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# **Post-translational modifications comparative identification and kinetic study of infliximab innovator and biosimilars in serum using capillary electrophoresis-tandem mass spectrometry**

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**ABSTRACT:** Despite reports indicating the potential impact of post-translational modifications on the activity of a monoclonal antibody, their prediction or monitoring post-administration remains a challenge. In addition, with the expiration of patents concerning the early generation of mAbs, the production of biosimilars is constantly increasing. Structural differences of biosimilars compared to the innovator product are commonly evaluated for the formulated product in the context of biosimilarity assessment. However, estimating their structural outcome after administration is particularly difficult. Due to the complexity of in vivo studies, there is a need to develop analytical strategies to predict PTMs consequently to their administration and their impact on mAbs potency. Here, we identified and evaluated the modification kinetics of 4 asparagine deamidations and 2 aspartate isomerizations of infliximab innovator product (Remicade®) and two biosimilars (Inflectra® and Remsima®) in vitro using serum incubation at 37°C. The methodology was based on a bottom-up approach with capillary electrophoresis hyphenated with mass spectrometry analysis for an unequivocal assignment of modified and unmodified forms. 2 asparagines demonstrated a gradual deamidation correlated with incubation time. The specific extraction efficiency was evaluated to determine possible changes in the antigen binding affinity of infliximab with the incubation. Results showed the possibility to achieve an additional aspect concerning biosimilarity assessment, oriented on the study of the structural stability after administration.

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## 1. INTRODUCTION

Monoclonal antibodies (mAbs) and their related formats, including bispecific antibodies and antibody-drug conjugates, are showing a tremendous interest as therapeutic treatments. As a result, mAbs represent the fastest growing category of pharmaceutical products. More than 100 therapeutic mAbs are currently approved, and 12 have been approved for the first time in 2022 [1]. In addition, several patents for the first generation of mAbs have recently entered the public domain. Therefore, the production of biosimilar mAbs, defined by regulatory authorities as an equivalent product containing the active substance of an original biopharmaceutical product, is of growing interest [2].

Based on immunoglobulin G (IgG), mAbs are tetrameric glycoproteins that exhibit a wide range of microheterogeneity, due to their inherent structure, resulting in a significant number of variants. Indeed, mAbs can undergo different types of post-translational modifications (PTMs) during protein expression or storage, including asparagine deamidation (deaN), aspartate isomerization (isoD), or N6glycosylation. Some PTMs, defined as hotspots, can potentially impact the physicochemical properties, the structure and the conformation of mAbs. As a consequence, their occurrence can alter the biological activity and the pharmacological properties of mAbs. For instance, in the case of glycosylation, different studies have shown reduced receptor binding for mAbs glycoforms exhibiting core-fucose N-glycans [3,4]. Similarly, a decrease in the antigen-binding affinity of IgG1 mAbs due to the deamidation of an asparagine residue located in the complement determining region (CDR) of the light chain (LC), has been described [5, 6]. In addition, changes in antibody-dependent cell-mediated cytotoxicity (ADCC) have been observed in the case of a specific deaN occurring in the constant domain (Fc) of a mAb [7–9]. Thus, the impact of PTMs on the biological activity of mAbs appears to depend on the localization and the nature of the modification. Therefore, PTMs require extensive characterization to identify their occurrence and to estimate their abundance, in order to ensure optimal efficacy and safety of the product. Regarding the development and approval of biosimilar candidates, the assessment of biosimilarity is achieved through detailed structural characterization and comparison to the innovator mAb. Especially, the comparison of the two products should emphasize the absence of differences concerning PTMs. In case of minor differences, further investigations are required to demonstrate the absence of significant impact on biological activity and toxicity in order for the product to be approved as a biosimilar [10].

Due to their structural complexity, significant research activities have been conducted in order to establish analytical methods that deliver a comprehensive characterization of mAbs structure, including PTMs. However, the described methods are strictly focused on production assessment or product stability studies [11,12]. Recent studies have shown that mAbs may undergo PTMs *in vivo* after administration of the product, which could potentially alter the efficacy of the therapeutic protein [5,13]. Thus, it appears crucial to characterize structural modifications of innovator mAbs, but also biosimilars, that will occur after injection of the product. Also, it is important to determine whether the modifications generated have an impact on the efficacy of the drug.

Infliximab (IFX) is a chimeric mAb targeting the tumor necrosis factor (TNF- $\alpha$ ). In addition to the innovator product, several biosimilars have recently been approved. Previous studies have evaluated the structural similarity between the formulation product of the innovator infliximab and the corresponding biosimilars [14,15], and after forced degradation of the mAbs [16,17]. However, studies predicting PTMs occurring after product administration are not yet described, mainly because they require structural analysis of mAbs present in serum, which contains important quantities of natural IgGs. Recently, we developed an analytical strategy based on capillary electrophoresis hyphenated to tandem mass spectrometry (CE-MS/MS), which allowed the absolute quantification and the characterization of mAbs PTMs extracted from serum using a specific purification protocol [18].

In the present work, the innovator product corresponding to infliximab (Remicade<sup>®</sup>) was characterized and compared to two biosimilar products (Inflectra<sup>®</sup> and Remsima<sup>®</sup>) concerning the occurrence of PTMs, after incubation in human serum at 37°C. After affinity extraction from serum samples, infliximab was characterized using CE-MS/MS to identify PTM hotspots and determine their modification levels. Four distinctive asparagine deamidations and two aspartate isomerizations, conventionally referred to as hotspots due to their potential impact on mAb properties, located on both the light chain (LC) and heavy chain (HC), were investigated. The study was designed to independently determine the modification kinetics for the different PTMs over time, using conditions that mimic the post-administration environment of infliximab. To prevent the introduction of any bias and/or artefactual PTMs due to sample treatment or analysis, an innovative normalization strategy adapted to the determination of PTM modification levels, was applied. CE-

MS/MS experiments allowed to achieve a comparative study of the different infliximab products regarding their stability after administration, demonstrating the need for this type of study to further extend biosimilarity assessment. Results obtained *in vitro* using model serum samples incubated under conditions that mimic the mAb environment were compared to samples collected from treated patients to demonstrate the relevance of the analytical approach in predicting the stability of the primary structure of mAbs after administration. Finally, the effect of a deamidation identified in the CDR region on the infliximab/TNF- $\alpha$  interaction was studied by means of specific extraction from serum.

## 2. MATERIALS AND METHODS

**2.1. Chemicals.** Chemical used were of high purity grade and purchased from Merck Sigma-Aldrich (Saint-Louis, MO, USA) if not stated otherwise. Ultrapure water used to prepare buffers and sample solutions was obtained using an ELGA Purelab UHQ PS water purification system (Bucks, UK). Infliximab innovator Remicade® (Merck Sharp and Dohme), biosimilar Inflectra® and Remsima® (Celltrion Healthcare) samples were EMA/ FDA-approved formulations purchased from the manufacturer. Stable-isotope labeled infliximab (SIL-IFX) internal standard was purchased from Sigma-Aldrich (Saint-Louis, MO, USA). RapiGest SF surfactant was purchased from Waters (Milford, MA). Streptavidin M-280 Dynabeads™ and biotinylated TNF- $\alpha$  were obtained from Fisher Scientific Invitrogen (Carlsbad, CA). Sequencing grade modified trypsin was purchased from Promega Corporation, (Madison, WI, USA). PBS (phosphate buffer saline) solution was purchased from Gibco ThermoFisher as tablets for reconstitution in water. Blank serums were provided by the French Institute of Blood (Paris, France). IFX stock solution at 1 g·L<sup>-1</sup> was prepared after reconstitution in H<sub>2</sub>O milliQ and stored at -20 °C.

**2.2. Infliximab structural stability study in serum.** A volume of 1 mL of human serum was spiked with Remicade®, Remsima® or Inflectra® at a final concentration of 10  $\mu\text{g}\cdot\text{mL}^{-1}$  and incubated at 37°C. Each sample was prepared in triplicate. A volume of 100  $\mu\text{L}$  of each serum sample was collected after 2, 6, 30, 50 and 90 days of incubation. Consequently, 4  $\mu\text{L}$  of SIL-infliximab internal standard solution at 0.25 g·L<sup>-1</sup> were added before proceeding immediately with sample preparation.

**2.3. Sample preparation.** Serum sample preparation was based on a protocol recently developed in our group [18]. Briefly, 217  $\mu\text{L}$  of Streptavidin M-280 Dynabeads<sup>TM</sup> ferromagnetic beads were preincubated with 148  $\mu\text{L}$  biotinylated TNF- $\alpha$  (10  $\mu\text{g}\cdot\text{mL}^{-1}$  in PBS) for 1 h at 20°C. Then, the serums, previously spiked with SIL-infliximab were added to the beads and left for 1 h at room temperature to allow any TNF- $\alpha$  antibody inhibitors to bind to the beads. A magnet was used to remove serum from the beads. Acidic dissociation of the mAbs from TNF- $\alpha$  was then performed by two consecutive washing steps using 50  $\mu\text{L}$  MeOH/H<sub>2</sub>O/FA: 48.5/48.5/3% solution. Supernatants were pooled and dried for 2 h at 35°C using a miVac DNA Concentrator System<sup>TM</sup>. The sample was reconstituted with 70  $\mu\text{L}$  of ammonium bicarbonate buffer (50 mM, pH 8) and digested. A denaturation-reduction-alkylation-digestion workflow, commonly used for the digestion of mAbs into peptides, was performed [19]. Finally, the peptide digests were evaporated at 30°C for 2 h, and then resuspended in 5  $\mu\text{L}$  ammonium acetate 100 mM, pH 4.

**2.4. Capillary electrophoresis-tandem mass spectrometry analysis.** Peptide mixtures obtained from sample preparation were analyzed using a CESI8000<sup>®</sup> capillary electrophoresis system (Sciex separations, Darmstadt, Germany) coupled to a Sciex 5600 TOF mass spectrometer (Darmstadt, Germany) operated by Analyst<sup>®</sup> software (Sciex, Darmstadt, Germany). The hyphenation of CE with ESI-MS was made through a sheathless interface, consisting of two capillaries in the CE system. The separation is performed in a bare fused silica capillary (total length 100 cm; 30  $\mu\text{m}$  i.d.), the outlet end of which was etched to make it porous using a home-made protocol [20]. A second capillary (total length 80 cm; 50  $\mu\text{m}$  i.d.) is used to maintain electrical contact at the porous interface with the background electrolyte (BGE) composed of 10% acetic acid. During the analysis, a transient isotachopheresis preconcentration step (t-ITP) was performed by injecting the peptide digest solubilized in a leading electrolyte of 100 mM ammonium acetate (pH 4), which is more conductive than the BGE. 68 nL of the peptide digest was injected hydrodynamically (5 psi, 100 s) and a voltage of 23 kV was applied to the separation capillary. For the ESI source parameters, the applied ESI voltage was 1.5 kV, the source heating temperature was 100 °C, and the curtain gas was set to 2. Experiments were performed in Top20 information-dependent acquisition (IDA, Sciex) with a total duty cycle of 1.9 s during 60 min of analysis. The mass/charge ( $m/z$ ) range was 100-2000 for MS and 50-2000 for MS/MS. The data were analyzed using Skyline software.

**2.5. Post-translational modifications characterization and extraction yield determination.** The presence of PTMs was systematically identified based on high-resolution  $m/z$  measurements provided by MS spectra and fragment characterization observed in MS/MS data collected from CE-MS/MS experiments. 6 distinct PTM hotspots were investigated: 4 asparagine deamidations (N57 (heavy chain: HC), N137 (light chain: LC), N158 (LC) and N387 (HC)) and 2 aspartate isomerizations (D283 (HC) and D404 (HC)) (localizations shown in Fig. S1). The proportion of each PTM was determined by integration of MS signals corresponding to unmodified peptides and their modified counterparts. To suppress any eventual artefactual modifications occurring during sample preparation, a PTM-specific normalization strategy previously implemented by our group was used [18]. Briefly, the SIL-infliximab internal standard added to the sample at the beginning of the workflow acted as a marker for endogenous modifications occurring during sample preparation and analysis (Fig. 1). For an amino acid (here denoted “L”) of infliximab, which incubated at 37 °C in serum (here denoted “sample”), its level of modification that occurred strictly during the *in vitro* incubation ( $L_{incubation}$ ) is expressed by Eq. (1). The references values (ref) are necessary as the initial structure of SIL might differ from infliximab.

$$L_{incubation} = \frac{L_{sample}^{IFX} - L_{ref}^{IFX} - L_{sample}^{SIL} + L_{ref}^{SIL} * L_{sample}^{IFX} * L_{sample}^{SIL} + L_{ref}^{SIL} - L_{sample}^{IFX} * L_{ref}^{SIL}}{1 - L_{ref}^{IFX} - L_{sample}^{SIL} + L_{ref}^{SIL} * L_{sample}^{IFX} * L_{sample}^{SIL}} \quad [\text{Eq. 1}]$$

With:

$L_{sample}^{IFX}$ : Abundance of the modification of “L” found in CE-MS for the infliximab compound, in spiked serum incubated at 37°C.

$L_{sample}^{SIL}$ : Abundance of the modification of “L” found in CE-MS for the SIL-infliximab compound, in spiked serum incubated at 37°C.

$L_{ref}^{IFX}$ : Abundance of the modification of “L” found in CE-MS for the infliximab compound, in spiked serum immediately analyzed.

$L_{ref}^{SIL}$ : Abundance of the modification of “L” found in CE-MS for the SIL-infliximab compound, in spiked serum immediately analyzed.

The determination of the extraction efficiency was based on the monitoring of a dedicated tryptic peptide of infliximab and SIL-infliximab, which possessed the particularity to be proteotypic for both compounds. The selected peptide was DILLTQSPAILSVSPPGER (LC01), located in the variable domain of infliximab (Fig. S1), and its specificity to this antibody was verified by a blast search using a human database [<https://blast.ncbi.nlm.nih.gov/Blast.cgi>]. The signal

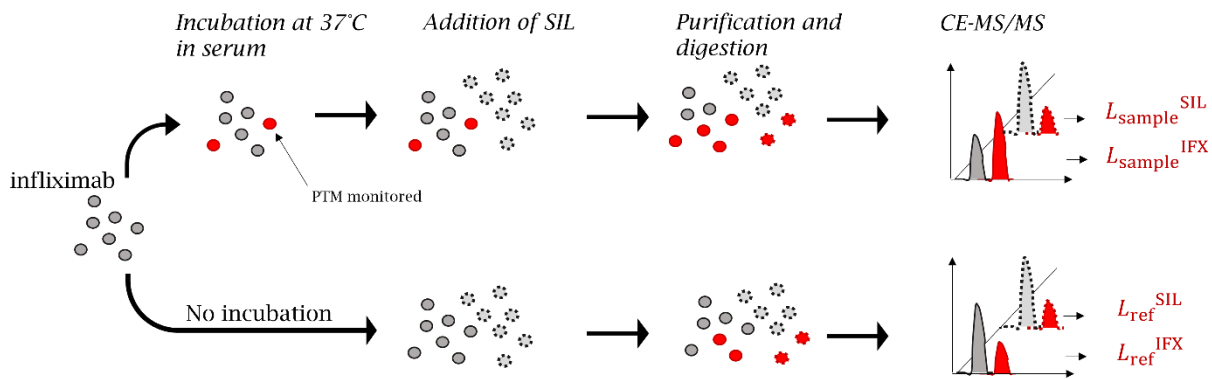


used for SIL-infliximab monitoring corresponds to the same LC01 peptide, incorporating  $^{13}\text{C}$  and  $^{15}\text{N}$  isotope labeling for the arginine (R) amino acid (+10 Da). The extraction efficiency was estimated by comparing the infliximab/SIL-infliximab signal ratio of the sample of interest (here infliximab-spiked serums that incubated at  $37^\circ\text{C}$ ) of the reference sample (immediately analyzed infliximab spiked serum) and was expressed by the Eq. (2):

$$\text{Extraction efficiency (\%)} = \frac{\left(\frac{A_{\text{LC01}}^{\text{IFX}}}{A_{\text{LC01}}^{\text{SIL}}}\right)_{\text{sample}}}{\left(\frac{A_{\text{LC01}}^{\text{IFX}}}{A_{\text{LC01}}^{\text{SIL}}}\right)_{\text{reference}}} \times 100 \quad [\text{Eq. 2}]$$

With  $\left(\frac{A_{\text{LC01}}^{\text{IFX}}}{A_{\text{LC01}}^{\text{SIL}}}\right)_{\text{sample}}$  the infliximab/SIL-infliximab signal ratio found in the spiked serum incubated at  $37^\circ\text{C}$ . And

$\left(\frac{A_{\text{LC01}}^{\text{IFX}}}{A_{\text{LC01}}^{\text{SIL}}}\right)_{\text{reference}}$  : infliximab/SIL-infliximab signal ratio found in the spiked serum immediately analyzed.

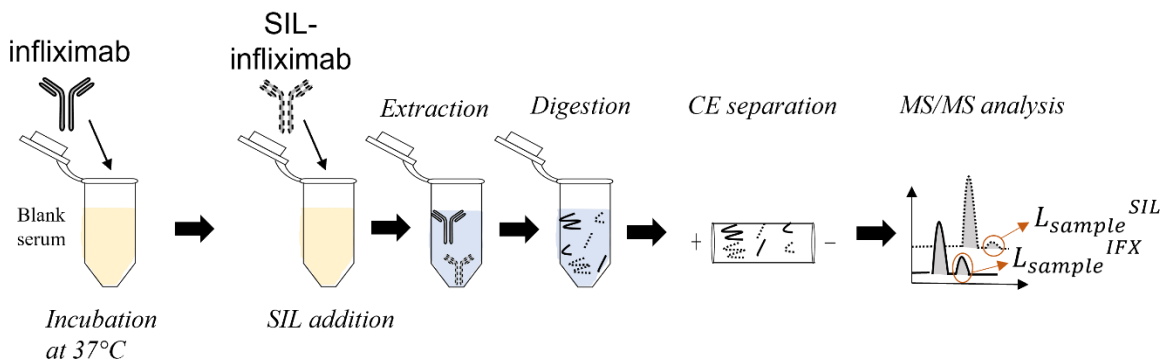


**Fig. 1.** Schematic representation of the normalization approach for the relative quantification of PTM levels occurring during incubation.

### 3. RESULTS AND DISCUSSION

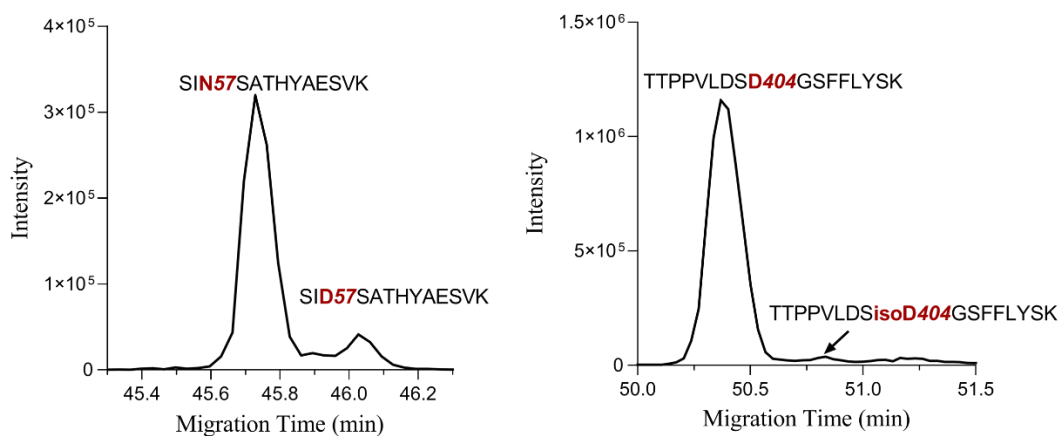
To investigate the possibility of studying *in vitro* the structural modification of infliximab after administration, the stability study was performed using blank serums spiked with either Remicade<sup>®</sup>, Inflectra<sup>®</sup> or Remsima<sup>®</sup>, incubated at  $37^\circ\text{C}$  to mimic physiological conditions. After incubation, as shown in Fig. 2, SIL-infliximab internal standard was added to the sample. Consequently, a specific extraction of both infliximab and SIL-infliximab was performed using TNF- $\alpha$  immobilized on ferromagnetic beads, followed by proteolytic digestion. Finally, the generated peptide mixture

was characterized by CE-MS/MS analysis (see Materials and methods section). Spiked serums analyzed immediately without incubation were used as references to compare the extraction yield and the level of PTMs of incubated samples. Concerning the relative quantification of PTM levels, the normalization strategy (Eq. (1)) using the SIL-infliximab as a marker was applied to account for possible artefactual reactions that could occur during sample preparation (Fig. S2).



**Fig. 2.** Schematic representation of the CE-MS/MS strategy used to identify post-translational modifications (PTMs) of infliximab after incubation at 37 °C in serum.

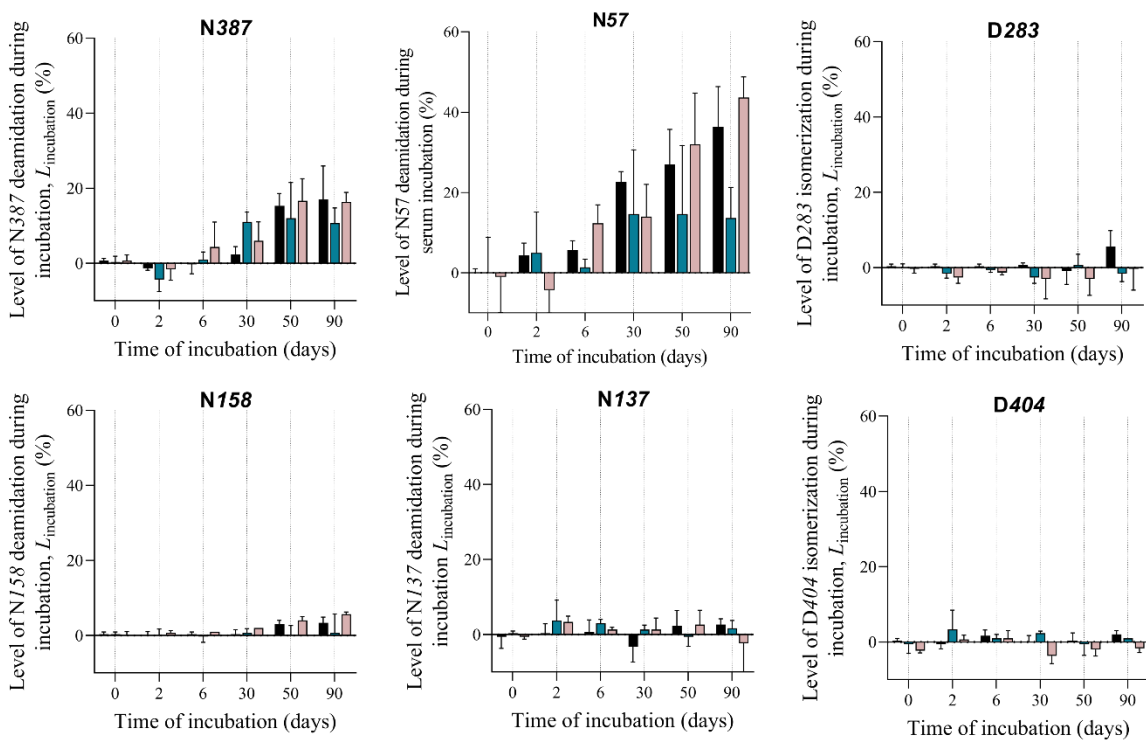
**3.1. Capillary electrophoresis separation.** CE-MS/MS data showed that the analytical workflow enabled the systematic identification of peptides presenting PTMs hotspots [14,21, 22]. Thus, the selectivity of CE separation, based on the electrokinetic mobility of the analytes, delivered a baseline separation of modified peptides from their unmodified counterparts. Therefore, CE-MS/MS analysis allowed reliable characterization of PTMs and relative quantification of modification levels for asparagine deamidation (deaN) as emphasized in Fig. 3A, which involved a mass difference of (+ 0.98 Da). In addition, aspartate isomerization (isoD), which did not involve a mass shift of the peptide could be identified after CE separation (Fig. 3B). Previous work in our group showed a lower electrophoretic mobility of the peptide with isoD than with D, due to the hydrodynamic radius change [23]. Without the implementation of CE separation prior to the MS analysis, the identification of the PTMs could be hindered or impossible due to isotopic profile overlapping on MS spectra between the modified peptide and the unmodified homolog.



**Fig. 3.** Examples of CE-MS/MS electropherograms exhibiting: A) Separation of a peptide presenting an asparagine deamidation (deaN57) from the unmodified homolog (N57). B) Separation of a peptide demonstrating an aspartate isomerization (isoD404) from the corresponding intact peptide (D404).

**3.2. Quantification of induced PTMs during *in vitro* storage.** For infliximab innovator and the corresponding biosimilars, the modification levels of 4 asparagine deamidations (N158 (LC), N137 (LC), N387 (HC) and N57 (HC)) and 2 aspartate isomerizations (D283 (HC) and D404 (HC)) were measured using CE-MS/MS analysis at different incubation time (Table S1). The median half-life of infliximab is approximately 14 days. However, the concentration of this mAb in patients with Crohn's disease was found to be highly variable between individuals, with some patients still presenting serum infliximab levels  $> 2.8 \mu\text{g}\cdot\text{mL}^{-1}$  after 14 weeks of treatment [24]. Therefore, the present study sought to evaluate the structural outcome of the mAb up to 90 days of incubation. Fig. 4 presents the evolution of PTMs abundance with incubation time. The results showed an absence of significant modification for the two aspartates studied, and in the case of the residues N158 and N137. Indeed, their level of modification induced during incubation, calculated from CE-MS/MS data, remained systematically below 10 % for the different infliximab products, even after 90 days. Concerning deamidation of the residue N387, the results showed a gradual increase of the residue deamidation over time up to 16.6 % after 50 days of incubation in serum for Remsima<sup>®</sup>, followed by a stabilization afterward (Fig. 4). The modification of this particular residue was attributed to the presence of an adjacent glycine in the amino acid sequence, which made the residue highly prone to deamidation at neutral to alkaline pH due to the possible formation of the cyclic imide [6,25]. Plus, the 3D structure of infliximab Fc part indicated that N387 is located on an external

helix exposed to the external environment, close to the area of binding with FcγRIII. Lu et al. have shown that deamidation of N325 reduces antibody-dependent cell-mediated cytotoxicity after 4 months of incubation at 40°C [7]. In the case of the residue N57, CE-MS/MS data demonstrated a gradually increasing level of deamidation, reaching 43.6 % for the Remsima® product after 90 days of incubation. The residue N57 is located in the CDR part of infliximab, so the modification of this residue could potentially hinder the affinity of the mAbs with the antigen due to its proximity during the interaction. Various studies have investigated the implication of this hotspot on the antigen-antibody interaction. For instance, Huang et al. observed a similar deamidation of the residue N55 for another IgG over time after administration to monkeys [5]. They noted a decrease in antigen binding affinity with this N55 deamidation.



**Fig. 4.** Modification levels of 6 amino acids generated after incubation of infliximab in serum at 37 °C. Black bars: Remicade®, blue bars: Inflectra® and pink bars: Remsima®. Error bars correspond to standard deviation of triplicates.

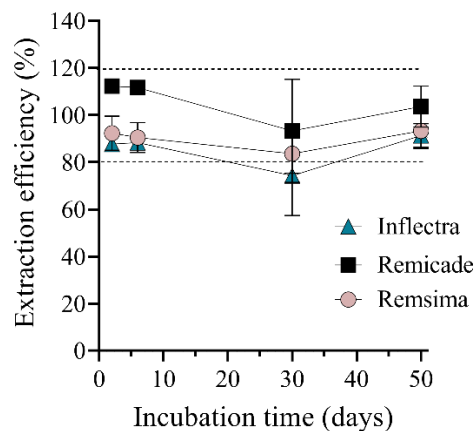
Regarding the assessment of biosimilarity, CE-MS/MS data obtained for the biosimilars showed a gradual modification of residues N57 and N387 in nearly equivalent proportions compared to the infliximab innovator product after 6 days when incubated in serum. However, it could be noted that Inflectra® showed lower modification levels for N57 than

the other compounds after this time point (Fig. 4). Although the kinetics of this deamidation should be confirmed with other batches of Inflectra®, the method has demonstrated its suitability for structural assessment of mAbs after administration. Therefore, the study allowed to point that both biosimilar products exhibited a similar structural evolution in comparison to the innovator product during incubation in the biological sample. In this case, the CE-MS/MS analysis allowed to demonstrate in the perspective of the outcome after administration, the structural equivalence of the studied biosimilars of infliximab compared to the innovator product in term of amino acids subjected to post-translational modification. Plus, the determination of the rate of these degradations is possible with this method but would require multiple batch experiments for biosimilarity assessment. CE-MS/MS analysis performed for the different products showed the occurrence of specific PTMs during the incubation of infliximab under conditions that mimic *in vitro* the environment of mAbs after administration. Thus, the strategy developed enabled to anticipate the occurrence of PTMs potentially affecting the mAbs after their injection, during their residence time, which is particularly long due to the extended serum half-life of IgG. In addition, the CE-MS/MS results highlighted the relevance of characterizing PTMs hotspots post-administration in order to attribute changes in the efficacy of the mAbs over time.

**3.3. Evaluation of TNF- $\alpha$  binding.** The presence of the identified PTMs may potentially affect the affinity of the mAbs for the corresponding antigen or receptors responsible for effector functions (*e.g.* FcRn, Fc $\gamma$ RI), especially in the case of the residue N57 which is located in the CDR of infliximab. The sample preparation developed for the CE-MS/MS analytical workflow was based on an affinity extraction step performed by the intermediate of recombinant TNF- $\alpha$  immobilized on ferromagnetic beads. Therefore, to further investigate the impact of the gradual deamidation of the amino acid N57 on the infliximab/TNF- $\alpha$  interaction, the efficiency of the bead extraction was evaluated (see Section 2.5).

As emphasized in Fig. 5, the CE-MS/MS analysis demonstrated an extraction efficiency systematically higher than 75%. The incubation time did not show a significant influence on the extraction efficiency over a period of 50 days. By extension, experiments showed that the infliximab/TNF- $\alpha$  binding could be maintained. Therefore, CE-MS/MS analysis allowed to correlate that these significant levels of N57 deamidation, resulting from prolonged incubation in serum, did not compromise the binding interaction of infliximab to its antigen. The experiment carried out during the

study was based on the estimation of the relative amount of infliximab bound using the SIL-infliximab, with a fixed amount of TNF- $\alpha$ , in order to avoid variations possibly due to the repeatability of the extraction.



**Fig. 5.** Achieved extraction efficiency of infliximab innovator product (Remicade®) or biosimilars (Inflectra® and Remsima®) for different incubation duration in blank serums at 37 °C. Error bars represent the standard deviation among triplicates. Dotted lines: bias of +/- 20% of the extraction efficiency compared to a sample without incubation (reference).

Thus, this approach allowed only the estimation of the actual occurrence of the infliximab/TNF- $\alpha$  interaction. As a consequence, although the infliximab/TNF- $\alpha$  interaction was maintained, the affinity of the interaction, represented by the dissociation constant, could still potentially be affected by the occurrence of PTMs in the CDR regions without completely abolishing the interaction. Concerning biosimilarity assessment, the infliximab innovator and the corresponding biosimilar demonstrated a similar evolution of the extraction efficiency over the studied period of time (Fig. 5). Data generated during the study showed that the different infliximab products underwent equivalent evolution when placed in conditions mimicking the environment of the mAbs after administration, further supporting their bioequivalence. As a result, the analytical workflow developed in this case allowed for an additional dimension of comparison in the context of biosimilarity assessment, with a reduced number of experiments.

**3.4. Comparison with *in vivo* values.** The relevance regarding the implementation of conditions mimicking the environment of the mAbs after administration to predict mAb stability was further investigated. Thereby, hotspots PTMs levels obtained for spiked serums incubated at 37 °C were compared with those values observed *in vivo* for the characterization of serums originating from treated patients, as previously reported [18]. The analysis of serums of patients after their administration with infliximab exhibited the absence of modifications at the residues D283, D404, N158

and N137, which is in full agreement with the CE-MS/MS results obtained under conditions simulating the environment of the mAbs (Fig. 4). In addition, the progressive deamidation of the residue N57 characterized by CE-MS/MS analysis in the case of incubated serum samples, was consistent with its evolution reported *in vivo* consequently to administration. In particular, a comparison of the deamidation kinetic of the residue N57 measured *in vitro* by the intermediate of incubated serums and *in vivo* in the case of samples originating from treated patients was performed. It allowed to demonstrate a significantly similar rate of modification of the protein (Fig. S3)) which validated the relevance of the conditions mimicking the environment of infliximab after administration. The gradual deamidation of the residue N57 was partially attributed to the effect of temperature, which is in agreement with previous reports [16]. A study recently investigated by Kato et al. has suggested that  $\text{H}_2\text{PO}_4^-$  and  $\text{HCO}_3^-$  ions are the principal catalysts for Asn deamidation *in vivo* [26]. Concerning the residue N387, comparison between *in vitro* and *in vivo* deamidation rate was less clear, as the maximum level of modification observed was 20 % (Fig. S4). Despite a higher dispersion among patients, it is possible to observe a similar tendency between the two studies.

The CE-MS/MS method demonstrated the possibility to independently characterize faint PTMs occurring on infliximab present in complex biological samples, in a precise and robust manner. The characteristics of the CE-MS/MS methods, supported by a specifically tailored sample preparation procedure, enabled to achieve an optimal sensitivity and to prevent any interference from natural IgGs or serum proteins. A novel analytical approach was developed in order to predict the occurrence of PTMs consequently to the administration, in the case of infliximab innovator and the corresponding biosimilars. The characterization of infliximab-spiked serums incubated at 37 °C allowed to identify stable residues alongside to the gradual deamidation of two distinctive asparagine residues over time. Without the necessity to perform additional experiments, the determination of the TNF- $\alpha$  affinity extraction efficiency showed that the N57 deamidation, located in the CDR of mAbs, did not involve significant alterations of the infliximab/ TNF- $\alpha$  interaction. Finally, comparison of N57 deamidation kinetics obtained from the analysis of model samples and serum samples demonstrated a relevant correlation between the two sets of experiments. Thus, the results allowed to emphasize the validity of using conditions mimicking the environment of mAbs in order to anticipate the evolution of their primary structure after administration, in a detailed manner.

#### 4. CONCLUSION

In the present work, we developed an analytical strategy implementing conditions that mimic the environment of mAbs after their administration over an extended period of time in order to predict *in vitro* the potential occurrence of primary structure degradation of mAbs. CE-MS/MS analysis enabled the simultaneous characterization of six PTM hotspots including deaN and isoD, for infliximab innovator and two corresponding biosimilar products in biological samples. Biosimilarity assessment usually performed on formulated products, the study allowed to investigate the different infliximab products from the perspective of their stability after administration, in order to further demonstrate their biological equivalence. Results allowed the identification of two distinct PTM hotspots presenting significant modification due to the environment, including deaN57 located in the CDR part of infliximab. CE-MS/MS data showed a similar evolution regarding modification levels between the infliximab innovator and the two biosimilars. Nonetheless, the excellent sensitivity of the method provided the possibility to distinguish faint differences in terms of modification levels. The comparison of the results obtained from the analysis of *in vitro* model samples and *in vivo* serum samples, originating from treated patients, proved to be in agreement regarding the affected residues and their respective modification kinetics. As a consequence, the comparison demonstrated the relevance of the *in vitro* analytical strategy developed, to predict the occurrence of PTMs and anticipate the primary structure stability of mAbs after administration. Concomitantly, the analytical strategy enabled to evaluate the influence of the N57 deamidation on the infliximab/TNF- $\alpha$  binding, which allowed to determine that the modification of the residue does not prevent the interaction within a 50-day incubation.

The study illustrated the importance of characterizing the evolution of the primary structure of a biosimilar candidate also in the context of its administration. Indeed, like in the case of infliximab, the evolution of the primary structure after administration to patients could be drastically different from the formulated product, which is usually used to perform stability studies using forced degradation. The *in vitro* analytical strategy developed provided a suitable approach in order to facilitate and anticipate this type of experiment, alleviating the effective administration of the product. It could also potentially be adapted to other mAbs in a straightforward manner, with the only requirement that the



adequate antigen is used for the specific extraction. Further studies evaluating the impact of structural degradation on other functional aspect of an antibody could help to better understand their outcome after administration. For instance, studies have showed that PTMs occurring near the Fc receptor region could impact the binding of the antibody to essential receptors such as FcRn or FcγRs, thereby affecting its serum half-life, or cytotoxicity [4,7,27,28].

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