

This method was applied to monitor the ziv-AFL charge variants in the clinical solutions at 4 and 0.6 mg/mL during storage in polyolefin bags for 14 days. Fig. 4 shows the (SCX)UHPLC/UV chromatograms for representative replicates of samples from ziv-AFL bags at Day 0, 7 and 14. These chromatograms have very similar profiles with the same peaks as on Day 0. No acidic or basic peaks appear. The retention times are also similar over time for all the samples (9.52 ± 0.03 min and 9.59 ± 0.05 min for ziv-AFL 4 mg/mL and 0.6 mg/mL bag samples respectively, estimated using results from the two bag replicates, and in this case two replicates of each bag). A more detailed discussion of this particular charge variant profile of ziv-AFL can be found in [22]. The fact that no changes were detected in the different charge variants of ziv-AFL indicates that no chemical changes had taken place in the clinical solutions.

3.8. *ELISA*

The ELISA method was developed and validated *ad hoc* for the quantification of the specific interaction between ziv-AFL and VEGF, as a mean of checking the functionality of this Fc-fusion protein [21]. Fig. 5 shows the results of the present stability study, in which Ziv-AFL-VEGF binding curves are represented over the 14-day study period for the two concentrations studied (including the results from the two bags). In the graphs, the absorbance ± SD is represented as a function of the concentration of ziv-AFL. Control samples (fresh samples prepared "there and then") were used for comparison purposes each check day and their results are shown in

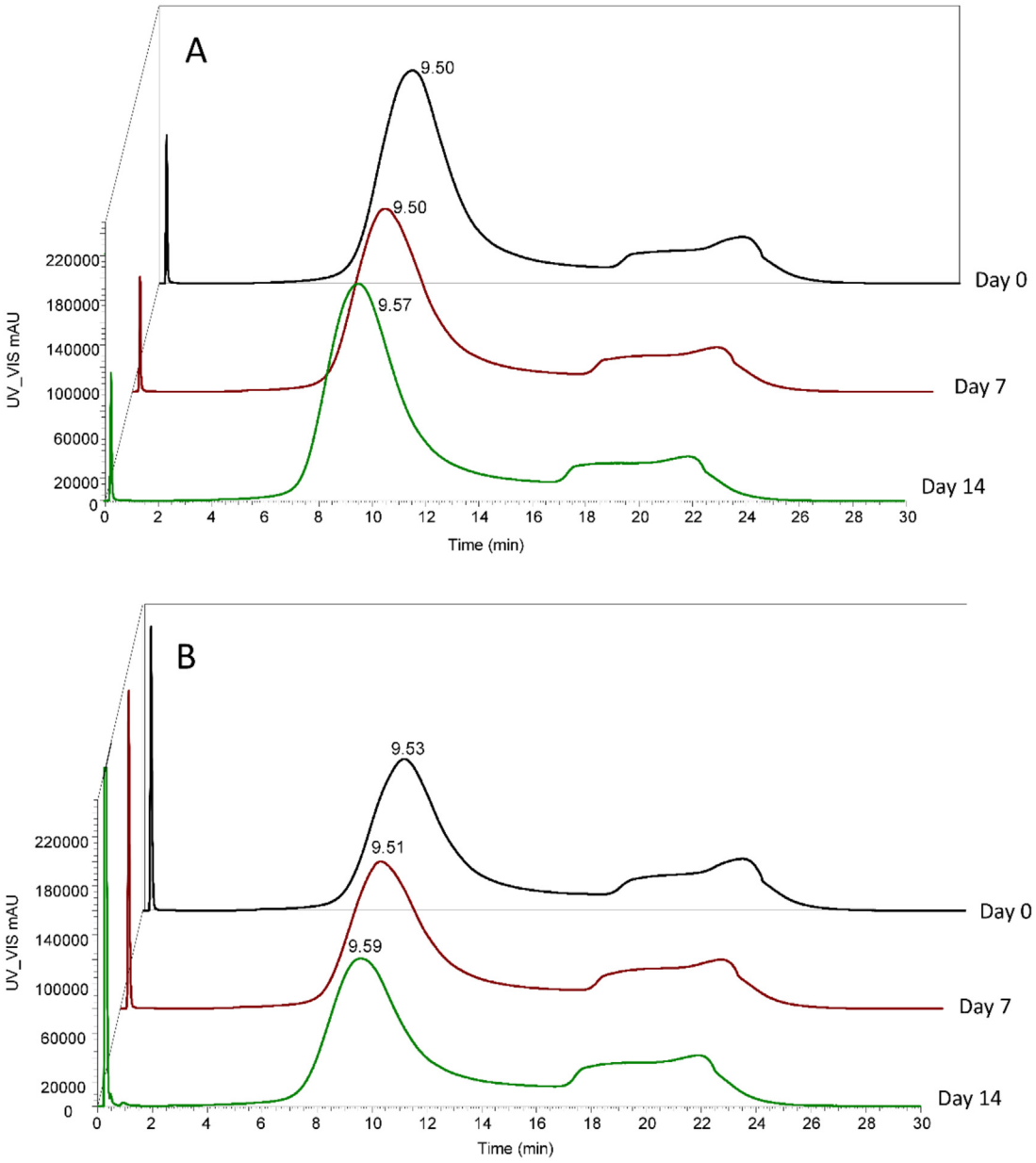

Fig. 4. Representative strong cation exchange high-performance chromatography with ultraviolet detection (SCX/UHPLC-UV) chromatograms of Ziv-AFL (Zaltrap®) solutions stored in polyolefin bags over the 14-day study period: (A) 4 mg/mL bag. (B) 0.6 mg/mL bag.

blue; for ziv-AFL 4 mg/mL bag samples the results are displayed in red while the 0.6 mg/mL samples appear in green. Broadly speaking, there is no significant decay in the absorbance of the samples analysed with respect to the control samples at any concentration (4 or 0.6 mg/mL ziv-AFL) or at any time during the study period (14 days), therefore confirming the functional stability of these clinical solutions for at least 14 days. As can be seen in Fig. 5, the behaviour of the immunoreaction ziv-AFL-VEGF was very similar to the control samples, as indicated by the similar tendency in the graph, with no significant differences (see error bars) between the particular experimental concentrations used to build the graph. Only two experimental points (indicated in Fig. 5 with an "*" in the 0.6 mg/mL bag sample on Day 1 and in the 4 mg/mL bag sample on Day 14) showed significant decay in the absorbance, which was attributed to an unexpected error while manipulating the samples. Nevertheless, these two experimental points do not affect the results, because no significant differences were detected in any of the other experimental points and the tendency of the graphs clearly indicated similar ziv-AFL-VEGF interaction in the control (fresh) samples and in the bag samples at concentrations of 4 and 0.6 mg/mL.

4. Discussion

A set of previously validated analytical methodologies [21,22] were selected for a comprehensive analysis of the stability of the complex Fc-fusion protein ziv-AFL in real hospital conditions of use – clinical solutions placed in polyolefin infusion bags – stored refrigerated at 2–8 °C for two weeks. In this paper, analytical similarity was assessed in terms of the values of the main CQA of the ziv-AFL samples over the 14 days of the study. Two clinical concentrations of ziv-AFL were evaluated: one at an intermediate level of clinical concentration (4 mg/mL) and the other at a lower level (0.6 mg/mL).

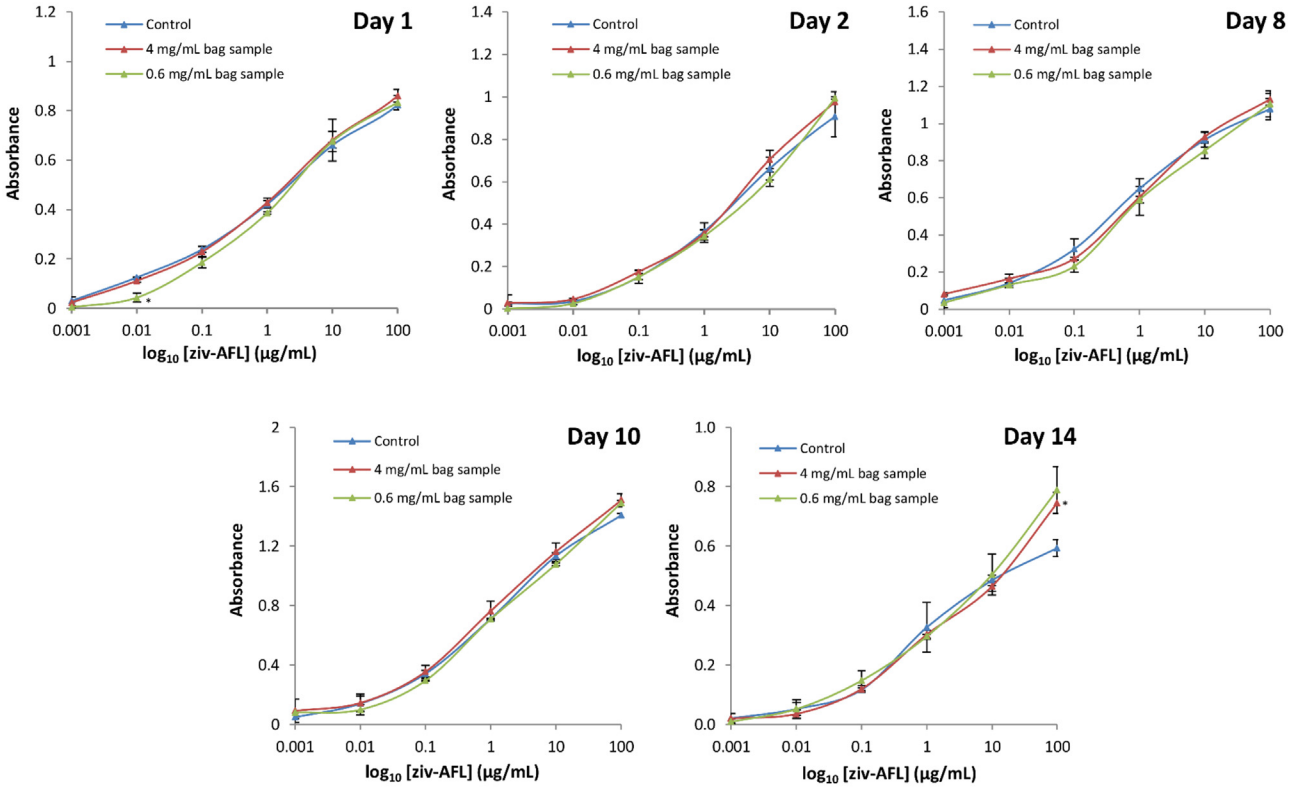

Fig. 5. Ziv-AFL (Zaltrap®)-VEGF non-competitive indirect ELISA graphs: absorbance represented as a function of ziv-AFL concentration (0.001-100 µg/mL) over the 14-day study period. Control samples (blue line); 4 mg/mL bag (red line) and 0.6 mg/mL bag (green line) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Aggregation was evaluated using three analytical methods: a) by analysing oligomers by SEC, b) by analysing larger soluble particulate populations up to 10 µm by DLS and c) by visual inspection. The results indicated that no changes had taken place in the initial particulate populations in the solutions and therefore that the clinical solutions remained unchanged. The SE-chromatographic profile indicates that oligomerization of ziv-AFL did not occur in any of the bag solutions over the 14-day study period. The particulate profile obtained from DLS indicated that aggregation did not take place in either of the bag solutions over the check period. The solutions remained clear in all visual checks. Therefore, no instability due to aggregation occurred in the 14-day study period in either of the solutions (4 or 0.6 mg/ mL), and the ratio between the natural ziv-AFL dimers and monomers remained the same throughout (around 2 % dimers/98 % monomers), an important finding given that in previous research on ziv-AFL it was demonstrated that the main degradation pathway in this therapeutic Fc-fusion protein was dimerization and the formation of high molecular weight aggregates (non-natural) [21]. Nonetheless, our results indicate that the controlled storage conditions applied in this study prevented aggregation.

The secondary structure of the protein was assessed by CD spectroscopy, and it was deduced that in both clinical solutions of ziv-AFL (4 and 0.6 mg/mL) the secondary structure remained stable over the 14-day period. The ziv-AFL CD spectra for the two samples remained unaltered as did the ellipticity. These results indicate that no β-amyloid aggregates were formed during the study period, as in recent research on the same protein we observed, using CD spectroscopy, a clear increase in the negative band associated with the same aggregation process [21]. Moreover, the estimation of the content of secondary structure using Dichroweb [25] indicated a high percentage of β sheet and random coil, which remained unaltered over the 14 days of the stability study.

The tertiary structure of ziv-AFL was assessed by estimating the tryptophan fluorescence spectral centre of mass. This remained constant over the 10 days tested in both ziv-AFL bag solutions. This confirms that the tryptophan environment in the protein conformation was unaltered. This finding, together with the results obtained from the intrinsic protein fluorescence test, allowed us to conclude that protein conformation in these solutions remained unaltered over the 10-day period.

Ziv-AFL is a very complex glycosylated Fc-fusion protein, which has a protein structure with a molecular weight of 97 kDa. According to the manufacturer, the glycosylation part increases the weight of the molecule by 15 % (up to 115 kDa) [21]. This complex glycan structure makes it difficult to develop analytical methods that can properly identify the different AFL charge variants [28]. However, the charge variant profile is a CQA that should be included in any study of stability. In previous research, a (SCX)UHPLC-UV method was shown to be capable of detecting changes in the charge variant profile of ziv-AFL, although it did not manage to identify or separate the different charge variants [28]. We therefore decided to use this method here to track changes in the charge variant profile of ziv-AFL in the clinical solutions, so as to assess the stability of these solutions over time. As these profiles remained constant, with high levels of similarity amongst all the samples regardless of the concentrations studied (4 or 0.6 mg/mL), this indicates that over the 14 days no chemical changes took place that might have affected the charge profile of ziv-AFL in these clinical solutions.

Finally, if we take into account the functionality of ziv-AFL measured as the capacity of the protein to bind to its target, i.e the VEGF; our results show that the Fc-fusion protein maintained its functionality over the 14-day study period, with no evidence of functional loss in either clinical solution. This is also coherent with the results obtained above using fluorescence spectroscopy (once the stabil-

ity of the other quality attributes had been demonstrated), in that a decrease in functionality is usually accompanied by a conformational change.

Our results demonstrate high physicochemical and functional stability of ziv-AFL during the 14-day stability study. The values for the CQA of the ziv-AFL samples were very similar to those for the control samples, i.e. the samples analysed immediately after preparation. The analytical results were also highly similar for the two concentrations tested, i.e. 4 mg/mL and 0.6 mg/mL.

5. Conclusions

In this study we analysed two concentrations (an intermediate concentration of 4 mg/mL in 0.9 % NaCl and a low concentration of 0.6 mg/mL in 0.9 % NaCl) of ziv-AFL clinical solutions prepared aseptically in polyolefin infusion bags and stored refrigerated in darkness. A specific set of analytical techniques were used to evaluate the main physicochemical CQA in addition to monitoring the functional integrity of the Fc-fusion protein over the 14-day test period by assessing its capacity to bind to its target, the VEGF. These tests revealed very similar results over the two-week period indicating a high degree of stability in both solutions. All the methods and strategies applied in this study had previously been validated to confirm their ability to detect changes in the structure or functionality of ziv-AFL. No changes were detected in the CQA that might impair the efficacy and safety of the medicine. These results will encourage hospital pharmacists to reuse Zaltrap® when stored in the conditions studied here.

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Author contributions

N.N. designed and supervised the work and wrote the paper with the contribution of J.H. and R.P.-R., who prepared figures; J.H. performed the spectroscopic experiments and ELISA supervised by S.C. including samples stress and figures supervising; R.P.-R. performed the chromatographic experiments; A.S. and J.C. designed and prepared samples and A.S. designed stability study and supervised stress of the samples, J.H., R.P.-R., A.S., S.C., J.C and N.N. discussed and approved final version of the manuscript.

Data Availability

The datasets generated and/or analysed during this research are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114209.

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