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Stability of infliximab solutions in different temperature and dilution conditions

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Key words

Infliximab; monoclonal antibody; Chemistry Techniques, Analytical; stability; dilution; temperature;

Highlights

- Storage at 25°C of diluted infliximab solutions did not increase aggregation levels
- Chemical instability of diluted infliximab was the main limiting factor at 25°C
- A single freeze-thawing cycle of diluted infliximab caused significant instability
- Diluted Infliximab solutions were physicochemically stable 90 days at 5°C

Abstract

Infliximab is a monoclonal antibody widely used for the treatment of inflammatory diseases. Over the past few years, many studies have assessed that monoclonal antibodies are prone to aggregation under stress conditions, such as temperature changes and agitation. The aim of this study was to assess stability of infliximab (Infliximab®) solutions at different concentrations. These solutions were separately submitted to three temperature conditions that are likely to happen during the drug dispensing system: -20°C, 5°C and 25°C. To perform a complete characterization of infliximab physicochemical and structural stability a wide range of analytical techniques were employed including: visual inspection, subvisible particles counting (HIAC), dynamic light scattering (DLS), size exclusion chromatography (SEC), cation exchange chromatography (CEX), and analysis of primary, secondary and tertiary structure. When stored at 25°C and 5°C CEX was the main limiting factor, while SEC showed only some acceptable variation. After a single freeze-thawing cycle, the amount of subvisible particles was significantly increased. Some variations were also visible in CEX and hydrodynamic diameter was increased after thawing 10 mg/mL samples. In regard of these results, infliximab (Infliximab®) solutions should not be used after a single freeze-thawing cycle between reconstitution and administration to the patient. The results showed a stability at 5°C of up to

14 days for 10 mg/mL solutions and 90 days for 0.4 and 2 mg/mL solutions, whilst samples stored at 25°C were stable only 7 days in original glass vials and 30 days when diluted.

1. Introduction

For several years now, treatments based on biological molecules have become ever more established as key therapeutic approaches. Infliximab is an IgG1 monoclonal antibody (mAb) that binds and inhibits transmembrane and soluble forms of Tumor Necrosis Factor alpha (TNF- α). It is widely used in rheumatology for the treatment of rheumatoid arthritis in association with methotrexate, spondylitis ankylosing and psoriatic arthritis, and in gastroenterology for Crohn's Disease and ulcerative colitis and in dermatology for psoriasis. In 2013, infliximab CT-P23 became the first biosimilar monoclonal antibody approved by the EMA [1] and by the FDA in 2016 [2].

In a stability study one of the keys is to quantify the active substance and highlight any type of degradation that could alter the integrity of the studied molecule, and analytical techniques therefore play an essential role in this kind of studies. Yet, the stability of monoclonal antibodies (mAbs) is rather complex to assess due to the multiples degradation pathways. Infliximab, like other mAbs and biopharmaceutical drugs, is susceptible to many factors that can affect its stability in solution, such as temperature [3,4], pH, oxidation [5] and agitation [6]. Degradation of mAbs can be physical or chemical more or less related to structural modifications. Even if the complete mechanism has not been fully described yet, monoclonal antibodies are also well known for their tendency to form aggregates under stress conditions [3,7]. Aggregation of mAbs occurs at different level and no lone analysis is able to completely evaluate the whole phenomenon. Different analytical techniques bring specific information about nanoparticles, subvisible particles or aggregates molecular weight for example. All these techniques supplement one another and should be associated in order to increase the level of evidence, and a recent European consensus recommends the use of several analytical techniques to assess their physicochemical stability [8]. Pharmaceutical industries have enhanced the formulation of their biological drugs to slow down quantitative and qualitative modifications and thus extend their stability, following ICH guidelines [9–11]. Forced degradation studies also give information upon degradation mechanisms but are not always representative of what happens in real use conditions [12]. There is a lack of data on the long term storage of the Mabs and such studies are difficult to perform due to the high cost of this kind of drugs, thus depriving healthcare practitioners of critical information about mAbs stability in clinical use conditions.

Stability of reconstituted and diluted infliximab solutions is set up to 24h for both biosimilars (according to summary of product characteristics of both REMSIMA®[3] and INFLECTRA®[4]), limited by a hypothetically short microbiological stability. But when prepared in controlled atmosphere area

microbiological stability is less of an issue, and overall stability depends on the limits of physicochemical stability.

The aim of this study was to evaluate the physicochemical stability of a biosimilar mAb (infliximab INFLECTRA®) for a wide concentration range at different storage temperatures, such as those possibly encountered in clinical situations for up to 90 days, using complementary analytical assays in order to understand the limits of mAb conservation in clinical settings.

2. Materials and methods

2.1 Materials and reagents

Inflectra® powder for solution for infusion (batch 2485036, expire on 09/30/2020) was provided for free of charge by Pfizer France SAS (Meudon, France). Sterile water for injection (Ecoflac®) was purchased from B Braun Medical (SAS, Boulogne Billancourt Cedex, France) and sodium chloride 0.9% infusion bags were purchased from Fresenius Kabi (Louviers, France). Sodium sulfate (Na_2SO_4), disodium phosphate (Na_2HPO_4), sodium azide (NaN_3), 4-Morpholinoethanesulfonic acid (MES), sodium chloride (NaCl), Guanidine Hydrochloride (GnHCl), Tris hydrochloride (Tris HCl), ammonium carbonate, dithiothreitol (DTT), iodoacetic acid, acetylated trypsin, sodium hydroxide (NaOH), hydrogen chloride (HCl), acetonitrile and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (Saint Quentin Fallavier Cedex, France). All reagents were certified of HPLC grade.

2.2 Preparation and conditioning

10 mg/mL infliximab solutions were sterilely prepared from Inflectra® powder for solution for infusion by adding water for injection to the medication vial, according to the manufacturer's instructions using a sterile metal needle (Blunt Fill Needle 18G x 1 ½", Becton Dickinson, Spain), under the laminar air flow of an ISO 4.8 microbiological safety cabinet. Solutions were then diluted to 0.4 and 2 mg/mL into 0.9 NaCl infusion bags.

2.3 Study design

The prepared units were then stored in the dark for up to 30 days for the 10 mg/mL solutions and 90 days for the 0.4 and 2 mg/mL solutions at controlled refrigerated temperature (Whirlpool refrigerator) at $5^\circ\text{C} \pm 2^\circ\text{C}$ or in a climate chamber (BINDER GmbH, Tuttlingen, Germany) at $25^\circ\text{C} \pm 2^\circ\text{C}$ and 60% residual humidity, until analysis. The units dedicated to the freeze-thawing study were frozen at $-20^\circ\text{C} \pm 2^\circ\text{C}$ for 60 hours.

For the studies at 5°C and 25°C, samples were analysed in triplicate at D0, D7, D14, D30 and D90, except for 10 mg/mL samples for which D90 was not performed. For the freeze-thawing study, samples were analysed at D0 and after 60 hours freezing after a complete thawing, assessed visually.

2.4 Analyses

Infliximab solutions were subjected to a series of analysis to investigate physical, chemical and structural stability. Total protein quantification was also performed. Before all analyses, the 10 mg/mL samples were diluted to 0.4 mg/mL of infliximab in sodium chloride solutions, except for subvisible particle determination for which the samples were analysed without dilution, and for peptide mapping analysis during which the samples were analysed at 2 mg/mL. The 0.4 mg/mL and 2 mg/mL infliximab solution samples were analysed directly without dilution.

2.4.1 Physical stability analysis

2.4.1.1 Visual inspection

The solutions were visually inspected under a white light in front of a matt black panel and a non-glare white panel. Aspect and colour of the solutions were noted, and a screening for visible particles, haziness, or gas development was performed.

2.4.1.2 Turbidimetry

Turbidity was evaluated by measuring the absorbance of the infliximab solutions at 320 nm and 350 nm using a spectrophotometer UV/VIS/NIR Jasco V – 670 (Jasco France, Bouguenais, France) equipped with a single monochromator, a deuterium lamp (range 190 - 350nm) and a halogen lamp (range 330 – 2700 nm). The results were analysed using the provided software (Spectra manager II)

An increase in the calculated aggregation index (AI) compared to the D0 reference is indicative of an increase of samples turbidity.

Equation 1 : Estimation of turbidity by calculation of Aggregation Index (AI). A corresponds to absorbance at specified wavelengths (280 and 350 nm)

$$AI = \frac{A_{350}}{A_{280} - A_{350}} \times 100$$

An AI value <10 was considered to be acceptable [13,14]

2.4.1.3 *Subvisible particles counting*

Subvisible particle counting was performed on a HIAC Royco 9703 (Hach Lange, Noisy le Grand, France) equipped with a HRLD 400 EC detector.

Four successive measurements were carried out on a volume of 5 mL, and the first run was discarded. The results were expressed as the number of particles of size superior to 10 μm and superior to 25 μm , per mL of solution. For the 10 mg/mL solutions, the triplicates from each condition were pooled together in order to have a sufficient volume to perform the analysis according to the European pharmacopeia monography (2.09.19)[15]. 10 mg/mL solutions were compared to the limits indicated in the European Pharmacopoeia 2.09.19 monography for small volume parenterals : <6000 particles /container (10 μm particles) and < 600 particles / container (25 μm particles). Whereas 0.4 and 2 mg/mL solutions were compared to limits indicated in the European Pharmacopoeia 2.09.19 monography for > 100 mL parenterals; <25 particles/mL equal to or greater than 10 μm and < 3 particles/mL equal to or greater than 25 μm [15]. Samples were considered to be conform if it didn't statistically increase when compared to D_0 values (Student test, $\alpha = 0.05$)

2.4.1.4 *Nanoparticles size determination*

Dynamic Light Scattering (DLS) allows the estimation of the size of protein aggregates between 0.3 nm and 10 μm . The particle size is obtained from the variation of intensity of light scattering due to Brownian motion of particles. Particle size was determined on 1 ml of each sample in clear disposable polystyrene cells by dynamic light scattering measurements, using a Zetasizer Nano ZS (Malvern Instruments SARL, Orsay Cedex, France). Each sample was automatically screened 3 times. The obtained size distribution was presented by percentage of intensity and must conform to reference (D_0) profiles. A variation in size of less than 3 nm of the main peak's hydrodynamic radius was considered as being acceptable. In parallel, the apparition of a peak different from infliximab main peak for which intensity percentage was superior to 10% was considered as being unacceptable.

2.4.1.5 *Size exclusion chromatography (SEC)*

Size exclusion chromatography allows the evaluation of monoclonal antibody aggregation or fragmentation. The chromatographic separation was performed by liquid chromatography (LC), using a LC-2010-A HT with integrated controller, pump, autosampler, oven and UV-VIS detector (Shimadzu Corporation, Marne la Vallée, France). Two LC separation columns TSK - GEL® G3000SWXL columns (7.8 mm x 30 cm x 5 μm , TOSOH Bioscience), purchased from Interchim (Montluçon, France) were used, mounted in series. A sample volume of 100 μl was injected into the system and then eluted using an isocratic flow (Na_2SO_4 0.1 M Na_2HPO_4 0.1 M and 0.05% m/w NaN_3 in water, buffered at pH 7) at a

flowrate of 0.6 mL/min, at a set temperature of 25 ° C. The detection wavelength was set up at 280 nm. Obtained SEC chromatograms must conform to reference (D₀) SEC chromatogram (monomer ≥98%). However, apparition of other species (oligomer or fragment) was followed, when applicable.

2.4.2 Chemical stability analysis

2.4.2.1 pH and osmolality

pH measurements were made using a SevenMulti™ pH-meter with an InLab™ Micro Pro glass electrode (Mettler-Toledo, Viroflay, France). Osmolality was measured for each solution using an osmometer Model 2020 Osmometer® (Advanced instruments Inc, Radiometer, SAS, Neuilly Plaisance).

pH measurements were considered to be acceptable if they did not vary by more than 1 pH unit from initial measure. Osmolality measurements are to be interpreted with regards to the potential modification of other parameters and for the purpose of this study were considered to be acceptable if they did not vary by more than 10 mosmol/kg from D₀ values.

2.4.2.2 Cation exchange chromatography (CEX)

Cation exchange chromatography is used to separate different isoforms of infliximab. The chromatographic separation was performed by liquid chromatography (LC), using a LC-2010-AHT with integrated controller, pump, autosampler, oven and UV-VIS detector (Shimadzu Corporation, Marne la Vallée, France). All analyses were performed on a TSKgel CM-STAT column (4.6 mm x 100 mm x 7 µm, TOSOH Bioscience), purchased from Interchim (Montluçon, France). Buffer A was prepared with 20mM MES, and buffer B was prepared with 20 mM MES, 0.5 M NaCl. For each buffer the pH was adjusted to 5.6 using 1N NaOH solution. The injected sample volume was of 100 µL and elution parameters were the following: flow rate of 1 mL/min using a gradient composed of 98% of phase A and 2% of phase B reaching 50% phase A and 50% phase B in 35 minutes, then the column was equilibrated with the initial mobile phase for 10 minutes between each analysis. Analysis temperature was set at 35°C. The detection wavelength was set up at 280 nm.

Obtained CEX chromatograms must conform to reference (D₀) CEX chromatogram. The distribution of each ionic variant was expressed in percentage of total area under curve (AUC). Any variation under 3% was considered to be acceptable.

2.4.3 *Structural analysis*

2.4.3.1 *Peptide mapping*

Peptide mapping allows the study of infliximab's primary structure. 500 μL of the solution (250 μL for the 2 mg/mL solutions), were denatured with GnHCl (final concentration 6M) in 0,119 M Tris HCl buffer at $\text{pH}=8.5$ and reduced with 2 mM DTT. Denaturation and reduction were performed together for 60 min at 37°C . Samples were then alkylated by 50 mM iodoacetic acid for 15 min at room temperature protected from light. Tris HCl buffer was removed by using a spin filter 10 kDa molecular weight cut-off (Millipore, Molsheim, France). After 20 min centrifugation at 4000 rpm, supernatant was completed up to 3.5 mL with 100 mM ammonium carbonate buffer ($\text{pH} = 7.6$). Spin filters were centrifugated again 20 min at 4000 rpm. The digestion step was performed with an acetylated trypsin ratio 1:20 (Trypsin / Infliximab, weight/weight) for 14 h overnight at 37°C . Before chromatographic analysis, samples were recovered from digestion step and completed to 400 μL with 0.1% TFA in water. The reaction was quenched by storing samples at 5°C inside the refrigerated autosampler, and 20 μL of each sample were injected into the column.

The chromatographic separation was performed by liquid chromatography (LC), using a LC-2030C with integrated controller, pump, autosampler, oven and UV-VIS detector (Shimadzu Corporation, Marne la Vallée, France). The separation was performed on a C18 Nucleosil column (250 mm x 3 mm, 5 μm , Macherey Nagel, France). Mobile phase was composed of 0.1% TFA in water (phase A) and 0.09% TFA in acetonitrile (phase B). Elution started with a gradient 95% phase A and 5% phase B. A linear gradient was run from 5% to 40% of phase B in 140 min. The flowrate was set to 1 ml/min and column oven temperature at 40°C . The detection wavelength was set up at 215 nm. A postrun treatment was done by subtracting the chromatogram of the trypsin solution.

Obtained peptide mapping chromatograms must conform to reference (D_0) chromatogram. Minor and unreproducible variations in the peptide mapping profile (eg. slight retention time variations, intensity variations) were considered to be acceptable. Major reproducible differences, like the appearance or disappearance of a peak, were considered to be unacceptable.

2.4.3.2 Second derivative Fourier Transform Infrared Spectroscopy (FTIR)

The secondary structure of infliximab was monitored by second derivative FTIR in amide I region (1600 – 1700 cm^{-1}) using an Avatar 320 (Nicolet, Thermo Electron, Courtaboeuf Cedex, France) equipped with a transmission cell with 25 mm BaF_2 windows. All acquisitions were treated with Omnic software. For FTIR analysis, samples of each concentration were concentrated up to 40 mg/mL using a spin filter 10kDa molecular weight cut-off (Millipore, Molsheim, France).

Obtained FT-IR spectra must conform to reference (D_0) profiles. A shift in maxima's and minima's wavenumbers inferior to 2 cm^{-1} was considered to be acceptable.

2.4.3.3 Second derivative UV spectroscopy

Tertiary structure was studied by second derivative UV spectroscopy. All spectrums were acquired on a Spectrophotometer UV/VIS/NIR Jasco V-670 (Jasco France, Bouguenais, France), using 1 cm pathlength quartz cuvettes and the measurement wavelength range was set between 250 and 350 nm. All acquisition data were treated with Spectra manager II software.

Obtained second derivative ultraviolet spectra must conform to reference (D_0) spectra. A shift in maxima's and minima's wavelengths inferior to 2 nm was considered to be acceptable.

2.4.4 Total protein quantification

Protein content per vial was estimated by measuring absorbance at 280 nm [16] of Inflectra® 10 mg/mL solutions diluted to 0.4 mg/mL. All data were acquired on a spectrophotometer UV/VIS/NIR Jasco V-670 (Jasco France, Bouguenais, France), using 1 cm pathlength quartz cuvettes. Protein content was calculated using the Beer-Lambert law. According to the manufacturer instructions, protein content was considered to be acceptable when between 90% and 110% of theoretical concentration.

2.4.5 Data analysis – acceptability criteria

The study was conducted following methodological guidelines issued by the International Conference on Harmonisation for stability studies (ICH guidelines for stability [9,11] and specifications for Biotechnological/Biological products [10]), Bardin et al., 2011 [8], the European Pharmacopeia [17] and recommendations issued by the French Society of Clinical Pharmacy (SFPC) and the Evaluation and Research Group on Protection in Controlled Atmosphere (GERPAC) [18]., French Society and European Society of Oncology Pharmacy[19]. NHS guidance [20] and INFLECTRA® specifications (provided by Pfizer®) were also taken into account.

3. Results

3.1 Visual inspection

At D₀, all infliximab samples were limpid, colourless and no visible particles were observed. Throughout the study, no evolution was noticed for the samples stored at 5°C and 25°C. After complete thawing, no evolution from D₀ was noticed with any storage condition.

3.2 Turbidity

Throughout the study, for each concentration and conservation condition, the AI remained within acceptability criteria, with a maximum of 1.761, 0.640 and 0.660 for respectively the 10 mg/mL, 2 mg/mL solutions and 0.4 mg/mL infliximab solutions. For every condition, AI values remained below acceptability limit even if there was an important variation between samples.

3.3 Subvisible particles counting

Larger than 10 µm subvisible particle count was out of acceptability criteria only after freezing-thawing of 0.4 mg/mL infliximab solutions (Figure 1). At D₀, >25 µm subvisibles particles counting revealed 9 ± 4 particles/mL for 0.4 mg/mL infliximab solutions, 1 ± 0 particles/mL for 2 mg/mL infliximab solutions and 24 particles/mL in 10 mg/mL infliximab solutions. The number of particles was more important with 10 mg/mL samples than with diluted solutions, respectively 127 and 24 particles/mL superior to 10 µm and superior to 25 µm. For each temperature and analytical time, the number of particles/mL did not significantly increase.

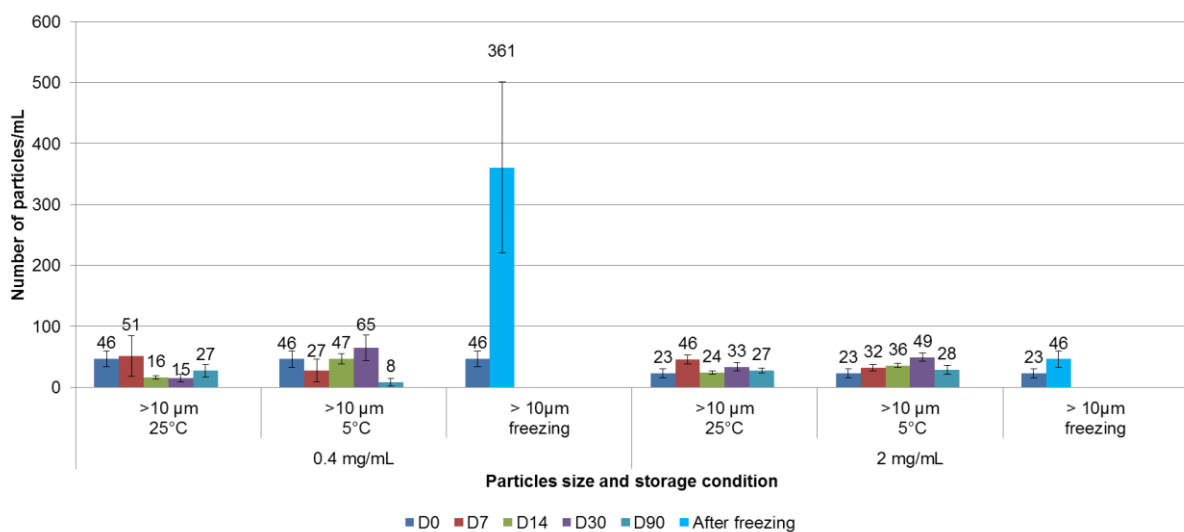


Figure 1 : Number of particles > 10 µm for 0.4 mg/mL and 2 mg/mL infliximab solutions in 0.9% NaCl infusion bags at each storage temperature (mean ± SD, n=3)

The counting of subvisible particles of size superior to 10 μm and superior to 25 μm in diluted solutions (0.4 and 2 mg/mL) was over European Pharmacopeia limit (respectively 25 particles /mL and 3 particles /mL) while it remained within European Pharmacopeia limits for the 10 mg/mL solutions as their nominal volume was lower than 100 mL (respectively 6000 particles/container and 600 particles/container).

3.4 Nanoparticles size determination

Infliximab nanoparticles sizes estimated by DLS are shown in Table 1. Hydrodynamic diameter was out of acceptability criteria only after freeze-thawing of 10 mg/mL infliximab solutions, as an increase of 3.17 nm was noticed after a single freeze-thawing cycle.

Table 1: Infliximab nanoparticles hydrodynamic diameter evolution over time and storage conditions for each studied concentration estimated by DLS (ND: not determined).

| Storage time (days) | 10 mg/mL | | 2 mg/mL | | 0.4 mg/mL | | Mean PDI \pm SD | |
|---------------------|-------------------------|------------|-------------------------|------------|-------------------------|------------|-------------------|--|
| | mean size \pm SD (nm) | | mean size \pm SD (nm) | | mean size \pm SD (nm) | | | |
| Freezing | | | | | | | | |
| D0 | 12.22 | \pm 2.66 | 16.83 | \pm 5.06 | 12.58 | \pm 2.49 | 0.202 \pm 0.048 | |
| D3 | 15.39 | \pm 5.76 | 17.68 | \pm 4.86 | 14.38 | \pm 5.00 | 0.190 \pm 0.043 | |
| 5°C | | | | | | | | |
| D0 | 13.45 | \pm 2.69 | 16.83 | \pm 5.06 | 12.58 | \pm 2.49 | 0.203 \pm 0.049 | |
| D7 | 12.66 | \pm 3.01 | 16.22 | \pm 4.91 | 13.56 | \pm 3.94 | 0.160 \pm 0.023 | |
| D14 | 14.91 | \pm 5.80 | 16.81 | \pm 5.54 | 12.62 | \pm 2.82 | 0.193 \pm 0.049 | |
| D30 | 13.58 | \pm 5.63 | 16.53 | \pm 5.29 | 12.98 | \pm 3.76 | 0.189 \pm 0.048 | |
| D90 | ND | | 17.78 | \pm 5.83 | 12.82 | \pm 3.81 | 0.156 \pm 0.035 | |
| 25°C | | | | | | | | |
| D0 | 13.45 | \pm 2.69 | 16.83 | \pm 5.06 | 12.58 | \pm 2.49 | 0.210 \pm 0.041 | |
| D7 | 12.88 | \pm 3.88 | 16.41 | \pm 4.63 | 12.61 | \pm 3.27 | 0.160 \pm 0.023 | |
| D14 | 13.76 | \pm 4.86 | 16.65 | \pm 5.01 | 12.72 | \pm 2.82 | 0.170 \pm 0.052 | |
| D30 | 12.73 | \pm 3.48 | 16.72 | \pm 5.33 | 13.23 | \pm 4.48 | 0.174 \pm 0.045 | |
| D90 | ND | | 17.40 | \pm 5.61 | 13.00 | \pm 2.92 | 0.162 \pm 0.025 | |

3.5 Size exclusion chromatography

The main peak, corresponding to infliximab monomeric form (elution time (et) = 27.3 min \pm 0.02 min) remained above 98% of total Area Under Curve (AUC) throughout the studies for all conditions. A second peak (et = 23.2 min \pm 0.2 min) corresponding to a high molecular weight polymer (HMWP) was observed from D₀ onwards but did not significantly vary overtime for any of the conditions. However, a fragment was detected with a et = 33.6 min \pm 0.1 min, and its AUC percentage increased overtime for samples of all three concentrations when stored at 25°C, but not for those stored at 5°C and at -20°C (the peak remained unquantifiable) (see supplementary data, Table A). As an example, SEC chromatograms of the 0.4 mg/mL infliximab solutions at D₀ are compared to chromatograms of the last analytical of each storage condition (D₉₀ for storage at 5°C and 25°C and D₃ after freezing) in Figure 2. The main peak corresponding to infliximab monomeric form represented 99.29% \pm 0.03 % of total AUC. At D₉₀ of the 25°C storage, the fragmented form reached its maximum of 0.48% \pm 0.08% of total AUC while no variation from D₀ was observed after storage at 5°C or -20°C.

Monomeric form at D₀ for infliximab solutions at 2 mg/mL and 10 mg/mL were respectively of 99.15% \pm 0.00% and 99.19% \pm 0.02% and the increase in fragmented form was observed for all 25°C storage conditions with respective values of 0.13% \pm 0.00% and 0.10% \pm 0.00% at D₉₀ while no modification was observed after storage at 5°C or -20°C.

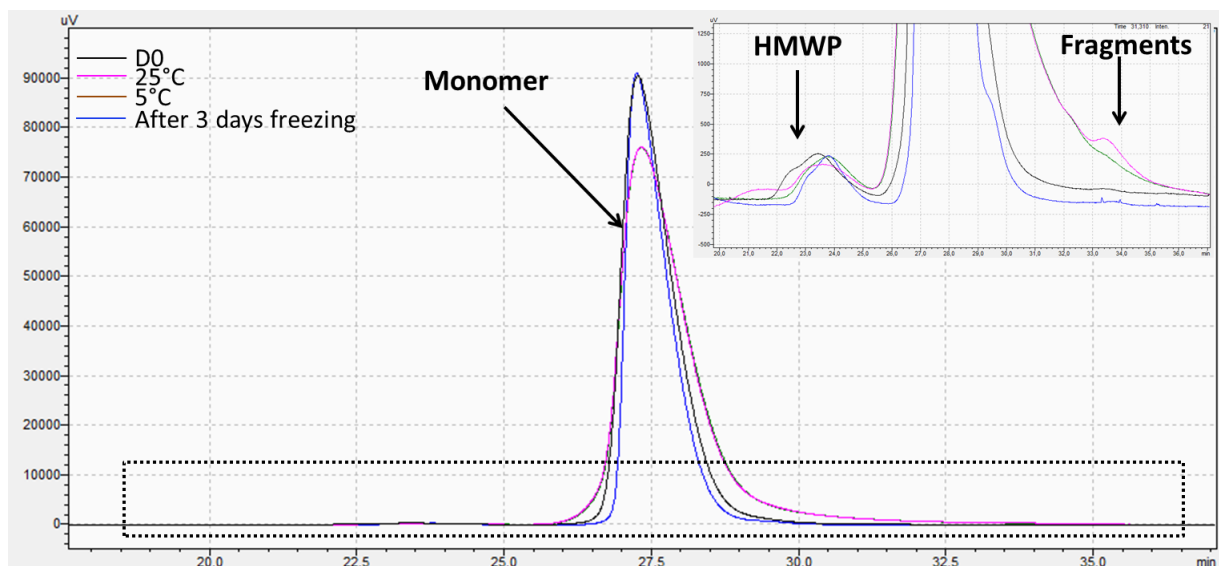


Figure 2 : SEC profile evolution of 0.4 mg/mL infliximab solutions at D₀ compared to D₉₀ after a 90 days storage at 5°C and 25°C and after a freeze-thawing cycle of 3 days

3.6 pH and osmolality

Initial pH values for the different conditions are shown in Table 2. For all studies, no variations of more than ± 0.27 pH units and 4.33 mosmol/kg were observed from D_0 values onwards. Throughout the study and for both conservation conditions, obtained pH and osmolality results meet acceptability criteria (no significant variation).

Table 2 : pH and osmolality values at D_0 for every conditioning condition (mean \pm SD, n=3)

| | Mean pH at $D_0 \pm$ SD | Mean osmolality at $D_0 \pm$ SD mosmol/kg |
|----------------------------------|-------------------------|--|
| 10 mg/mL in original glass vials | 6.98 \pm 0.07 | 280.33 \pm 3.06 |
| 0.4 mg/mL in NaCl bags | 6.92 \pm 0.05 | 280.33 \pm 0.58 |
| 2 mg/mL in NaCl bags | 7.09 \pm 0.01 | 261.67 \pm 1.53 |

3.7 Cation exchange chromatography

CEX chromatograms presented a specific profile with 6 different peaks. None of the studied conditions led to new acidic or basic variants appearance. As an example, CEX results of 0.4 mg/mL infliximab solutions are shown in Figure 3. A significant decrease of peak 4 (-5.36%) and increase of peak 1 (+5.47%) was simultaneously observed at D_{90} when sample were stored at 25°C. Modifications until D_{30} included were not significant. When stored at 5°C, peak proportion of total AUC did not vary by more than 2.75%. A similar evolution profile was observed for other storage and concentration conditions. The 10 mg/mL solutions at 25° condition showed a coupled decrease of peak 4 (-3.34%) and increase of peak 1 (+3.77%) from D_{14} included.

After a freeze-thawing cycle, a decrease of peak 4 and an increase of peak 1 percentage of total AUC was observed when 10 mg/mL infliximab samples were frozen for 3 days. CEX chromatograms of 0.4 mg/mL Infliximab solutions did not show any significant variation from D_0 (Figure 3. B).

When stored at 5°C, all three studied infliximab concentrations remained within acceptability criteria throughout the study (no variation of variants percentage).

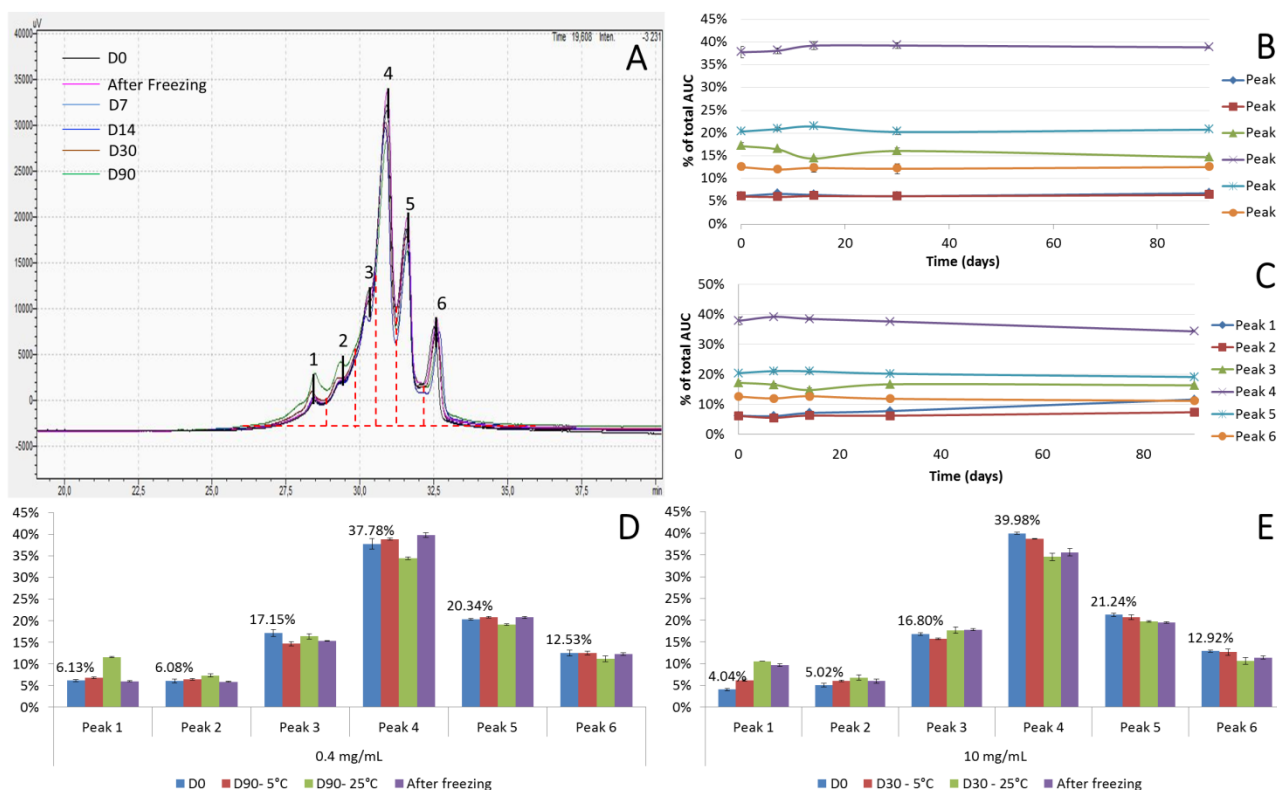


Figure 3 : Cation Exchange chromatograms at each analytical time of 0.4 mg/mL infliximab solutions stored at 25°C (A), evolution of peaks percentage from total AUC (mean \pm SD, n=3) for the 5°C (B) and 25°C (C) storage condition, comparison of each peaks percentage of total AUC at the final analytical time for each storage condition of 0.4 mg/mL infliximab solutions (D) and 10 mg/mL infliximab solutions (E).

3.8 Peptide mapping

Peptide mapping chromatograms of 10mg/mL infliximab solutions at D₀ is shown in Figure 4. Despite being very similar, minor modifications from D₀ profile were observed at D30 for both temperature conditions (supplementary data, figure A). After freezing, peptide map was qualitatively different from D₀ (supplementary data, figure B)

For all other conditions of temperature and concentration, peptide map was not significantly different from D₀ at any time.

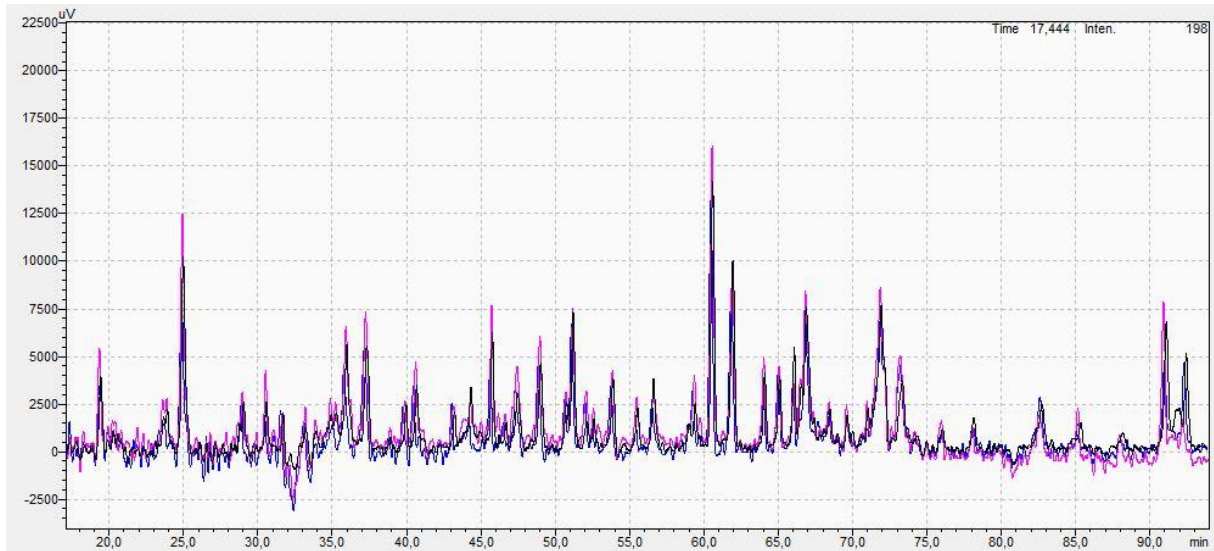


Figure 4 : peptide mapping chromatogram of 10 mg/mL infliximab solutions at D₀. (n=3, black, blue and pink chromatograms corresponding to the triplicate)

3.9 Second derivative FT-IR

Second derivative FTIR spectra of 0.4 mg/mL infliximab solutions at D₀ and compared to those at the final analytical time of each condition is shown in Figure 5. No modification was observed from D₀ to D₉₀ or after a freeze-thawing cycle, nor for any other concentration and storage conditions at any time.

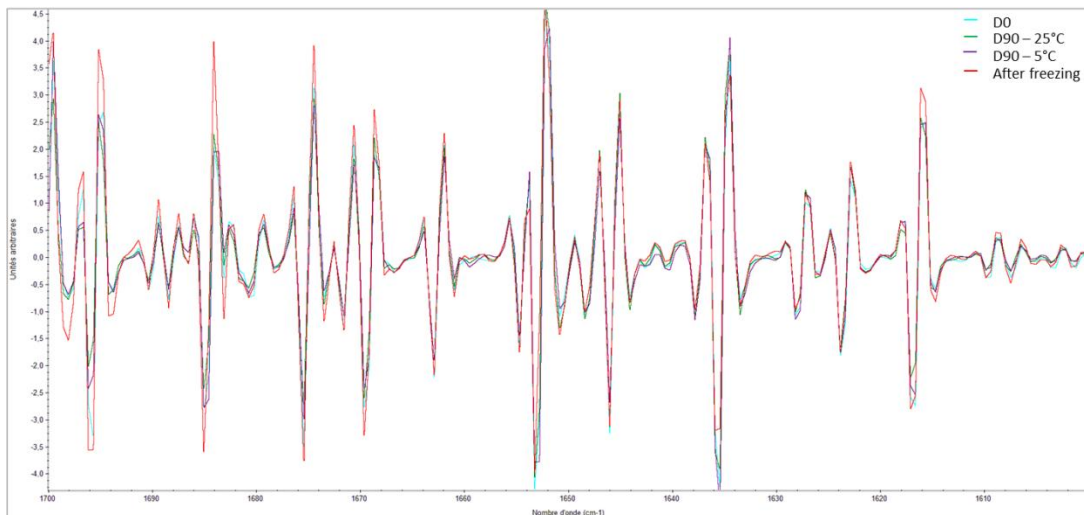


Figure 5 : Second derivative FTIR spectra of 0.4 mg/mL infliximab solutions at the final analytical time of each temperature condition.

3.10 Second derivative UV spectroscopy

Second derivative UV spectra of 0.4 mg/mL infliximab solutions at D₀ and compared to those at the final analytical time of each condition is shown in Figure 6. No modification was observed from D₀ to D₉₀ or after a freeze-thawing cycle, nor for any other concentration and storage conditions at any time.

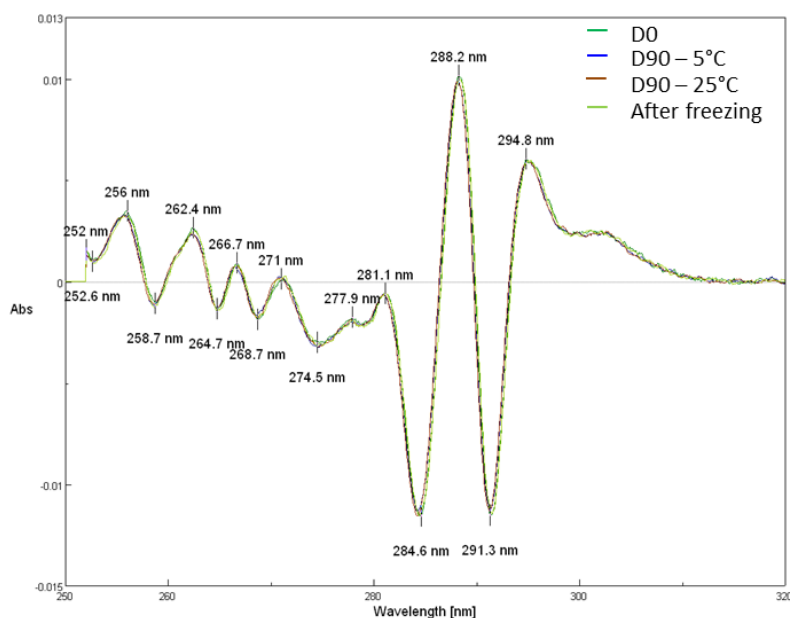


Figure 6 : Second derivative UV spectra of 0.4 mg/mL infliximab solutions at the final analytical time of each temperature condition.

3.11 Total protein quantification

Quantification for every preparation and storage conditions is shown in Figure 7. For 0.4 mg/mL infliximab solutions concentration was respectively of 0.39 ± 0.01 mg/ at D₀. For 2 mg/mL infliximab solutions, concentration was 1.75 ± 0.04 mg/mL and for 10 mg/mL infliximab solutions, the result is expressed as the amount of protein/vial and was of 9.05 ± 0.79 mg/vial at D₀.

Over the study, for each concentration and temperature condition, results remained between 90% and 110% of theoretical concentration.

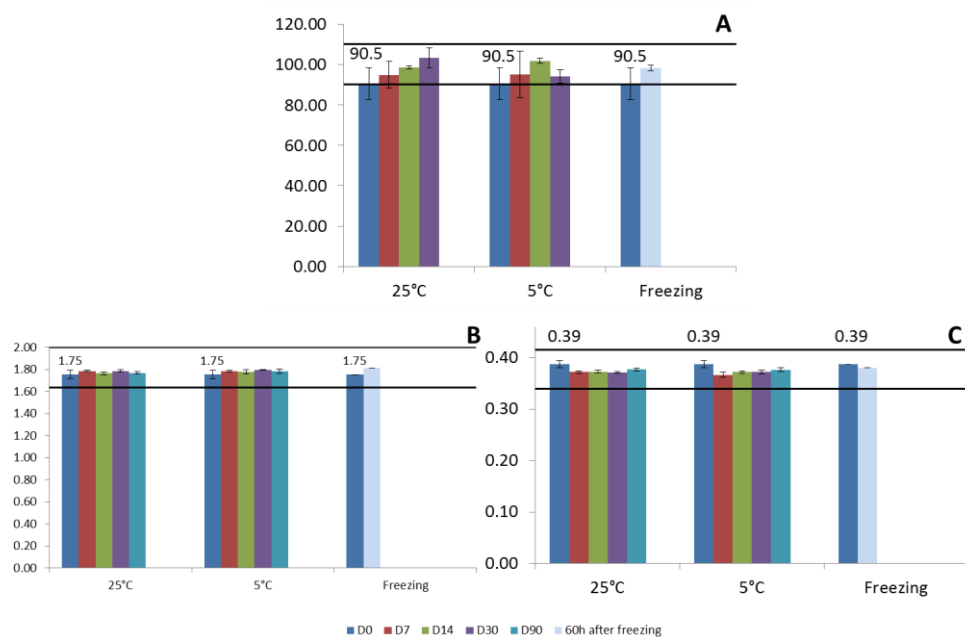


Figure 7 : Total protein quantification of infliximab solutions: 10 mg/mL (A), 0.4 mg/mL (B), and 2 mg/mL (C) for each conservation temperature condition

3.12 Synthesis

All signs of instability observed are presented in Table 3.

Table 3 : Summary of stability result for each analytical technique and conditions.

| | 10 mg/mL | | | 2 mg/mL | | | 0.4 mg/mL | | |
|-------------------|----------|--------------|------------------|----------|-----|------------------|-----------|-----|------------------|
| | Freezing | 5°C | 25°C | Freezing | 5°C | 25°C | Freezing | 5°C | 25°C |
| Physical | | | | | | | | | |
| Visual exam | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Turbidity | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| HIAC | ✓ | ✓ | ✓ | * | ✓ | ✓ | * | ✓ | ✓ |
| DLS | * | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| SEC | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | | | oligomers at D14 | | | oligomers at D14 | | | oligomers at D14 |
| Chemical | | | | | | | | | |
| pH/osmo | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| CEX | * | ✓ | * 7 days | ✓ | ✓ | * 30 days | ✓ | ✓ | * 30 days |
| Structural | | | | | | | | | |
| Peptide mapping | * | * 14 days | * 14 days | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| SD-FTIR | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| SD-UV | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

✓ : Absence of instability at the end of the study

* : Instability observed

x Days : last analytical time without instability

4. Discussion

Our study presents new information about the stability of concentrated and diluted infliximab solutions, pertinent to regular pharmaceutical and clinical use. A large panel of analytical techniques was used to assess the effect of storage temperature and dilution on the physicochemical stability of infliximab solutions as recommended by the international guidelines [11–17]. In forced degradation studies physical degradation is usually the main issue [21], even if it can be associated with chemical degradation under some conditions [22]. However, in this long term storage study, chemical stability appeared to be the most critical point, as after 90 days storage at 25°C, important chemical modifications and minor physical instabilities were revealed by CEX and SEC for both 0.4 and 2 mg/mL solutions, however storage at 5°C allowed infliximab to remain physicochemically unchanged for up to 90 days included. Freezing caused physical degradation (increased particle levels) for all three concentrations. Stability appeared to be dilution dependent, in addition to temperature dependent. For the 10 mg/mL infliximab solutions, obtained data is in favour only a 7 days physicochemical stability when stored at 25°C after reconstitution with water for injection in original glass vials. When stored at 5°C, the data is in favour of an extended stability of 14 days. 0.4 mg/mL and 2 mg/mL infliximab solutions diluted in NaCl and stored in Polyolefin (Polypropylene PP) bags were stable up to 30 days at 25°C and 90 days at 5°C

Aggregate formation is normally the main critical point limiting mAbs stability in solutions, and has also been shown to occur after freeze-thawing stress of monoclonal antibodies [23]. Four analytical techniques, that complement each other, were used to assess the aggregation level of infliximab solutions. Sub-visible particles over 10 µm and over 25 µm were counted by light obscuration (HIAC) and nanoparticles between 0.3 nm and 10 µm were assessed by DLS. SEC was also used to explore aggregation, since this Liquid Chromatography (LC) method is able to separate mAbs monomeric form from the polymeric form (dimeric or more) and fragments. To complete aggregate formation study, turbidity was assessed by calculating an aggregation index based on absorbance at 280 nm and 350 nm. Without aggregation, absorbance at 350 nm is almost null, but when aggregates are presents in solution, the UV beam is deflected leading to an increase in absorbance. Those four analytical techniques allowed us to have a global vision of infliximab's aggregation potential, but mAbs stability is not only limited to physical issues. Chemical stability was assessed by CEX which is an LC method separating mAbs charge variants, mainly resulting from the manufacturing process. Osmolality and pH are part of the chemical stability and were followed because of the clinical impact they could have during the infusion to the patient. Beside the clinical aspect, pH variations can be responsible of mAbs degradation [24], especially leading to a potential aggregation. In this work primary structure was assessed by performing a peptide map, a chromatographic separation of

peptides stemming from an enzymatic digestion. Secondary and tertiary structures were characterized by spectroscopic methods, respectively SD-FTIR and SD-UV. However, circular dichroism and fluorescence spectroscopy could have been employed as alternative techniques to assess secondary structure and conformational changes. However, it has been shown that even if circular dichroism can provide complementary information about secondary structure, it is not more efficient than SD-FTIR to highlight small conformational changes that are likely to happen during a stability study [25].

Visual examination has not shown any differences throughout the studies when samples were stored at 5°C. This result was confirmed by turbidity and subvisibles particles counting analysis, which did not show any increase overtime, indicating the absence of large aggregate formation. The number of particles larger than 10 µm and 25 µm was over generally above European Pharmacopeia acceptability criteria [15] for 0.4 and 2 mg/mL solutions, even at D₀, but since the manufacturer recommends to infuse infliximab with a infusion set equipped with an inline 0.22 µm filter which would retain the particles, the clinical impact of such subvisible particles might be negligible as they would be retained before administration. DLS results did not highlight the formation of dimer or smaller than 1 µm aggregates since no particles with a significant higher hydrodynamic diameter were detected. As previous studies have shown before [26], hydrodynamic diameter was lower with 0.4 mg/mL samples than with 2 mg/mL (10 mg/mL samples were diluted to 0.4 mg/mL before analysis which explains why they were not larger than 2 mg/mL particles).

SEC results highlighted the presence of HMWP from D₀ regardless of the conditions, probably dimers and other size variants that were hardly separable. Those HMWP have already been highlighted by studies [26] made on another infliximab biosimilar (REMSIMA®). SEC also detected a fragment formation, which was present but under the quantification limit at D₀ for the 0.4 mg/mL concentrations. Yet, this fragment increased overtime for every concentration condition at 25°C, but not at 5°C. Despite increasing fragment percentages, the monomeric form remained over 99% of total AUC. Without official recommendations for acceptable degradation of monoclonal antibodies, the critical threshold used was the one set up by the manufacturer (monomer >98% of total AUC). None of the analytical techniques used for physical stability evaluation indicated the formation of aggregates during any of the studied conditions. Previous internal intentional degradation studies (data not shown) highlighted that some mAbs degradation products (especially fragments) were hardly separable by SEC. The use of two columns mounted in series allowed us to increase selectivity of the SEC method without using smaller particles packed columns, which even if they possess several advantages like better peak resolution and separation between aggregates and native protein peaks [27], require the use of ultra-high pressure chromatographic systems, whilst using UV detection that was the only option available at our laboratory. However, fluorescence detection based SEC (or CEX)

would avoid some of the limits of UV based detection, especially by increasing sensitivity which is major issue for monoclonal antibodies

Infliximab presented a CEX profile with 6 peaks sufficiently resolved, the 7th that had been described in other studies [26,28] was visible (between peak 5 and 6 of these studies) but not quantifiable. Mobile phase pH was adjusted to 5.6 and could explain the differences observed between our chromatograms and other studies. Indeed, acidic variants were better resolved with a pH at 5.6 while basic variants were better visualized at pH = 6.5 [29]. In order to make our CEX results comparable to studies already performed with the infliximab biosimilar REMSIMA, the CEX analysis was performed by injecting the intact antibody. However, an enzymatic digestion by papain which split Fc and Fabs of monoclonal antibodies might provide more information about the localisation of potential charge variants. Moreover, the papain digestion could also increase the selectivity of the method by increasing the separation between variants. Another way of increasing specificity is to separate variants with a semi-preparative column, then analyse each fraction one by one.

Charge variants were present even at D0 and corresponded to post-translational modifications resulting in infliximab with zero, one or two C-terminal Lysine associated with a variation of charged glycans [28]. An equilibrium between all those variants was observed at D0. During the studies, no new acidic or basic variants appeared, but the variation observed (increase of acidic variant 1) for all concentration conditions when stored at 25°C was a sign a chemical instability. The 3% threshold corresponded to the limit where variation was considered to be significantly different from analytical variations. Our studies have shown that chemical instability was the principal stability limitation of infliximab solutions during long term storage at 25°C. Significant modifications arose faster with the 10 mg/mL conditions (14 days against 90 days for other conditions), so diluting a mAb solution may be a way to increase chemical stability. However, dilution will also diluted excipients, some of which are known to have a stabilizing effect, like surfactants [24]. On the other side, 10 mg/mL samples were reconstituted in water for injection while samples at lower concentrations were diluted in 0.9% NaCl bags, and it had been shown that the ionic strength may interfere with monoclonal antibodies stability [30].

Modifications in the primary structure were observed through peptide mapping analysis. A minor modification was noticed only in the 10 mg/mL in glass vials conditions after D₃₀. Even though we were not able to accurately characterize the sort of modifications of the peptide map (oxidation, deamidation) without using LC-MS method, LC was sensitive enough to perform a qualitative comparison between samples at D0 and samples after storage. A variation in primary structure is often a cause of physical instability, fragmentation or aggregation. Considering the absence of instability it is not surprising that primary structure remained unchanged during the study for all

concentration and temperature conditions. However, if the units had been exposed to light during the study, maybe certain oxidation modifications might have noticed, as light has been shown to catalyse some modifications [31].

Secondary and tertiary structures were assessed respectively by SD-FTIR and SD-UV spectroscopy and no sign of instability was highlighted. Variations observed with CEX could have been related to a structural modification, but they were either below the sensitivity limits of spectroscopic methods either not related, as Jasper C. et al [23] have shown in FTIR. However, Jasper C. et al. [25] did show that variations in SEC and CEX can be not related to a modification in SD-FTIR spectrum. Previous studies made with other infliximab biosimilars [27,31] assessed the stability of diluted solutions over a study period of 30 days. This study has shown that infliximab diluted in NaCl polyolefin bags were physically and chemically stable up to 90 days when stored at 5°C, and 30 days at 25°C.

Physical and chemical signs of instability were detected when stressing 10 mg/mL infliximab solutions by freeze-thawing. Variations of nanoparticles hydrodynamic diameter were observed in DLS when infliximab samples were frozen at 10 mg/mL but were absent when for 0.4 mg/mL and 2 mg/mL solutions. This could be explained by aggregation (possible formation of dimers or other HMWP) slightly impacting overall particle size, without modifying any other structural aspect. This result could have been confirmed by SEC but the amount of formed aggregates was probably under the limit of detection (LOD) of the assay. Chemical stability was assessed by CEX and highlighted a correlated variation of two peaks corresponding to the main peak with no C-terminal Lysine [26] (peak 4) and an unidentified acidic variant (peak 1). These modifications were not correlated to a pH variation and suggested a chemical instability that could be part of a possible aggregation mechanism predicted by DLS. To complete physicochemical analysis, a structural analysis was performed. Secondary and tertiary structures were assessed respectively by SD-FTIR and SD-UV spectroscopy. None of these analytical techniques showed any sign of instability. Peptide mapping was used to characterize primary structure, and highlighted an unexpected change in the complete mapping of infliximab solutions at 10 mg/mL in glass vials. An analysis of the peptide map by LC-MS could have allowed us to determine what kind of degradation the peptides underwent and determine if the modification of the peptide map was related or not to the potential aggregation phenomenon.

However, infliximab solutions at 0.4 and 2 mg/mL did not show any sign of chemical instability and hydrodynamic diameter did not vary after freezing. HIAC results suggested an increase in subvisible particles after freeze-thawing of 0.4 and 2 mg/mL infliximab solutions, whilst subvisible particles levels remained unchanged for 10 mg/mL solutions. This increase was correlated with a slight increase in turbidity (but remaining within acceptability criteria) and with SEC results, as HMWP increased in both

cases (again remaining within acceptability criteria), and could therefore be in favour of mild aggregation. Alternatively, subvisible ice residues in the solutions after the thawing protocol cannot be excluded and may have caused the increased subvisible particle levels, however such a phenomenon wouldn't explain the alteration of the other parameters.

Aggregation phenomenon is well known for occurring after freeze-thawing stress of monoclonal antibodies [23]. 10 mg/mL solutions were reconstituted with water for injection only, thus their ionic strength was negligible. It has already been shown that an increase in salt concentration increases monoclonal antibodies Antigen-binding fragment [32], this results suggested that ionic strength could also prevent aggregation for infliximab solutions after freeze-thawing. The level of aggregation has been shown to be dependent of antibody concentration [23]. So the potential aggregation observed with 10 mg/mL infliximab solutions could be a combination of both ionic strength and concentration differences compared to the 0.4 and 2 mg/mL solutions, in a freeze-thawing stress situation.

An accidental freezing could happen during conservation, and yet the results of this study suggested a limited stability and no use after freezing should be accepted, especially for 10 mg/mL infliximab solutions. Further studies will allow determining the instability mechanism by performing harder stress conditions and increasing suspected instability signs.

The global impact of ambient temperature storage was observed for all conditions, as CEX and SEC chromatograms highlighted respectively a chemical and physical instability of infliximab molecules when samples were stored at 25°C. However, differences in stability were also spotted between concentrated (10 mg/mL) and diluted solutions (0.4 and 2 mg/mL) for the same temperature condition. Those differences could be explained by the sample concentration and the reconstitution solvent (for 10 mg/mL solutions the solvent was water for injection, whereas NaCl 0.9% was the main diluent for the 0.4 mg/mL and 2 mg/mL solutions). It has been shown that ionic strength has a stabilizing effect on the conformational and chemical stability of the fab fragment [33], and it can therefore be hypothesized that a possible way of increasing 10 mg/mL infliximab stability would be to prepare it with a saline solution and not with water for injection. The container itself (glass vials or polyolefin bags) may also play a role in infliximab solutions stability.

The assessment of physicochemical and structural properties is a good indicator to estimate mAbs stability, and we can suppose that infliximab binding to TNF will not be affected if physicochemical and structural properties are unchanged. Further biological effectiveness and immunogenicity studies will be needed to confirm that administration to patients is completely safe

after long term storage. The reconstitution of infliximab solutions was performed under aseptic conditions, minimizing the risk of contamination but microbiological studies will be needed to extend long term storage recommendations. In clinical use conditions, stability of reconstituted and diluted infliximab solutions is set up to 24h for both infliximab biosimilars (according to summary of product characteristics of both REMSIMA®[34] and INFLECTRA®[35]), limited by a hypothetically short microbiological stability. Due to the high cost of infliximab, an extended stability is relevant for hospital pharmacies and particularly in centralized reconstitution units since infliximab solutions preparation could be optimized, and drug wastage reduced. Studies performed on another infliximab biosimilar (REMSIMA®) have shown that infliximab diluted solutions were stable at least 7 days when diluted in NaCl bags [29,36], but freshly reconstituted vials, and solutions in infusion bags diluted from an aged vials haven't been studied to our knowledge.

5. Conclusion

A complete characterization of an infliximab biosimilar physicochemical and structural properties was performed during this study. This stability study highlighted that chemical instability was the main stability limitation. The impact of storage temperature was confirmed, as infliximab solutions were more stable when stored at 5°C than at 25°C; diluted solutions at 0.4 and 2 mg/mL were more stable than 10 mg/mL solutions. Both dilution and temperature had an impact on stability, but their respective roles remain to be precisely determined.

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