Modeling study of long-term stability of the monoclonal antibody infliximab and biosimilars using liquid-chromatography–tandem mass spectrometry and size-exclusion chromatography–multi-angle light scattering

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Abstract
Monoclonal antibodies (mAbs) represent a dynamic class of biopharmaceutical products, as evidenced by an increasing number of market authorizations for mAb innovator and biosimilar products. Stability studies are commonly performed during product development, for instance, to exclude unstable molecules, optimize the formulation or determine the storage limit. Such studies are time-consuming, especially for mAbs, because of their structural complexity which requires multiple analytical techniques to achieve a detailed characterization. We report the implementation of a novel methodology based on the accelerated stability assessment program (ASAP) in order to model the long-term stability of mAbs in relation to different structural aspects. Stability studies of innovator infliximab and two different biosimilars were performed using forced degradation conditions alongside in-use administration conditions in order to investigate their similarity regarding stability. Thus, characterization of post-translational modifications was achieved using liquid-chromatography–tandem mass spectrometry (LC-MS/MS) analysis, and the formation of aggregates and free chain fragments was characterized using size-exclusion chromatography–multi-angle light scattering (SEC-MALS-UV/RI) analysis. Consequently, ASAP models were investigated with regard to free chain fragmentation of mAbs concomitantly with N57 deamidation, located in the hypervariable region. Comparison of ASAP models and the long-term stability data from samples stored in intravenous bags demonstrated a relevant correlation, indicating the stability of the mAbs. The developed methodology highlighted the particularities of ASAP modeling for mAbs and demonstrated the possibility to independently consider the different types of degradation pathways in order to provide accurate and appropriate prediction of the long-term stability of this type of biomolecule.

Keywords Monoclonal antibody · Biosimilar · Stability study · Stability modeling · Mass spectrometry · Multi-angle light scattering

Introduction
Monoclonal antibodies (mAbs) and their related therapeutic agents such as fusion proteins, bispecific antibodies (BsAbs) [1] or antibody–drug conjugates (ADC) [2] are meeting with unprecedented success as biopharmaceutical products. Currently, more than 100 therapeutic mAbs are approved worldwide, with 10 newly authorized products reported in 2020 alone [3]. Their therapeutic applications have been focused mainly in oncology and for the treatment of immune disorders; however, their application is continuously broadening, as recently illustrated with the development of treatments for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [4]. Also, with several patents reaching the public domain, the development of biosimilars that define
an equivalent product containing the active substance of an original biopharmaceutical product is gaining growing interest [5].

mAbs represent tetrameric glycoproteins based on immunoglobulin G, which naturally exhibit a wide variety of variants. Because of their structural complexity, extensive research activity has been devoted to developing analytical methods able to provide a detailed characterization of their structure [6, 7], which requires a panel of analytical techniques in order to analyze the different aspects of the protein [8, 9]. In addition, mAbs can undergo different types of post-translational modification (PTM) and/or structural alterations, for instance asparagine deamidation, methionine oxidation or aggregation. The modifications potentially altering the properties, the quality and the safety of mAbs are referred to as critical quality attributes (CQA) [10, 11]. Therefore, they need to be characterized in order to maintain modification levels within appropriate limits during the production process and also during stability studies. In the same manner, biosimilarity assessment requires a complete characterization of the biosimilar candidate to provide a comprehensive comparison with the innovator product over the different levels defining their structure. The comparison should also demonstrate the absence of significant differences between the two products regarding CQA; otherwise, the absence of impact in terms of clinical activity and toxicity should be demonstrated [5]. Several studies have described the comparison between innovator mAbs and biosimilar candidates; however, they focused on assessing the biosimilar produced [12–14]. Recently, interest has increased in the implementation of forced degradation studies in order to evaluate the biosimilarity of mAbs with regard to their stability [15, 16].

Early during the drug development process, important efforts are made to develop the most stable formulation. Indeed, it is important to limit the risk of stability issues and endogenous degradation. The conventional methodology for investigating their stability consists of subjecting mAbs to various stress conditions including temperature, oxidation, light or extreme pH. This enables the evaluation of major degradation pathways and selection of the most stable formulation [17]. This methodology is used for small chemical drugs [18–20] and for the development of therapeutic mAbs [21]. Nevertheless, the application of forced degradation does not allow the precise prediction of degradation during the shelf-life of the product. To address these limitations, different modeling approaches, referred to as risk-based predictive stability (RBPS) or accelerated stability assessment program (ASAP), have been recently developed [22]. These modeling approaches are based on accelerated stability studies and statistical modeling in order to predict the long-term stability of the drug [23]. Because this type of study necessitates extensive computational modeling [24], Waterman et al. [25, 26] helped to popularize ASAP studies with the recent introduction of a software program (ASAPprime®, FreeThink Technologies) enabling the implementation of this approach. The ASAP methodology is based on a modified Arrhenius equation which links the degradation rate of a compound to the temperature and the relative humidity. Typically, during ASAP studies, the pharmaceutical product is exposed to different temperatures, typically between 50 °C and 80 °C, and various relative humidity levels for 3 to 4 weeks. ASAP studies have generally been used to predict the stability of small chemical drugs [27, 28], and the approach has recently been used to predict the stability of peptides [29, 30]. It has also been used in regulatory submissions for advanced shelf-life prediction in support of stability studies, especially for clinical trials [31].

In this work, the innovator product corresponding to infliximab was characterized alongside two different types of biosimilar products in the context of forced degradation in order to assess their biosimilarity with respect to stability. Several PTM hotspots including methionine oxidation and asparagine deamidation were characterized using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In addition, size-exclusion chromatography coupled to multi-angle light scattering analysis (SEC-MALS-UV/RI) was used to characterize aggregates and free chain fragmentation. Consequently, the ASAP approach was applied in order to perform the modeling of asparagine deamidation and free chain fragmentation of infliximab concomitantly. The quality of the models generated for the different alterations of the mAbs was evaluated in order to investigate the possibility of obtaining long-term prediction regarding the stability of mAbs. Finally, the ASAP models developed for the different types of alterations were compared with experimental data generated from stability studies. The stability studies were performed using in-use conditions by reconstitution of infliximab in IV bags followed by storage for 3 months in order to show the adequacy of this approach for long-term prediction of therapeutic mAbs.

**Materials and methods**

**Chemicals** Chemicals used for the experiments were systematically of analytical grade or high-purity grade. Ultrapure water used to prepare buffers and sample solutions was obtained using a Milli-Q Reference A+ water purification system purchased from Merck Millipore (Billerica, MA, USA). LC-MS grade H₂O and acetonitrile (ACN) used for ultrahigh-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) experiments were purchased from VWR Chemicals (Fontenay-sous-Bois, France), respectively. Commercial products of infliximab innovator Remicade® (Merck Sharp and Dohme) and the respective
EMA/FDA-approved biosimilars Remsima® (Celltrion Healthcare) and Flixabi® (Biogen) were purchased from their respective manufacturers. Dithiothreitol (DTT) and iodoacetamide (IAM) were purchased from Sigma-Aldrich (Breda, the Netherlands). Trypsin enzyme was purchased from Promega (Madison, WI, USA).

**Infliximab reconstitution and stability study** First, the vials of infliximab were reconstituted using water for injection followed by gentle shaking, based on the guidelines of the manufacturers which were common for Remicade®, Remsima® and Flixabi®. Reconstitution was performed to a final concentration of 10 mg/mL. Consequently, the stability samples were prepared from the reconstituted solution by dilution of infliximab in an intravenous bag containing NaCl 0.9% (Freeflex, Fresenius Kabi or Ecoflac, B. Braun) to a final concentration of 1 mg/mL. Several intravenous bags were prepared and stored under the following stability conditions for 3 months: 4 °C, 25 °C exposed to light.

**Forced degradation study** Forced degradation samples of infliximab were prepared and characterized in order to identify the major degradation product and to evaluate biosimilarity between the three references of infliximab. High-temperature stress was performed as follows: 1 mL of reconstituted infliximab of each reference (1mg/mL) was conditioned in 1.5 mL vials, hermetically closed, and stored at 40 °C in temperature stability chambers (Binder, Tuttlingen, Germany) for 1, 2 and 3 months. Oxidative stress was performed as follows: 1 mL of reconstituted infliximab of each reference (1mg/mL) was diluted to 0.5 mg/mL with H₂O₂ 0.1% (final concentration: 0.05%). After 24 or 48 h, filtration of the samples was performed using a 30 kDa Amicon centrifugal filter (Merck, Molsheim, France) to remove the H₂O₂ and to concentrate the sample to a final concentration of 1 mg/mL.

**Stability prediction of Remicade® using ASAP modeling** Remicade® solution was conditioned in 1.5 mL vials, hermetically closed, and stored as described in Table 1 in order to perform the degradation study. Remicade® accelerated degradation was performed in temperature stability chambers (Binder, Tuttlingen, Germany). For each condition, all vials were placed in the chambers at the same time, and were removed after appropriate time of stress, to be stored at 4 °C before analysis. Temperature of the chambers was monitored in real time in order to avoid any excursion of temperature. Statistical and stability predictions were performed using the software ASAPPprime® version 5.0.3 (FreeThink Technologies, Branford, CT, USA). Stability prediction was realized for the following CQA: deamidation and fragmentation. For deamidation, the default fit method was used. For fragmentation, the diffusion method was used. Confidence intervals were calculated through the Monte Carlo approach: the number of Monte Carlo simulations was set at 2500. The limit of specification for deamidation was 5.0%, and 1.0% for fragmentation.

**Tryptic digestion of monoclonal antibody samples** For one sample, a volume equivalent to a theoretical quantity of 50 μg of mAbs was sampled and desalted against milli-Q H₂O using a 30 kDa Amicon centrifugal filter (Merck, Molsheim, France) in order to remove salts. Afterward, 20 μL of ammonium bicarbonate 50 mM (pH 8.0) was added to the sample. The sample was incubated with mild stirring at 40 °C for 10 min. DTT was added to a final concentration of 10 mM and incubated at 80 °C for 20 min. After cooling down to room temperature, IAM was further added to the sample mixture to a concentration of 10 mM followed by incubation of the sample for 20 min at room temperature in the dark in order to enable alkylation of the thiol residues. A 1 μL volume of trypsin (0.5 μg/μL) was added to the mixture which was left for incubation at room temperature for 3 h. Another volume of 1 μL was added and digestion was performed overnight by incubation at 37 °C. Following digestion, a volume of 24 μL of formic acid (FA) 0.1 M was added and the sample was left at RT for 1 h. Finally, the samples were diluted to a final concentration of 0.5 μg/μL using H₂O (0.1% FA). Digested samples were stored at 5 °C prior to UPLC-MS/MS analysis.

**Ultrahigh-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis** The described UPLC-MS/MS method was previously developed by our research group [32, 33]. Peptide mixtures obtained from tryptic digestion were separated by UPLC (ACQUITY, Waters, Manchester, UK) using a reversed-phase C18 stationary phase (BEH C18 1.7 μm, 2.1 × 150 mm) from Waters (St Quentin-en-Yvelines, France) directly coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The mobile phases were composed of 0.1% formic acid (FA) in water (mobile phase A) and 0.1% FA in acetonitrile (mobile phase B). Peptide separation was carried out using a gradient from 5% to 80% B over 38 min and maintained at 80% B for 3 min, at a flow rate of 100 μL/min. The sample volume used for UPLC-MS/MS
experiments was systematically 10 μL. The LTQ Orbitrap XL MS was equipped with a heated electrospray ionization source (HESI-II) from Thermo Scientific (Bremen, Germany). ESI source parameters were set as follows: ESI voltage of 4.0 kV, sheath gas flow rate of 40 and an auxiliary gas flow rate value of 12. ESI nebulizer temperature was set to 300 °C. Capillary voltage and tube lens were set to 35 V and 90 V, respectively. MS/MS experiments were performed in a Top5 data-dependent acquisition (DDA) composed of one full MS scan over the mass/charge (m/z) range of 150–2000 followed by five sequential MS/MS scans realized on the five most intense ions detected at a minimum threshold of 500 counts. Full MS scans were collected in profile mode using the high-resolution FTMS analyzer (R = 60,000) with a full-scan AGC target of 1E6 and microscans = 1. The ion trap was used in centroid mode at normal scan rate to analyze MS/MS fragments. The MSn AGC target was set to 1E4 with microscans = 3. Ions were selected for MS/MS using an isolation width of 2 Da, then fragmented by collision-induced dissociation (CID) using a normalized CID energy of 35, an activation Q of 0.25 and an activation time of 30 ms. The default charge state selected was z = 2. Using these parameters, the total duty cycle was determined to be 0.65 s. Parent ions were excluded from MS/MS experiments for 60 s in case ions triggered an event twice in 15 s using an exclusion mass width of ±1.5 Th. The instruments were controlled using Xcalibur 2.1.0 SP1 Build 1160 (Thermo Scientific, Bremen, Germany).

**MS/MS data analysis** Data obtained from UPLC-MS/MS experiments were analyzed using Xcalibur Qual Browser 2.2 SP1.48 (Thermo Scientific, Bremen, Germany). Purely proteolytic peptides (no miscleavages) were determined in silico considering the tryptic digestion of the amino acid sequence of infliximab. For each proteolytic enzyme, conventional cleavage rules were applied and carbamidomethylation of cysteine (+57.0215 Th) considered as a systematic modification. With regard to the occurrence of PTMs, asparagine deamidation was characterized as a potential modification considering a mass shift of +0.9840 Da, and methionine oxidation by taking into account a mass shift of +15.9949 Da. Peptide identification and PTM characterization were achieved manually from the conjunction of intact peptide mass measurements in full MS and MS/MS peptide fragments attribution using a mass tolerance of less than 5 ppm in MS and 0.05 Th in MS/MS. Relative modification levels were determined for PTM using the peak areas corresponding to the MS signal for the intact peptide and the modified equivalent.

**Size-exclusion chromatography–multi-angle light scattering (SEC-UV/RI-MALS)** The described SEC-UV/RI-MALS method was previously developed by our research group [32]. Intact mAb samples were characterized by size-exclusion liquid chromatography (SEC) using a Biozyn SEC column (300 x 4.6 mm, 1.8 μm) purchased from Phenomenex (Le Pecq, France). Experiments were performed using a Prominence HPLC-UV system (Shimadzu; Marne-la-Vallée, France) equipped with an RID-20A refractive index detector (Shimadzu, Marne-la-Vallée, France) and a miniDAWN TREOS II multi-angle light scattering instrument (MALS) acquired from Wyatt Technology (Santa Barbara, CA, USA). The MALS instrument was equipped with a 658 nm laser and light scattering measurements were performed simultaneously at 49°, 90° and 131°. The MALS was also equipped with a COMET ultrasonic actuator (Wyatt Technology) which enables sonication of the MALS flow cell between each analysis in order to prevent any deposit formation. The mobile phase used for SEC-UV/RI-MALS analysis was composed of 50 mM phosphate buffer (pH 6.8) and 300 mM NaCl using a flow rate of 200 μL/min. An injection volume of 20 μL was used, the UV absorbance detection was performed at a wavelength of 280 nm and the analysis time was 35 min. Data were collected and processed using Astra ® version 7.2 software (Wyatt Technology). Relative levels of aggregation and free chain fragmentation were estimated from 280 nm absorbance chromatograms using the peak area compared to the sum of all the peak areas corresponding to the different forms of the mAbs.

**Results and discussion**

**Infliximab biosimilarity assessment in relation to stability**

In order to study the biosimilarity between the infliximab innovator (Remicade®) and the corresponding biosimilar products (Remsima®, Flixabi®) in terms of stability, the products were subjected to different stress conditions. The primary structure of the mAbs was then systematically characterized using LC-MS/MS analysis (see “Materials and methods”) in order to identify PTM hotspots and estimate the level of modification. In addition, the aggregation and/or chain fragmentation of the mAbs was characterized using SEC-MALS-UV/RI analysis.

For the freshly reconstituted products, LC-MS/MS analysis enabled sequence coverage of 94.9% to be systematically achieved (Fig. S1). This shows the robustness of the method and the possibility for near complete characterization of the primary structure, providing the opportunity to identify all the PTM hotspots described for infliximab. Therefore, LC-MS/MS data enabled the unambiguous identification of one deamidation and nine different oxidation hotspots. As emphasized in Fig. 1A, the level of oxidation initially ranged from 0.7% to 5% depending on the residue.
Levels of oxidation were found to be similar for the different residues except for heavy chain (HC) M18 and M255 residues in addition to light chain (LC) M55, which exhibited higher modification levels for the biosimilar products. The residue M18 is located in the mAb variable domain, and the oxidation of the amino acid M255 is reported to lead to lower affinity with the FcRn receptor [34]. Therefore, the occurrence of such modifications can alter the biological properties of the mAbs. It is important to characterize the occurrence and the proportion of PTMs in the context of biosimilarity assessment, which in this case showed only slight differences. The residue N57 is located in the complementarity-determining region (CDR) of infliximab HC, and therefore deamidation into aspartic acid would have a major impact on the epitope interaction. Also, this residue has been described as being sensitive to deamidation [12]. The level of N57 deamidation was between 1.5% and 2.4% initially for the different products (Fig. 1B). Note that the PTM levels observed for the reconstituted products are consistent with those in other studies [12, 14, 35, 36]. Similarly, the reconstituted products were characterized using SEC-MALS-UV/RI. SEC-MALS results presented in Fig. 1C show that initially the proportion of aggregates represents less than 1% of the total forms, whereas the proportion of aggregates is slightly higher in the case of the two biosimilar products.

Consequently, forced degradation was performed on the three references of infliximab in order to identify potential degradation of the mAbs and to assess their biosimilarity with regard to their stability. To accelerate the oxidation of residues, the different samples were incubated in the presence of H₂O₂, whereas mAbs were exposed to relatively high temperature in order to generate deamidation and aggregation. Results of LC-MS/MS experiments demonstrated an increase in oxidation in the case of M55, M18, M255 and M431, as presented in Fig. 2A, whereas no significant modification could be observed for other residues characterized (Fig. S2). Thus, the oxidation levels increased rapidly, with modification levels ranging from 39.8% to 76.0% after incubation for 24 h, depending on the residue, and systematically above 62.5% after 48 h of incubation. Regarding deamidation in the CDR, the residue N57 exhibited modification levels that gradually increased over time, with values ranging from 1.9% to 3.8% after 5 days of exposure to a temperature of 40 °C, and from 21.9% to 24.5% after 90 days of incubation (Fig. 2C). The tridimensional structure of infliximab described in the literature was compared to the LC-MS/MS data in order to characterize the differences between the modified residues and the amino acids which remained intact. As emphasized in Fig. 2B, the amino acid residues prone to modification were located at the periphery of the protein, and therefore significantly exposed to the environment. In addition, this observation suggests that amino acids buried deeper inside the tertiary structure of the protein may be less sensitive to endogenous modification. Regarding the oxidation and deamidation hotspots characterized, the data showed similar modification levels among the different products corresponding to infliximab. Therefore, LC-MS/MS experiments showed the relevant similarity of the different infliximab products with regard to their stability under stress conditions in addition to the similarity of their tertiary structure. Indeed, the modified residues are consistent between the different samples, indicating similar exposure to the environment. In the case of SEC-MALS analysis, the proportion of mAb aggregates remained constantly below 1% irrespective of the duration of the stress (Fig. 2D). In contrast, exposure to a temperature of 40 °C led to the fragmentation of infliximab in free heavy chains and light chains, with a proportion of 5% of fragments after 90 days (Fig. 2E). In this case as well, the innovator and biosimilar products corresponding to infliximab demonstrated similar levels of fragmentation throughout the duration of the temperature stress. Consequently, the fragmentation into

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Fig. 1 Proportions of oxidation (A) and N57 deamidation (B) determined from LC-MS/MS data and levels of aggregation (C) measured from SEC-MALS-UV/RI experiments in the case of Remicade® (red), Remsima® (green) and Flixabi® (blue) present after reconstitution.
free HC and LC was determined to be the major degradation pathway regarding size variants of infliximab.

**Infliximab stability and biosimilarity assessment for in-use conditions**

Following the assessment of the different infliximab products with respect to stress stability, the in-use stability of infliximab was evaluated using different conditions, this time after reconstitution in intravenous (IV) bags. The conditions were selected to reflect different scenarios regarding storage temperature, the type of IV bag or the exposure to light (see “Materials and methods”) over a period of 3 months. In addition to assessing the stability of the different products corresponding to infliximab in real-life conditions, this study aimed to provide evidence regarding the possibility of anticipated preparation in a hospital unit in order to manage a continuously growing number of patients. The results obtained from LC-MS/MS experiments concerning the residues sensitive to oxidation are presented in Fig. 3. At a storage temperature of 4 °C, oxidation appeared globally limited, with levels ranging from 0.5% to 4% depending on the residue. Moreover, the oxidation levels did not show a significant increase upon storage at 4 °C even for an extended time (Fig. 3A). Similarly, when the IV preparations were maintained at 25 °C in the dark, oxidation levels did not exhibit any increase, and the modification levels were comparable to the data obtained at 4 °C (Fig. 3B). In the case of IV bags kept at 25 °C exposed to light, the sensitive methionine previously identified showed a gradual increase in oxidation levels, with values ranging from 0.5% to 12.9% in the case of M255, which showed the highest oxidation levels (Fig. 3C). The oxidation of residue M255 is reported to significantly decrease the affinity between the mAbs and the FcRn receptor which is responsible for the preservation of IgG proteins in the endosome [37]. Therefore, the results obtained show that prolonged exposure to light is likely to impact the serum half-life of infliximab. Interestingly, the
methionine and tryptophan residues, which were not modified during the stress degradation, also did not exhibit any modification in the IV bag samples. Therefore, this comparison shows that H₂O₂ represents a relevant oxidation stress assay capable of indicating residues prone to modification. For the different conditions, the biosimilar products generally exhibited similar behavior regarding oxidation hotspots regardless of the type of IV bag. However, in the case of the infliximab innovator, LC-MS/MS data showed that M55 and M255 demonstrated increased oxidation when the mAbs were reconstituted in IV bag B. For instance, in the case of M255, the level of oxidation was 5% after incubation when the mAbs were reconstituted in IV bag A, while the level was 13% if the product was solubilized in IV bag B (Fig. 3C). IV bag A is composed of polypropylene whereas IV bag B is constituted of low-density polyethylene. Therefore, this result could be linked to an interaction between the additives of the IV bag and the mAbs.

With regard to the deamidation of the amino acid hotspot N57, the results presented in Fig. 4A show that the modification of the residue was not significantly increased when the sample was stored at 4 °C, with modification levels ranging from 1.5% to 2.7%. On the contrary, the samples subjected to a temperature of 25 °C demonstrated a gradual increase in the modification level from 1.5% to 5%. In the case of the deamidation, light exposure did not influence the kinetics of the modification, indicating that temperature is the major factor in this case. Meanwhile, the study of the higher order structure using SEC-MALS-UV/RI analysis did not demonstrate infliximab fragmentation for the samples maintained at 4 °C, whereas the samples subjected to a temperature of 25 °C over an extended period exhibited fragmentation of the proteins in free chains (Fig. 4B).

As a consequence, the experiments performed concomitantly using LC-MS/MS analysis and SEC-MALS-UV/RI allowed us to investigate the stability of the different products corresponding to infliximab with respect to different PTM hotspots and aggregation/fragmentation. The study using stressed conditions allowed us to identify the residues prone to modification, especially for oxidation and deamidation. Thus, the study demonstrated that the occurrence of oxidation is driven by the media solubilizing the mAbs. In addition, data demonstrated that residues accessible to the solvent are rapidly modified, in contrast to the amino acid buried in the structure of the protein, which remains intact. For deamidation and free chain fragmentation, the degradation of the protein was attributed to the effect of temperature. The investigation of the infliximab products reconstituted using in-use conditions demonstrated that the residues from modification during real-life conditions were the same as those modified using stressed conditions. The results showed that no significant modification was observed when samples were stored at 4 °C for a period of 3 months. The innovator product and the corresponding biosimilar demonstrated important similarity in terms of stability; however, it is important to note that the innovator samples exhibited significantly higher oxidation for the residues M55, M18, M431 and M255 when the product was reconstituted in IV bags composed of polyethylene, which has not been described before. Such results clearly emphasize that biosimilarity assessment should not be restricted to a simple structural characterization but should also investigate the stability of biosimilar candidates over the different levels characterizing the structure of the mAbs, if possible in conditions as close as possible to real-world use conditions. Indeed, to our knowledge, this is the first study evaluating the evolution of PTM hotspots during hospital in-use conditions of infliximab. Also, it is the first time that biosimilarity assessment was performed in the context of hospital in-use conditions.
Infliximab stability prediction using ASAP modeling

After the stability study, an accelerated stability assessment program (ASAP) was realized for infliximab in order to evaluate the possibility of employing this type of approach in predicting the long-term stability of therapeutic mAbs. Experimentally, the ASAP model is built by subjecting the studied product to different temperature conditions and various relative humidity levels in the case of a solid formulation. The level of the degradation product (DP) generated depending on the conditions is then used to determine the modified Arrhenius equation parameters as illustrated in Eq. 1:

\[ \ln(k) = \ln(A) - \frac{E_a}{RT} + B \times RH \]  

(1)

where \( k \) represents the degradation rate, \( \ln(A) \) the pre-exponential factor, \( E_a \) the activation energy, \( T \) the temperature, \( R \) the gas constant, \( B \) the moisture sensitivity factor and \( RH \) the relative humidity.

Thus, the model is established by artificial generation of the considered DP, close to the specification limit in each stress condition [26], which makes it possible to overcome the heterogeneous kinetics of degradation [25]. The specification limit corresponds to the maximum amount of degradation product allowed for the therapeutic product. For small chemical synthetic drugs, the limit of specification is generally defined from 0.05% to 1.0% by the regulatory authorities depending on the posology of the molecule [38]. For biotherapeutic products, the limit is defined on a case-by-case basis for each drug based on the regulatory guidelines or the information described in the scientific literature [39]. The time necessary to reach the specification limit is referred to as the isocconversion time. The model developed can then be used to predict the level of DP that would be observed during long-term stability study in the envisaged storage temperature and relative humidity. Note that in order to be able to perform ASAP modeling, it is essential to characterize the DP beforehand and benefit from an analytical method which allows it to be analyzed without any interference. Infliximab and mAbs in general are extremely complex macromolecules which may undergo several modifications simultaneously [9]. Thus, it is usually difficult to predict the long-term stability for biotherapeutic products because of their inherent complexity and the non-Arrhenius behavior for quality attributes such as aggregation [40]. However, the aggregation pathways seem to be significantly mAb-dependent [41]; for instance, a recent study was able to model aggregation using a thermodynamic equation with other types of mAbs [42].
For the long-term stability study of infliximab, the ASAP approach was performed concomitantly for different degradation processes in order to investigate the possibility of modeling the stability of the mAbs regarding different aspects defining the structure of the protein. As illustrated in Eq. 1, the implementation of the modified Arrhenius equation makes it possible to study the degradation influenced by temperature and/or humidity. The results obtained previously for stability studies showed that oxidation of methionine hotspots were influenced by the level of oxygen and the exposure to light, whereas the storage temperature had no influence on the level of their oxidation when increased from 4 °C to 25 °C (Fig. 3). On the contrary, the stability study showed that for the different infliximab products, the deamidation of N57 and the fragmentation in free chains are impacted by the temperature (Fig. 4). Therefore, the ASAP modeling was envisaged only for these two types of degradation. Thus, a vial of infliximab corresponding to the marketed formulation was reconstituted and consequently split into equal-volume samples, which were subjected to temperature ranging from 30 °C and 45 °C for up to 30 days. During the incubation, the samples were regularly characterized regarding N57 deamidation using LC-MS/MS analysis and concomitantly regarding chain fragmentation of mAbs by the intermediate of SEC-MALS-UV/IR analysis. Note that the incubation temperature was limited, because above 45 °C, rapid precipitation generating non-soluble particles could be observed. The specification limits were fixed at a value of 5.0% in the case of deamidation and 1.0% for free chain fragmentation. The monograph of the pharmacopoeia for infliximab did not mention the limits in terms of acceptable PTM levels; therefore, the specification limits were determined using minimal values considering modification levels previously described in the literature. In addition, 5% modification was considered as a maximum acceptable level of PTMs, in order to maintain 95% of the original form as is commonly acceptable [16, 43].

As emphasized in Fig. 5A, the deamidation of the residue N57 demonstrated a gradual increase from 0.7% initially up to 8.6% for incubation at 45 °C for 15 days. Results also showed an increase in the kinetics of the reaction when the incubation temperature was increased. With regard to the fragmentation of infliximab in free chains, no fragments were initially detected (Fig. 5B). Subsequently, the proportion of free chains increased over time when the mAbs were exposed to increasing temperatures up to 1.6% and incubated at 40 °C for 30 days. In this case as well, the kinetics of the free chain fragmentation increased when the temperature was higher, showing that the degradation process is impacted by the temperature. For each temperature condition, the isoconversion time was calculated or extrapolated from the experimental data. A linear regression was used in the case of N57 deamidation (Fig. S3) and a diffusion regression (Fig. S4) for mAb free chain fragmentation, which is consistent, as the two processes of degradation showed different evolutions (Fig. 5). The isoconversion times and isoconversion ratios obtained for the different conditions are detailed in Table 2. The isoconversion ratio corresponds to the ratio between the latest measurement time and the calculated isoconversion time. It enables us to estimate the extent

| Temperature condition | Deamidation | Fragmentation |
|-----------------------|-------------|---------------|
|                       | Isoconversion time (days) | Isoconversion ratio | Isoconversion time (days) | Isoconversion ratio |
| 30 °C                 | 48.4 ± 12.2 | 0.6           | 72.9 ± 14.9 | 0.4 |
| 35 °C                 | 35.6 ± 3.5 | 0.8           | 43.0 ± 8.1 | 0.7 |
| 40 °C                 | 21.4 ± 2.8 | 1.4           | 10.9 ± 2.0 | 2.8 |
| 45 °C                 | 8.3 ± 1.5  | 1.8           | 5.8 ± 1.1 | 2.6 |

Table 2 Isoconversion time and isoconversion ratio calculated from ASAP stress samples for deamidation of the amino acid N57 and infliximab chain fragmentation for the different temperature conditions

![Fig. 5](A) Proportion of N57 deamidation estimated from LC-MS/MS analysis and of (B) infliximab chain fragmentation determined from SEC-MALS-UV/RI analysis for the ASAP stress samples. The red discontinuous lines indicates the specification limit in each case.
of the extrapolation required to determine the isoconversion time. Thus, it tends to be closer to 0.0 when an important extrapolation is required to calculate the isoconversion time. The value is above 1 when the specification limit is reached during the experiment and extrapolation is not necessary. Therefore, the isoconversion ratio makes it possible to limit excessive extrapolation in order to build a valid model. Generally, isoconversion ratios lower than 0.1 are considered out of range, and the data should be excluded from the model. For the N57 deamidation hotspot, the lowest isoconversion ratio calculated was 0.62 at 30 °C, whereas for the free chain fragmentation, the lowest isoconversion ratio was 0.41 (Table 2). Thus, the data obtained using the different conditions were compatible with the model for infliximab N57 deamidation and free chain fragmentation.

Using the experimental data from the different conditions, the parameters of the Arrhenius equation were calculated for the deamidation of the amino acid N57 and the fragmentation of the mAbs in free chains, as summarized in Table 3. Different parameters were considered in order to evaluate the adequacy of the various ASAP models. The correlation coefficient $R^2$ between the model and the experimental data was 0.934 for the deamidation and 0.968 in the case of the free chain fragmentation, thus demonstrating validity (Table 3).

| Model parameters | Deamidation | Fragmentation |
|------------------|-------------|---------------|
| Ln A             | 34.05 ± 6.22| 52.43 ± 5.80  |
| $E_a$ (kcal/mol) | 22.06 ± 3.85| 34.25 ± 3.58  |
| B                | NA          | NA            |
| $R^2$            | 0.934       | 0.968         |
| $Q^2$            | 0.576       | 0.898         |

Indeed, regarding adequacy, the ASAP model is considered to be valid when $R^2 > 0.9$ [44]. The cross-validation coefficients $Q^2$ were also calculated yielding a value of 0.576 for deamidation and 0.898 for free chain fragmentation. Due to their respective characteristics, the value of $Q^2$ is expected to be lower than $R^2$; however, it is the difference between the two values which makes it possible to further demonstrate the adequacy of the model compared to the experimental data. For the two types of degradation considered, the values of both $R^2$ and $Q^2$ proved to be satisfactory. In addition, as presented in Fig. 6, the residual plots corresponding to the DP formation rate ln (k) were satisfactory compared to other studies using the ASAP methodology [29], with residues of ln k systematically lower than ±0.4.

Previous stability studies of infliximab using forced degradation enabled the identification of different alterations of the mAbs. In addition, they identified the degradation processes which could potentially be modeled using an ASAP approach. Thus, the characterization of the samples subjected to different temperature conditions showed the possibility for concomitant modeling of the deamidation of the residue N57 and the fragmentation of the mAbs in free chains. The ASAP models established for the different degradation types were shown to be in agreement with the experimental data, confirming the validity of the model based on common practice regarding ASAP prediction. In addition, the relevance of the models clearly indicates that N57 deamidation and free chain fragmentation are thermodynamically driven alterations of infliximab. The ASAP models further highlighted the possibility of performing mAb stability prediction using the ASAP approach over several degradation processes which may occur concomitantly on the structure of the mAbs. From that perspective, the methodology developed in this work is particularly interesting because it provides the opportunity to perform comprehensive stability prediction regardless of...
the structural complexity of the mAbs. To the best of our knowledge, this is the first time that ASAP modeling was performed simultaneously with success for degradation over different levels defining the structure of the mAbs, primary structure for the deamidation and tertiary structure in the case of free chain fragmentation.

**Comparison between stability prediction and long-term stability data**

In order to evaluate the performance of the model, the stability prediction generated using the described ASAP models was compared to the data obtained during the in-use stability study of infliximab with respect to the deamidation of the residue N57 and the free chain fragmentation of the mAbs (Fig. 4). The elaboration of the ASAP model takes into account the variability of the generated model and eventually the variability of the analytical method in order to predict the minimum and maximum quantity of DP generated over time for a designated condition (Fig. 7). Regarding deamidation of the residue N57, at 4 °C the level of deamidation predicted using the model was systematically below 2% and the prediction described a near absence of increase concerning the modification level. However, the level of deamidation determined during the in-use stability study was systematically over the predicted level, even in the initial conditions directly after reconstitution (Fig. 7A). Nevertheless, the predicted and experimental evolution appears similar. This observation was attributed to the fact that infliximab vials used to perform the ASAP modeling and the in-use stability study came from different batches. Indeed, after reconstitution the two batches exhibited different initial deamidation levels of N57: 0.7 % ± 0.1 for the ASAP batch and 1.5 % ± 0.1 for the batch used for in-use stability samples (Fig. 7A). If a correction of the ASAP prediction is applied based on the initial mismatch concerning the deamidation level, all the experimental points of the in-use stability study are comprised in the prediction showing an outstanding correlation. For the storage conditions at 25 °C and 40 °C, the predictions described a gradual increase of the deamidation level after 90 days up to 3% for a temperature of 25 °C (Fig. 7B) and 4% at 40 °C (Fig. 7C). The predictions generated from the ASAP model demonstrated different slopes depending on the temperature applied. The experimental data are showing a relevant correlation with the prediction for the different case in term
of deamidation levels and evolution over the duration of the study. Therefore, the correlation of the results obtained from the ASAP prediction and experimental data demonstrated that the ASAP modeling is able to predict the evolution of the modification level of the residue characterized.

Concerning free chain fragmentation, the prediction generated for a temperature of 4 °C indicated a calculated level < 0.02% after 6 months (Fig. 7D) which, considering the performance of the SEC-MALS-UV/RI method, corresponds to an absence of fragmentation. Experimentally, when stored at a temperature of 4 °C, free chain fragmentation of infliximab was not observed, which is in agreement with the prediction of the model. For the conservation conditions at 25 °C, the prediction achieved from the model initially exhibited an absence of fragmentation followed by a constant increase over the duration of the experiment to a maximum value of 0.1% after 6 months. From the comparison with the experimental data, the fragmentation appears to be slightly overestimated by the model prediction (Fig. 7E). The difference in terms of the proportion of free chains was attributed to the extremely low levels generated during the first 60 days of the study, ranging from 0% to 0.015%, which can be difficult to accurately estimate from SEC-MALS-UV/RI chromatograms. When the proportion of free chains was further increased, the experimental data correlated with the model prediction. In addition, the larger discrepancy between the prediction and the experimental data represented 0.01%, which remained negligible and placed the prediction on the safer side compared to the level of free chains effectively characterized (Fig. 7E). Williams et al. reported a similar conclusion in a study using ASAP for small chemicals drugs [27]. As illustrated in Fig. 7F, for a temperature of 40 °C, the model prediction showed a steeper increase in the proportion of free chains above 0.25% after a period of 6 months. The experimental data showed a consistent correlation with the prediction provided by the model regarding the proportion of free chains and the evolution over time. Note that the experimental results from the in-use condition samples exhibited minor differences compared to the prediction of the model, which in this case as well represented a maximal difference of 0.01%, which is lower than the standard deviation provided by the analytical method. Indeed, in addition, the experimental data showed significant variability between the sample triplicates (Fig. 7F). As a consequence, the correlation of the model developed from the ASAP approach with the data obtained from the analysis for the samples stored using in-use conditions was found to be relevant for the long-term fragmentation of the infliximab in free chains.

Finally, the ASAP approach was successfully used to model two different types of alteration occurring on the infliximab mAbs. Thus, the required experiments were performed over a period of 30 days and allowed for the concomitant modeling of the deamidation of the residue N57 and the fragmentation of infliximab into free chains, for a period longer than 6 months (Fig. 7). For the two different alteration processes, the comparison between the prediction generated from the ASAP model and the experimental data obtained from long-term storage of infliximab reconstituted in IV bags demonstrated a relevant correlation. Also, the evolution of mAb degradation over time was relevant with the modeling. Therefore, the results achieved showed the possibility of using an ASAP model in order to predict the stability of mAbs under conventional storage conditions. In addition, the long-term stability prediction generated from the ASAP model could be eventually considered in order to optimize the composition of the formulation regarding mAb stability and help to determine the expiration time of the product. The implementation of the ASAP approach for the long-term stability study of infliximab highlighted the crucial requirements. Indeed, before the development of the model, it is essential to perform a complete characterization of the different degradation products generated by the therapeutic protein investigated. This makes it possible to identify the major degradation products as a preliminary approach, in addition to identifying the degradation processes which can be modeled by a modified Arrhenius equation due to the impact of temperature and/or humidity. In a similar manner, the implementation of the ASAP approach requires an analytical method able to unambiguously separate the investigated DP and accurately estimate the level. In particular, results achieved for the ASAP modeling concerning infliximab demonstrated that the accuracy, sensitivity and variability of the analytical method is crucial for providing a valid model and may influence the correlation with the experimental data. ASAP models could be established concomitantly for two different types of structural alterations of infliximab. Therefore, the workflow implemented in this study showed the methodology required to perform ASAP modeling of therapeutic mAbs. It may be possible to extend the ASAP modeling of mAbs to a wider range of modifications known to influence the characteristics of the protein such as aspartic acid isomerization and N-terminal glutamic acid cyclization in order to provide a comprehensive stability model regarding these complex macromolecules. As such, capillary zone electrophoresis coupled to tandem mass spectrometry (CE-MS/MS) represents a relevant technique for characterization of a large number of PTMs simultaneously [45].

Conclusion

In the present study, an innovator product corresponding to infliximab and two approved biosimilars were characterized during forced degradation and stability studies after
reconstitution of the products in IV bags, in order to assess their similarity in the context of degradation. The characterization of several PTM hotspots, including deamidation and oxidation, was realized using LC-MS/MS, and SEC-MALS-UV/RI was used to characterize aggregates and free chain fragmentation. For oxidative stress and temperature stress conditions, results demonstrated high similarity between the reference and the biosimilar products. During the stability study, experimental data for the most part exhibited similar evolution. However, the innovator infliximab demonstrated significantly higher levels of oxidation when the product was stored in IV bags composed of polyethylene and exposed to light. This result underlines the need to consider different possible impacts of content–container interaction when performing such stability study and biosimilarity comparison. Consequently, the ASAP approach was implemented in order to perform the simultaneous modeling of infliximab N57 deamidation and mAb fragmentation in free chains. The application of the ASAP methodology allowed us to successfully model the evolution of infliximab concerning the different degradation processes over a period of up to 6 months. Finally, the results obtained from the long-term stability study of infliximab reconstituted in IV bags were compared to the evolution of infliximab predicted from ASAP models to determine the relevant correlation between the evolution of mAbs predicted from the ASAP models and the long-term stability results in the case of deamidation and free chain fragmentation. The methodology developed in this study showed the possibility of elaborating the ASAP model of different degradation processes concomitantly, over the different levels defining the structure of the mAbs, in order to address the structural complexity of the protein. The present study helps to demonstrate the possibility of using such an approach to predict the stability not only of small molecules but also of biopharmaceutical products. The results highlight the current limitations of modeling based on a modified Arrhenius equation. In addition, this study allowed us to define the consecutive steps required for the implementation of an ASAP model for mAbs.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

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