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Simultaneous quantification and structural characterization of monoclonal antibodies after administration using capillary zone electrophoresis-tandem mass spectrometry

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ABSTRACT

Monoclonal antibodies (mAbs) are demonstrating major success in various therapeutic areas such as oncology and the treatment of immune disorders. Over the past two decades, novel analytical methodologies allowed to address the challenges of mAbs characterization in the context of their production. However, after administration only their quantification is performed and insights regarding their structural evolution remain limited. For instance, clinical practice has recently highlighted significant inter-patient differences in mAb clearance and unexpected clinical responses, without providing alternative interpretations. Here, we report the development of a novel analytical strategy based on capillary zone electrophoresis coupled to tandem mass spectrometry (CE-MS/MS) for the simultaneous absolute quantification and structural characterization of infliximab (IFX) in human serum. CE-MS/MS quantification was validated over the range 0.4–25 $\mu g \cdot mL^{-1}$ corresponding to the IFX therapeutic window and achieved a LOQ of 0.22 $\mu g \cdot mL^{-1}$ (1.5 nM) while demonstrating outstanding specificity compared to the ELISA assay. CE-MS/MS allowed structural characterization and estimation of the relative abundance of the six major N-glycosylations expressed by IFX. In addition, the results allowed characterization and determination of the level of modification of post-translational modifications (PTMs) hotspots including deamidation of 4 asparagine and isomerization of 2 aspartate. Concerning N-glycosylation and PTMs, a new normalization strategy was developed to measure the variation of modification levels that occur strictly during the residence time of IFX in the patient's system, overcoming artefactual modifications induced by sample treatment and/or storage. The CE-MS/MS methodology was applied to the analysis of samples from patients with Crohn's disease. The data identified a gradual deamidation of a particular asparagine residue located in the complementary determining region that correlated with IFX residence time, while the evolution of IFX concentration showed significant variability among patients.

1. Introduction

Monoclonal antibodies (mAbs) and their derivatives, such as fusion proteins or antibody-drug conjugates, have been very successful as therapeutic treatments and have contributed significantly to the emergence of the biopharmaceutical industry. Their field of application was initially focused on oncology, but therapeutic mAbs have also proven to be particularly relevant for the treatment of various types of immune disorders. For instance, Crohn's disease is an inflammatory bowel disease resulting from overexpression of the tumor necrosis factor (TNF-α), a pro-inflammatory cytokine of the immune system[1–4]. In order to reduce inflammation and limit the manifestation of clinical symptoms in patients, several mAbs have been successfully used as therapeutic treatments [5,6], of which infliximab (IFX) is currently the most widely used [7–9]. IFX is a chimeric immunoglobulin G (IgG1) containing a murine variable region, with a molecular mass of 147 kDa [10]. IFX

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inhibits TNF-α activity by direct interaction, or by triggering antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity [11–13].

As the introduction of IFX for the treatment of Crohn's disease is relatively recent, knowledge about the evolution of mAbs after their administration to patients remains relatively limited. Thus, clinical practice suggests that patients may respond differently to treatment in several aspects [14,15]. After administration of an equivalent dose, some patients have shown significantly higher clearance, resulting in IFX concentration < 1.2 $\mu g \cdot mL^{-1}$ after 14 days, while in a small proportion of the treated population, the mAbs can be detected in the serum several months after administration [16]. Similarly, approximately 1/3 of patients failed to show any clinical improvement and some populations exhibited a loss of clinical response over time that remains unexplained to date [15,17]. Previous in vitro studies have shown that post-translational modifications (PTMs) can affect the biological activity of mAbs [18]. In the case of therapeutic mAbs, asparagine deamidation [19], aspartate isomerization and N-glycosylation [20,21] have been identified as hotspots with respect to their impact on mAb bioactivity. PTMs located in CDR parts of mAbs may potentially affect their antigen binding affinity [22,23], whereas modifications localized in Fc parts could affect their cytotoxic activity [24,25] or serum half-life time [26,27]. Therefore, it might be suggested that poor clinical response could potentially be triggered by certain PTMs occurring in vivo. *In vivo* PTMs of mAbs was observed after administration to monkeys [28], but it remained unclear whether they could be responsible for loss of response.

Currently, clinical follow-up of patients during treatment only includes monitoring of serum IFX concentration which is performed exclusively using ELISA immunoassay [29]. ELISA assays are fast and easy to use, however they may exhibit specificity issues, potentially leading to false positive or negative results [30,31], and cannot provide information regarding the structure of IFX [32]. In addition, mAb quantification, typically in the range of 0.5–14 $\mu g \cdot mL^{-1}$, achieved using ELISA methods may potentially exhibit uncontrolled biases because of the absence of internal standard to normalize eventual component loss during washing steps for example [33].

Mass spectrometry (MS) has gradually taken a leading role in the structural characterization and quantification of proteins due to its optimal specificity, sensitivity and structural information [34,35]. Liquid chromatography hyphenated to tandem mass spectrometry (LC-MS/MS) represents the most commonly used technique for the analysis of peptide mixtures [36]. The use of heavy-isotope labeled internal standards enabled the accurate and robust quantification of proteins in biological matrices using MS through normalization of experimental variabilities due to sample preparation and analysis [37]. Capillary zone electrophoresis (CZE) has recently demonstrated to be particularly relevant for peptide separation. Indeed, the electrophoretic migration generated in CZE enables the separation of analytes with different charge states in solution and/or different hydrodynamic radii [38]. Thereby, CZE is suitable for the separation of short or polar peptides, such as glycopeptides, in parallel with longer and more apolar ones in the same analysis [39]. Therefore, the hyphenation of CZE to MS has recently proven to be particularly relevant for the characterization of the primary structure of mAbs [40–42], with the possibility to achiev 100% sequence coverage using a single analysis [39].

In the present work, we have designed and implemented a novel CZE-MS/MS method for the absolute quantification and simultaneous characterization of the primary structure of IFX in serum samples from Crohn's disease patients. A specific sample preparation protocol was developed to extract IFX from biological samples while ensuring optimal compatibility with the subsequent CZE-MS/MS analysis. A full method validation was performed to demonstrate the characteristics of the developed CZE-MS/MS method. Furthermore, the CZE-MS/MS data were used to characterize the N-glycosylation of IFX in biological samples by differentiating the structure and relative abundance of glycans. The data could also be used to characterize different PTMs for which an innovative normalization methodology was incorporated to avoid the introduction of bias regarding the estimation of the modification levels. Finally, the CZE-MS/MS analytical workflow was applied to a series of serums from patients treated with IFX for Crohn's disease, highlighting that the structural characterization of mAb after administration could be essential to understand variations in clinical response. The aim of the study was to provide an accurate analytical strategy delivering in biological samples, simultaneous quantification of IFX and PTMs characterization post-administration. The CE-MS/MS strategy could be further applied in a cohort study for instance to investigate if clinical loss of response might be associated with specific PTMs.

2. Materials and methods

Chemicals used were of high purity grade and purchased from Merck Sigma-Aldrich (Saint Louis, MO, USA) if not stated otherwise. IFX (IFX, Remicade®) samples are EMA/FDA-approved formulation purchased from the manufacturer (Merck Sharp and Dohme). RapiGest SF surfactant was purchased from Waters (Milford, MA). biotinylated TNF-α from R&D System was obtained from Biotechne. Sequencing Grade Modified Trypsin was purchased from Promega Corporation, (Madison, WI, USA). PBS solution (Phosphate buffer saline) was purchased from Gibco ThermoFisher as tablets for reconstitution in water.

2.1. Standards and QCs with blank serums

Blank serums were provided by the French institute of blood (Paris, France) after extraction and purification from total blood. Calibration samples and quality control (QC) samples were prepared by spiking known concentrations of IFX to the blank serum for method evaluation.

2.2. Serum from Crohn's disease patients

Serum samples from 24 patients were obtained from the gastroenterology department of Saint-Louis hospital (Paris, France). The patients had been diagnosed with Crohn's disease for at least 3 months and were receiving regular IFX injections. Gender, age and time since last IFX administration prior to serum collection were indicated. 100 µL of serum per patient was collected, and the SIL-IFX internal standard (1 µg) was added.

2.3. Sample preparation

Spiked serums used as calibrants, and patient serums were prepared in exactly the same manner. For IFX affinity purification, 217 µL of Streptavidin M-280 Dynabeads™ (Fisher scientific Invitrogen, Carlsbad, CA) were washed with PBS solution. 148 µL of biotinylated TNF-α (10 $\mu g \cdot mL^{-1}$) was added and the mixture was left at RT for 1 h. Excess biotinylated TNF-α was removed by three consecutive washes with PBS. Afterward, a volume of 100 µL of serum was added to the functionalized magnetic beads and the mixture was left at RT for 1 h to ensure optimal capture of IFX and SIL-IFX by the immobilized TNF-α. After incubation,

the serum was removed and 50 µL of MeOH/H_2O/FA mixture (48.5/48.5/3; v/v/v) was added before incubation for 10 min. The supernatant was collected, and this step was repeated. Supernatants were pooled and dried for 2 h using a miVac DNA concentrator system™ (Genevac), at 35 °C. The sample was reconstituted in 70 µL of 50 mM ammonium bicarbonate (pH 8). The last step followed a denaturation-reduction-alkylation-digestion protocol commonly used for protein digestion [43]. Extracted mAbs were denatured with 5 µL of RapiGest SF™ at 0.1%, at 80 °C for 10 min. Consequently, 10 µL of 50 mM dithiothreitol was added and the sample was left at 80 °C for 20 min to reduce disulfide bridges. The solution was cooled to room temperature, and 10 µL of 50 mM iodoacetamide was added. The sample was incubated in the dark for 20 min to perform cysteine alkylation. 1 µL of trypsin 0.25 $g{\cdot}L^{-1}$ was introduced to the sample and left at 20 °C for 2 h. 1 µL of trypsin was again added prior incubation at 37 °C overnight. Afterward, 1 µL of formic acid 98% was added to quench the reaction. The peptide mixtures were finally evaporated and reconstituted in 5 µL ammonium acetate 100 mM, pH 4.

2.4. CZE-ESI-MS/MS

Experiments were performed using a CESI8000® Capillary Electrophoresis system (Sciex separations, Darmstadt, Germany) coupled by mean of a sheathless CE-ESI-MS interface to a Sciex 5600 TTOF mass spectrometer (Darmstadt, Germany) operated by Analyst® software (Sciex, Darmstadt, Germany). A detailed description of this interface was provided by Haselberg et al. [44]. Separation was performed using a homemade bare-fused silica capillary (total length 100 cm; 30 µm i.d.), the outlet end of which was etched with hydrofluoric acid [45], resulting in a 2-cm porous tip positioned inside the cannula of the sheathless interface. The cannula was filled through a second capillary (total length 80 cm; 50 µm i.d.) with background electrolyte (BGE) consisting of 10% acetic acid to maintain electrical contact between the CE inlet and outlet electrodes [46]. Concerning CZE-ESI-MS/MS conditions, a preconcentration step by transient isotachophoresis was implemented by injecting the peptide digest reconstituted in a leading electrolyte of ammonium acetate (pH 4), which is more conductive than the BGE. The analytes were therefore first focused in the capillary and then separated by zone electrophoresis. 68 nL of sample was injected hydrodynamically (5 psi, 100 s) and a voltage of 23 kV was applied. The total analysis time is 60 min. The ESI source parameters were set as follows: ESI voltage 1.5 kV, while gas supplies (GS1 and GS2) were turned off, source heating temperature 100 °C, and curtain gas value 2. Experiments were performed in Top20 information-dependent acquisition (IDA, Sciex), and the accumulation time was 250 ms for MS scans and 80 ms for MS/MS scans, leading to a total duty cycle of 1.9 s. Mass/charge (m/z) range was for 100–2000 in MS and 50–2000 for MS/MS. SIL-IFX with arginine and lysine residues labeled with (^{13}C; ^{15}N) was used as an internal standard for quantification to correct for variability originating from sample preparation. DILLTQSPAILSVSPGER (LC01) was selected as the peptide for quantification because it is strictly specific to IFX, which was verified using a blast with a human database [https://blast.ncbi.nlm.nih.gov/-Blast.cgi], and the absence of known PTMs. LC01 was therefore located in IFX variable part and was found to be specific to this mAb. Data were obtained as.wiff and were analyzed through Skyline software. The peak ratio (IFX/SIL-IFX) were calculated using this software.

2.5. CE-MS/MS quantification method validation

The quantification method was validated using blank serum spiked with known concentrations of IFX and stable-isotope labeled infliximab (SIL-IFX) internal standard. Statistical validation of the method was performed in order to comply with both European medicines agency (EMA) and food and drug administration (FDA) guidelines [47,48]. Indeed, both guidelines are mainly similar, but there are minor differences regarding the threshold of acceptance criteria or methodology [49]. Therefore, for each parameter, the most stringent criteria and the largest number of replicates were systematically applied. For linearity assessment, 5 calibration curves were carried out over 6 concentration points over a time span of 3 months. QC samples spiked with IFX at 4 different concentrations (QC1:0.5; QC2:1.5; QC3:12 and QC4:19 $\mu g{\cdot}mL^{-1}$) were analyzed, representing a total of 12 replicates for each QC level, divided into 4 separate batches run on different days. The trueness and precision calculated from the QC sample replicates were expressed as percent bias, and relative standard deviation (RSD%), respectively. LOD and LOQ were estimated by signal-to-noise ratio (>3 for LOD and >10 for LOQ). The selectivity of the method was determined using 6 blank human serum samples by extraction of the m/z corresponding to the LC01 peptide and expressed as a percentage of the IFX's LOQ signal. Specificity was tested in triplicate using serum spiked with adalimumab, etanercept and cetuximab at 5 $\mu g{\cdot}mL^{-1}$, alongside with IFX at 19 $\mu g{\cdot}mL^{-1}$, and compared to serum spiked with IFX alone. Matrix effects were studied using the post-extraction addition protocol. MS signal of IFX (1.5 $\mu g{\cdot}mL^{-1}$) in a standard solution of H_2O, was compared to a post-extraction solution spiked with the same concentration. The matrix factor of IFX (IFX peak area in the presence of matrix divided by IFX peak area in the absence of matrix) was divided by that of SIL-IFX. The stability of the peptide mixture was evaluated by 3 freeze and thaw cycles. After each freeze/thaw cycle, samples were analyzed with CE-MS/MS. Stability was also determined for storage in the autosampler (10 °C). Three replicates were stored in the autosampler and injected into the CE-MS/MS instrument every 2 h. The effect of hemolysis, lipemia and icterus on IFX quantification was evaluated. IFX-free serums with these characteristics were provided by the Lariboisière hospital (Paris, France). IFX concentration back-calculation was evaluated after spiking at 19 $\mu g{\cdot}mL^{-1}$ and the same samples were then analyzed by ELISA assay using a Promonitor® IFX kit, following the supplier's instructions. Correlation between ELISA and CE-MS/MS quantification results was determined using linear regression analysis.

2.6. Relative quantification of PTMs

The m/z of ions corresponding to peptides containing PTM hotspots were extracted from the MS and MS/MS data. Thus, 6 glycans (G0F, G1F, G2F, G0FN and M5) were characterized (Table S1 and Fig. S1) in addition to 6 amino acid PTMs: 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)). The localization of the different PTMs on IFX amino acid sequence was represented in Fig. S2. They were selected because they represented PTM hotspots of the studied mAbs [23,50,51]. For a monitored amino acid (e.g. labeled "L"), its level of modification, obtained by CE-MS/MS analysis, in IFX and SIL-IFX compounds, was determined for spiked serums ($L_{s,CE-MS}^{IFX}$ and $L_{s,CE-MS}^{SIL}$) and patient serums ($L_{p,CE-MS}^{IFX}$ and $L_{p,CE-MS}^{SIL}$).

The normalization strategy was developed to remove possible endogenous structural changes due to sample preparation and/or storage. The proportion of induced PTM is given in Table 1 for the 3 different steps that IFX and SIL-IFX undergo during their lifetime. Table 1 also includes (last row) the final level of this PTM, measured by CE-MS/MS, which corresponds to the sum of each step. It can be noted that the sample preparation affects similarly IFX and SIL-IFX in the same sample but differently in two distinct samples ($L_{prep\ A}$ and $L_{prep\ B}$) to address errors associated with pipette volumes or CE-MS/MS analysis. L_{blood} represents the proportion of the studied amino acid that underwent through modification during the residence time in the patient's body. As

Table 1
Expression of the different steps potentially affecting the PTM levels of IFX and SIL-IFX.

		PTM level generated at each stage of mAbs life			*PTM level measured by CE-MS/MS analysis*
		Production	Patient administration	Sample preparation	
Spiked serums (s)	IFX	L_{prod}^{IFX}		$L_{prep\ A} \times (1- L_{prod}^{IFX})$	$L_{s,CE-MS}^{IFX} = \sum steps$
	SIL-IFX	L_{prod}^{SIL}		$L_{prep\ A} \times (1-L_{prod}^{SIL})$	$L_{s,CE-MS}^{SIL} = \sum steps$
Patient serums (p)	IFX	L_{prod}^{IFX}	$L_{blood} \times (1- L_{prod}^{IFX})$	$L_{prep\ B} \times (1- L_{prod}^{IFX}) \times (1-L_{blood})$	$L_{p,CE-MS}^{IFX} = \sum steps$
	SIL-IFX	L_{prod}^{SIL}		$L_{prep\ B} \times (1-L_{prod}^{SIL})$	$L_{p,CE-MS}^{SIL} = \sum steps$

L_X^Y: Level of modification of the PTM "L", for the analyte "Y" (IFX or SIL-IFX) that occurred in the step "X"
$L_{s,\ CE-MS}^Y$: Level of modification of the PTM "L", for the analyte "Y" (IFX or SIL-IFX) that is observed in CE-MS/MS analysis of a healthy serum spiked with IFX and SIL-IFX
$L_{p,\ CE-MS}^Y$: Level of modification of the PTM "L", for the analyte "Y" (IFX or SIL-IFX) that is observed in CE-MS/MS analysis of a patient serum spiked with SIL-IFX

only infliximab is administrated to patients, the corresponding SIL-IFX case is grayed out. Normalization consists of extracting L_{blood} from data collected by CE-MS/MS (last row), which led to the Eq. (1), using Mathematica® software (Wolfram).

Level of PTM of an amino acid "L_{blood}" occurring in blood of patients (%) =

$$L_{blood} = \frac{L_{p,\ CE-MS}^{IFX} - L_{s,\ CE-MS}^{IFX} - L_{p,\ CE-MS}^{SIL} + \left(L_{s,\ CE-MS}^{IFX} \times L_{p,\ CE-MS}^{SIL}\right) + L_{s,\ CE-MS}^{SIL} - \left(L_{p,\ CE-MS}^{IFX} \times L_{s,\ CE-MS}^{SIL}\right)}{1 - L_{s,\ CE-MS}^{IFX} - L_{p,\ CE-MS}^{SIL} + \left(L_{s,\ CE-MS}^{IFX} \times L_{p,\ CE-MS}^{SIL}\right)} \quad (1)$$

Bilateral statistical tests were performed to determine whether the L_{blood} calculated for each patient's serum was significantly different from the 30 spiked serums used as controls. The p-value corresponding to the probability that this hypothesis is true was calculated by applying a Welch's two-tailed t-test. The critical value for the p-value to be considered unlikely was 0.05.

3. Results and discussion

3.1. CE-MS/MS method development and validation

The designed analytical workflow, represented in Fig. 1, consisted of the specific extraction of IFX and the stable-isotope labeled infliximab (SIL-IFX) internal standard from serum. The extraction was performed using recombinant TNF-α immobilized on in-house prepared ferromagnetic beads. Accordingly, the extracted IFX and SIL-IFX underwent digestion, and the resulting peptide mixture was then separated and analyzed by high sensitivity CE-MS/MS analysis. Method development was performed using blank serums spiked with known concentrations of IFX and SIL-IFX. The spiked serums were used to complete the validation of the CE-MS/MS quantification method and to demonstrate the ability to characterize different types of PTMs, including IFX N-glycosylation, Asn deamidation and Asp isomerization, from the same experiment.

Quantification of IFX was performed using a label-free method based on the MS signal corresponding to the LC01 peptide, which was selected because it is proteotypic for IFX (Table 2). For each CE-MS/MS analysis, unambiguous peptide identification was provided by high-resolution *m/z* ratio measurement in addition to the peptide fragment fingerprint (Fig. S3). Statistical validation of the CE-MS/MS quantification method was conducted to determine the performance of the analysis. The linearity of the method was demonstrated over the concentration range 0.4–25 $\mu g \cdot mL^{-1}$ (Fig. S4). This range corresponds to the concentrations typically observed in IFX-treated patients[52]. In addition, the CE-MS/MS analysis allowed to achieve a LOD of 0.06 $\mu g \cdot mL^{-1}$ (0.4 nM) and a LOQ of 0.22 $\mu g \cdot mL^{-1}$ (1.5 nM) respectively (Table 2). The method

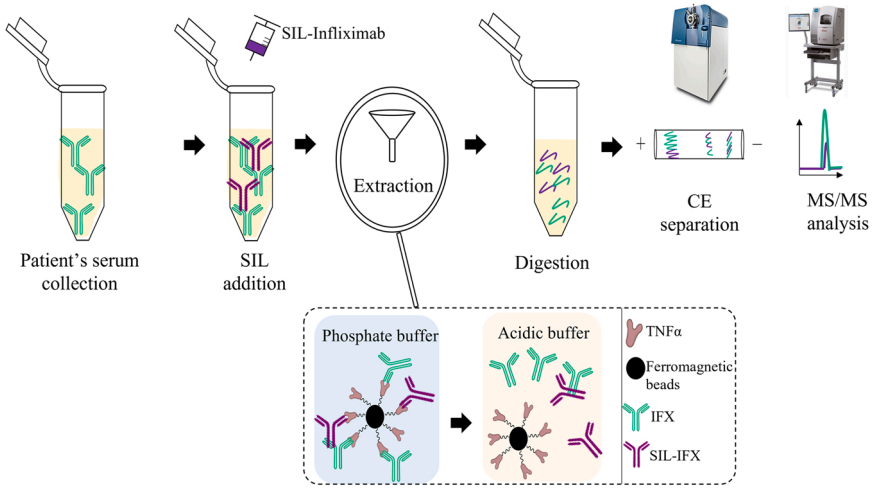

Fig. 1. Schematic representation of the analytical workflow developed for the absolute quantification and structural characterization of infliximab in human serum using CE-MS/MS analysis.

Table 2
Compilation of the results obtained for the statistical validation of the CE-MS/MS method for the absolute quantification of infliximab.

Sequence	DILLTQSPAILSVSPGER (LC01)			
Concentration range	0.4–25 µg·mL^{-1}			
Equation	0.1108x − 0.027			
R^2	0.989			
LOD	0.06 ± 0.02 µg·mL^{-1}			
LOQ	0.22 ± 0.08 µg·mL^{-1}			
Selectivity (n = 6)	blank signal ≤ 2 ± 2% IFX LOQ			
Specificity (% Bias)[a]	2.1 ± 1.3%			
Matrix effect[b]	≤ 11 ± 4%			
Level of QC	QC1 (0.5 µg·mL^{-1})	QC2 (1.5 µg·mL^{-1})	QC3 (12 µg·mL^{-1})	QC4 (19 µg·mL^{-1})
Intra-days statistics				
Precision (RSD %)[b]	5.8	2.0	3.1	4.2
Trueness (% Bias)[a]	-4.9	-11.1	0.8	-2.6
Inter-days statistics				
Precision (RSD %)	7.7	5.6	7.5	4.5
Trueness (% Bias)	7.0	-6.4	9.6	-0.9

[a] % bias: determined as: (measured conc. – nominal conc.)/nominal conc. * 100%.
[b] RSD: determined as: standard deviation/ABS(mean). * 100%.

was found to be selective as the interferences in blank serums remained less than 15% of the LOQ signal of IFX. The CE-MS/MS method was applied to serums spiked with other biotherapeutics (adalimumab, etanercept and cetuximab). The bias from the nominal IFX concentration for spiked serums was 2.1 ± 1.3%. Thus, it was concluded that CE-MS/MS quantification was not significantly affected by the presence of other biotherapeutics, although adalimumab and etanercept also target TNF-α. They were most likely extracted concomitantly during sample preparation, but could not further interfere as the LC01 peptide was strictly specific to IFX. The results highlighted the outstanding specificity of the IFX proteotypic peptide quantification delivered by the CE-MS/MS method. Trueness and precision were estimated using quality control (QC) samples (Table 2) for intra-day and inter-day replicates. RSD for replicates and biases from the nominal concentration were systematically below 15% underscoring the appropriate accuracy of the method. The experiments showed that matrix effects remained limited with values lower than 15% (Table 2). No carry-over could be observed when the analysis of the blank samples was performed immediately after the QC4 samples. Finally, the recovery was found to be above 85% after three consecutive freeze-thaw cycles, illustrating the stability of the sample. Similarly, storage of a sample for 15 h in the instrument autosampler did not influence the accuracy of the quantification with a bias from nominal concentration below 2% (Fig. S5). As a consequence, the characteristics of the CE-MS/MS method determined from the statistical validation showed the validity of the absolute quantification of IFX.

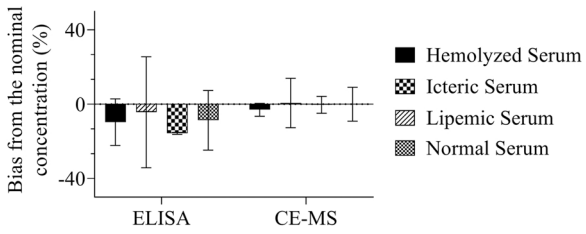

Fig. 2. Infliximab quantification accuracy and repeatability for different types of spiked serums (19 µg·mL^{-1}) measured using ELISA assay and CE-MS/MS analysis (n = 3). Hemolyzed serum typically contained products of red blood cells lysis, lipemic serum exhibited an important turbidity due to the accumulation of lipoproteins and icteric serum contained an excess of bilirubin.

Additional experiments were conducted to complement the method validation to further investigate the properties of the CE-MS/MS quantification method. CE-MS/MS quantification was performed on IFX-spiked icteric, hemolyzed and lipemic serums to assess the impact of sample type on the analysis. The different serum types considered represented common alterations that affect the general aspect of the serum and may alter matrix effects due to the presence of endogenous interfering compounds in the serum [53]. The results yielded an average bias from the nominal concentration of 3.0%, 0.3% and 0.7% for hemolyzed, icteric and lipemic serums, respectively (Fig. 2). Therefore, quantification by CE-MS/MS demonstrated excellent accuracy that was not affected by the type of serum analyzed. The same samples were also quantified using a commercial ELISA assay. As emphasized in Fig. 2, the experiments exhibited significantly higher biases of 9.7%, 4.3% and 15.6% for hemolyzed, lipemic and icteric serums respectively. It is noteworthy to mention that the ELISA quantification showed a systematic underestimation of IFX even in normal serum.

Previous reports described the possibility of using CE-MS/MS to characterize mAbs amino sequence, N-glycosylation and amino acid PTMs in a single analysis [38]. CE-MS/MS analysis achieved a sequence coverage of 90%. Only a single peptide long peptide composed of 63 amino acids could not be identified due to poor ionization, however it did not present any PTMs hotspots (Fig. S2). From this observation, we studied the possibility of characterizing IFX N-glycosylation in serum samples simultaneously with quantification using CE-MS/MS data. Indeed, CE-MS/MS quantification method is derived from a quantitative proteomics methodology, hence the collected MS and MS/MS data also include IFX peptides exhibiting PTMs. IFX contains a single N-glycosylation site located in the HC constant domain of the mAb. Characterization of the N-glycosylation was achieved through the intermediate of the glycopeptide EEQYNSTYR [54]. CE-MS/MS data demonstrated robust characterization of the 6 major glycans for spiked serums with IFX concentrations above 4 µg·mL^{-1} (Fig. S1). The processing of the MS/MS spectra allowed systematic characterization of the glycosylation structure. MS signals corresponding to the glycopeptide bearing different glycans were considered in order to estimate the relative abundance of the different glycans. The CE-MS/MS glycoprofiles were established for IFX-spiked serum samples and compared to IFX commercial solution (Fig. 3). CE-MS/MS glycoprofiles showed no significant differences between the commercial IFX solution and the spiked serum

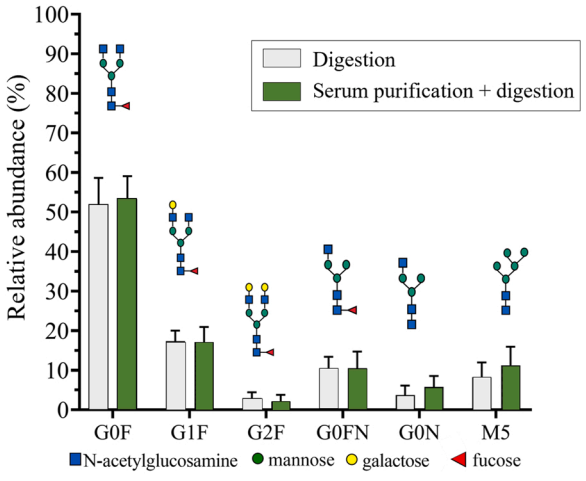

Fig. 3. Glycoprofile of IFX obtained using CE-MS/MS method developed for spiked serum (green bars, n = 30) and for IFX commercial product (white bars, n = 15). The structure of the glycan linked to the Asn of the EEQYNSTYR peptide is presented above each bar. Error bars presents the standard deviation among the replicates.

samples, demonstrating the reliability of the relative quantification of glycosylation delivered by the analytical workflow. In addition, the IFX glycoprofile was similar to previous reports performed on the commercial IFX product [54,55]. The level of performance achieved in the characterization of N-glycosylation was explained by the ability to partially separate glycopeptides bearing different glycosylation thanks to the electrokinetically driven separation of CZE (Fig. S1). As a result, the glycopeptides are gradually transferred to the ESI ionization source, resulting in optimal sensitivity. Finally, because the characterization was performed at the glycopeptide level, independent localization and characterization could be envisaged even in the case of mAbs incorporating multiple glycosylation sites.

Similarly to N-glycosylation, the possibility of performing hotspot characterization of amino acid PTMs simultaneously to IFX quantification was investigated. The study focused on 4 asparagine deamidations (N158 LC, N137 LC, N387 HC and N57 HC) and 2 aspartate isomerizations (D283 HC and D404 HC) mentioned in the literature to potentially affect mAb activity [56]. CE-MS/MS data obtained from IFX-spiked serums demonstrated the successful characterization of the 6 PTMs of interest in a reproducible manner, concomitantly to IFX quantification and N-glycosylation characterization. The selectivity of CE allowed a systematic baseline separation of modified peptides from their unmodified counterparts (Fig. S6), which enabled to perform the relative quantification despite MS isotopic profiles overlap. Indeed, asparagine deamidation involved a mass difference of only 0.98 Da whereas aspartate isomerization did not induce any mass difference. As a consequence, it is essential to separate the two peptides before their transfer to the MS instrument to provide optimal sensitivity and, in the case of isomerization, to distinguish peptides carrying different aspartate isomers. The MS signals corresponding to the intact peptide and its modified counterpart could be used to determine the levels of modification for each PTM independently. The modification levels measured for the different PTMs by CE-MS/MS analysis were compared for IFX-spiked serums and IFX commercial product. Results showed no significant differences between the IFX commercial product and serum samples spiked with IFX, demonstrating that serum purification did not introduce bias into the analysis (Fig. 4).

As a result, the developed CE-MS/MS analytical strategy demonstrated adequate accuracy and repeatability for the quantification of IFX in biological samples through extensive method validation. Especially, the analytical strategy delivered substantial specificity that resulted in the absence of interferences, even for samples containing other types of biopharmaceuticals or in the case of altered serum samples. Furthermore, the quantification of IFX by CE-MS/MS was found to be as efficient as, if not better than, the ELISA quantification which is currently the reference method for this type of analysis. To our knowledge, this is the first time that the CE-MS technique has been described for the absolute quantification of a protein. Concomitantly, the structure of the N-glycosylation could be unambiguously characterized and their relative abundance estimated from serum samples with IFX, using the same CE-MS/MS data. The major glycosylations successfully detected by the CE-MS/MS method represented more than 90% of the IFX content [54]. Since the characterization was performed at the glycopeptide level, independent localization and characterization could be envisaged even in the case of mAbs incorporating multiple glycosylation sites. Finally, the CE-MS/MS method has demonstrated the possibility of simultaneously characterizing faint amino acid PTMs in a relevant and accurate manner. For every quantitative and structural aspect considered, the performance of the CE-MS/MS method was fully compatible with the characterization of IFX for serum samples from patients treated for Crohn's disease. The ability to quantify a protein in a complex biological sample together with an advanced characterization of its primary structure has never been described before and represents a significant advance from an analytical chemistry perspective. This level of performance was achieved due to the excellent sample preparation protocol developed, which allowed optimal compatibility with CE-MS/MS analysis, in

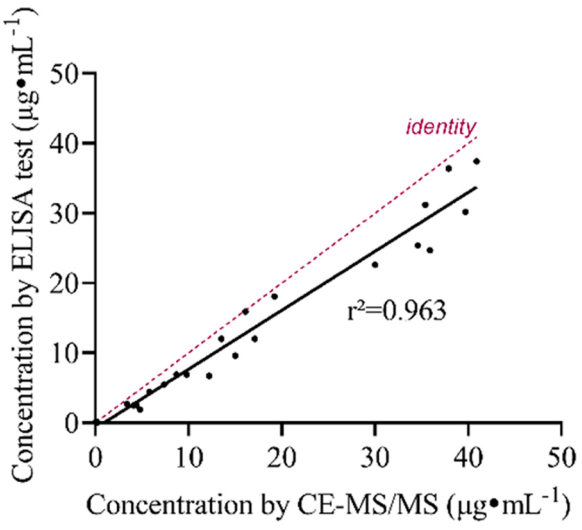

Fig. 5. Comparison of the quantification of IFX achieved in patient serums using ELISA assay versus CE-MS/MS analysis (n = 24). Linear regression is presented.

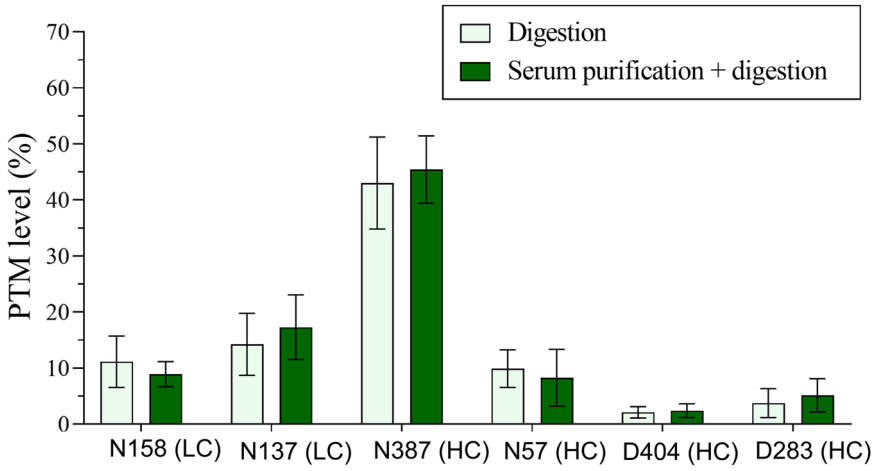

Fig. 4. Modification level determined using CE-MS/MS analysis for 4 asparagine deamidations and 2 aspartate isomerization in the case of IFX spiked serums (green bars, n = 30), or in IFX commercial product (white bars, n = 15). Error bars represent the standard deviation between replicates.

addition to the relevant characteristics of the CE separation mechanism and state-of-the-art CE-MS coupling. Quantification of IFX in patient serums has been performed by ELISA assays [57] or by LC-MS/MS [37,58,59], but never in conjunction with structural characterization. Structural assessment of IFX PTMs has mostly been done for biosimilarity assessment and production control [55,60,61]. This is the first time that such level of characterization is demonstrated under conditions consistent with analysis directly after drug administration to patients.

3.2. CE-MS/MS analysis of patient serums treated using IFX

The CE-MS/MS analytical workflow was consequently applied to 24 IFX-treated serums from patients diagnosed with Crohn's disease. The CE-MS/MS analysis was performed to quantify free IFX in patient serums in addition to the structural characterization in terms of N-glycosylation and amino acid PTMs. The CE-MS/MS data allowed the successful quantification of IFX for all patient samples (Table S2). Among the 24 patient serums characterized, 7 were found at concentrations above 25 $\mu g \cdot mL^{-1}$ and required prior dilution to fit within the calibration range. Patient samples were also quantified using a commercially available ELISA assay to confirm the validity of the CE-MS/MS results (Table S2). The comparison of CE-MS/MS and ELISA analyses, shown in Fig. 5, exhibited a good correlation (R^2=0.963) between the two techniques. However, a limited tendency towards lower quantification in the case of ELISA assay compared to CE-MS/MS analysis could be observed. This difference was consistent with the systematic underestimation provided by ELISA assay that could be observed during method development (Fig. 2). El Amrani et al. also described a similar trend when comparing LC-MS/MS analysis with the ELISA assay [37]. They attributed this discrepancy to the ELISA assay, which requires two distinct interactions of IFX, one with the plate, and the other with the detection reagent. Therefore, the experimental procedure could lead to a biased quantification in case of suboptimal interactions with either of the two required interactions. In contrast, the affinity extraction procedure designed in our case for sample preparation required only a single high-affinity interaction, in addition to the CE-MS/MS analysis which was systematically normalized with the SIL-IFX internal standard.

For the characterization of IFX N-glycosylation and amino acid PTMs, only 3 out of the 24 available samples exhibited an IFX concentration lower than 4 $\mu g \cdot mL^{-1}$ and were therefore not considered because the performance of the CE-MS/MS method could not be guaranteed, as demonstrated during method development. In addition, IFX may experience artifactual modifications, for example due to sample storage and/or preparation, which could potentially alter the reliability of the structural characterization. For example, endogenous deamidation of asparagine may be generated during proteolytic digestion due to the use of ammonium bicarbonate at mildly alkaline pH [62,63]. The digestion protocol was not modified because the alkaline pH provided the maximum enzymatic activity necessary to achieve an optimal LOQ. However, the objective of the CE-MS/MS method was to characterize the structural changes and to determine the levels of modifications occurring after administration to the patient. As a consequence, an innovative normalization method was developed to systematically subtract artifactual modifications and provide an accurate estimate of the levels of PTMs generated during the residence time of IFX in the patient's system. Fig. 6 summarizes the implemented normalization strategy. For each PTM, the level of endogenous modification was determined for IFX and

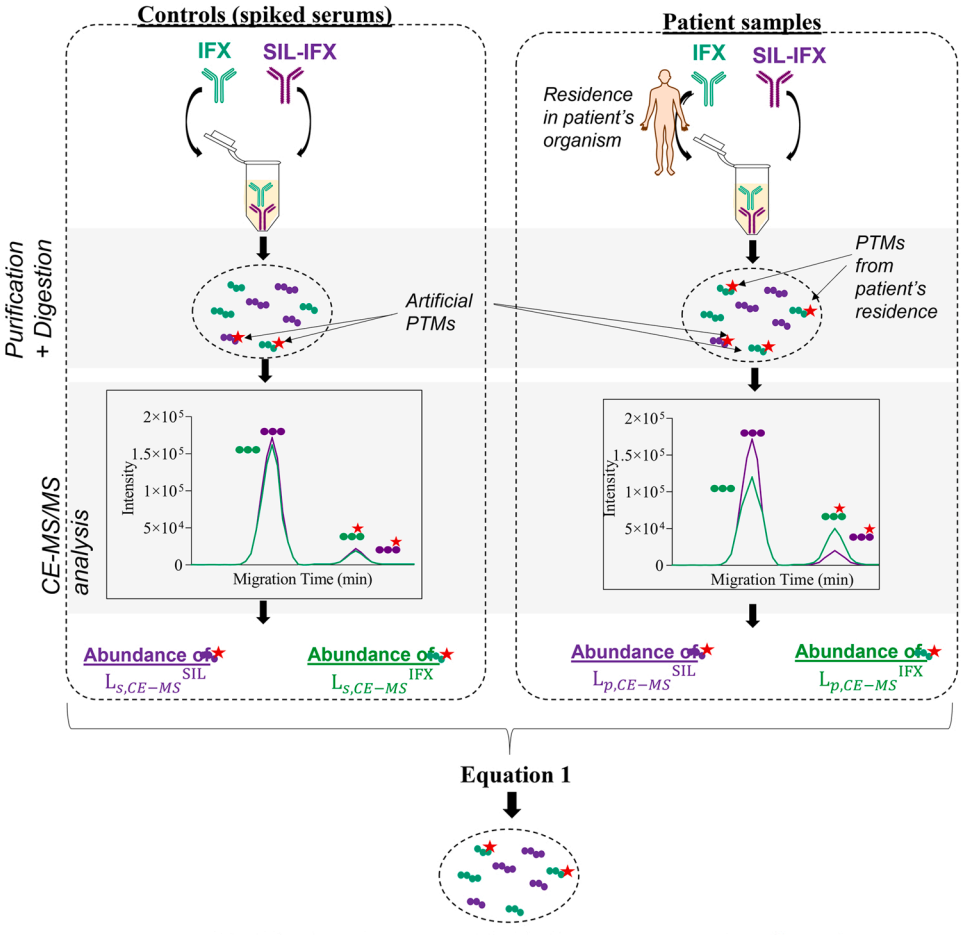

Fig. 6. Schematic representation of the procedure for the determination of the level of modification of an amino acid due to IFX residence in a patient's organism (L_{blood}). The strategy for normalization of PTM levels included CE-MS/MS analysis of the peptide of interest in the case of IFX ($L_{p,\ CE\text{-}MS}^{IFX}$) and SIL-IFX ($L_{p,\ CE\text{-}MS}^{SIL}$) in the patient's serum (left-hand panel) and similarly for IFX ($L_{s,CE\text{-}MS}^{IFX}$) and SIL-IFX ($L_{s,\ CE\text{-}MS}^{SIL}$) in spiked serums (right-hand panel) in order to obtain the parameters required to calculate L_{blood} using Eq. 1 (Cf. experimental section, and Fig. S7 and S8 for SIL-IFX information).

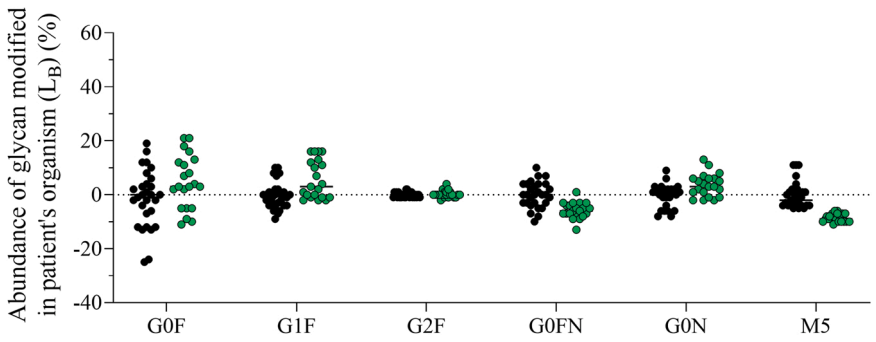

Fig. 7. Glycan relative abundance variations for IFX during residence in patient's blood after normalization measured using CE-MS/MS analysis. Green dots = patients' serums (n = 21). Black dots = controls (n = 30) that did not incubated in a human's blood.

Table 3
P-values calculated from Welch's t-test applied on patient serums compared to IFX spiked samples used as controls (degree of freedom = 22, α = 0.05). Data of the two groups were compared for each PTMs monitored in this study, with p-value < 0.05 indicating a significant difference between the two groups.

	p-value
N-glycosylation	
G0F	1.0E-01
G1F	5.0E-04
G2F	1.4E-01
G0FN	1.8E-07
G0N	2.0E-03
M5	6.3E-08
Amino acid PTMs	
N57	1.5E-07
N158	2.4E-01
N137	3.9E-01
N387	2.3E-01
D283	3.7E-01
D404	7.9E-01

SIL-IFX internal standard using CE-MS/MS analysis on calibration samples. Afterward, the modification level strictly due to IFX residence in the system of the patient could be calculated by comparing the CE-MS/MS analysis of the calibrants and patient serums. The mathematical expression of each parameter (Fig. 6) enabled the establishment of Eq. 1, which provides a systematic normalization the level of modification occurring during the residence time of IFX in the patient's organism (cf. paragraph 2.7).

Concerning IFX N-glycosylation, CE-MS/MS analysis characterized the 6 major glycosylations for serum samples from IFX-treated patients, and the MS data could be used to perform their relative quantification (Table S3). Using the CE-MS/MS data, the variation of the abundance of each glycosylation during the IFX residence time in the organism of patients was calculated by applying the Eq. 1 (Fig. 7). Regarding patient samples, the overall dispersion between the different samples was consistent with the dispersion of the IFX-spiked serums used as controls and reflected the standard variation of the CE-MS/MS method observed previously (Fig. 3). For the different types of glycans, a Welch's t-test was conducted to determine whether the relative abundance was significantly different in the patient serums compared to the control samples, without assuming equal variances [64]. The results, compiled in Table 3, demonstrated that the hypothesis that the two groups were statistically similar was rejected for G1F, G0N, G0FN and M5, indicating a statistical difference (p-value < 0.05). Especially, patient samples presented 6–12% lower M5 abundance than the mean value of control samples. In the case of G0FN proportion, patient serums were approximately 5% lower than controls. Thus, the CE-MS/MS results indicate that the glycodistribution of IFX was slightly altered after administration, independent of the patient. This observation could potentially be due to several IFX glycoforms exhibiting faster clearance, leading to more rapid elimination of the concerned glycans. Goetze *et al.* have shown that high-mannose glycan have faster serum clearance than other glycan forms, which is in agreement with the results observed Fig. 7 [65].

Regarding asparagine deamidation and aspartate isomerization, CE-MS/MS analysis demonstrated consistent characterization of the different PTM hotspots for the 21 patient serums considered (Table S4). As with N-glycosylation, Eq. 1 was applied for normalization to estimate the level of modification occurring during the residence time of IFX in the patient's system. The results, represented in Fig. 8, showed the absence of significant levels of modification for the amino acids N137, N158, N387, D283 and D404. In addition, Welch's t-tests yielded p-values > 0.05, proving that the control samples and patient serums were not significantly different (Table 3). Several studies have investigated the conditions affecting PTMs, such as pH, temperature or humidity. Typically, harsh conditions were used to generate significant

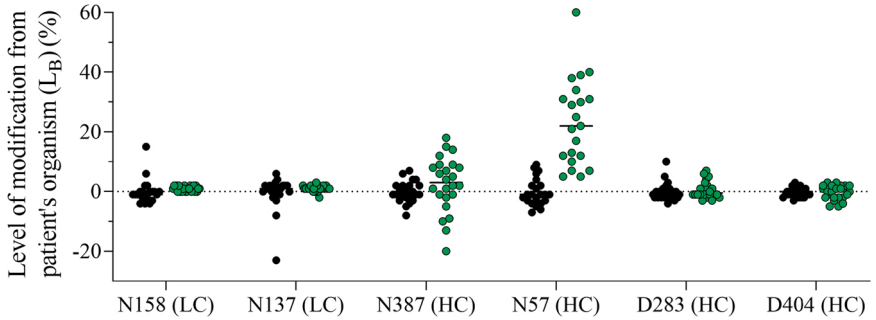

Fig. 8. Proportion of PTMs induced to IFX during residence in patient's blood $\mathbf{L}_{blood}$, (n = 21) (green dots) compared to the values found in spiked serum taken as controls (black dots). For asparagine (N), the modification monitored was deamidation, and for aspartate residues (D) the modification was isomerization.

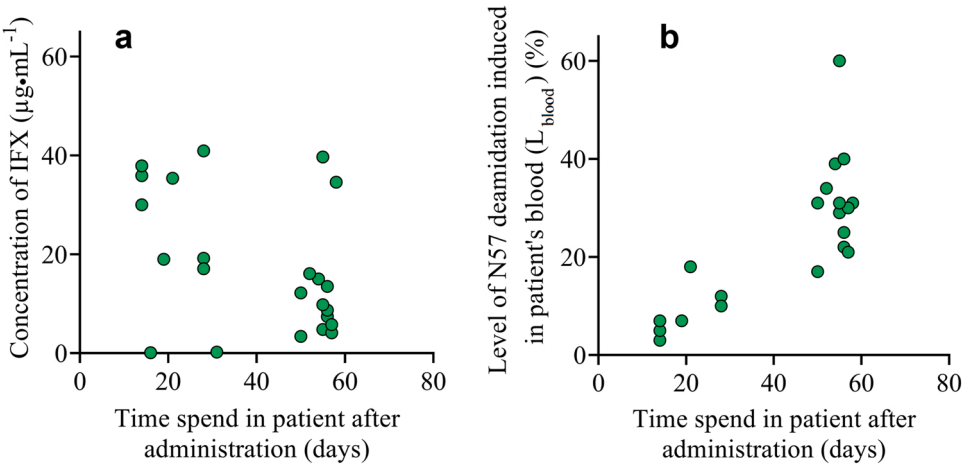

Fig. 9. (a) Evolution of the concentration of Infliximab in patient serums with anteriority of the administration to patient (n = 23). (b) Evolution of the deamidated level of N57 with anteriority of the administration to patient (n = 21).

degradations in a short amount of time, but did not necessarily reflected conditions after administration [36,55]. Therefore, environment conditions of IFX consequently to administration did not affect some hotspot PTMs that are nevertheless observed under extreme conditions.

In the case of N57 deamidation, the analysis of patient serums exhibited significantly higher levels of modification and important dispersion compared to the spiked serums (Fig. 8), which was confirmed by a p-value indicating significant differences (Table 3). Thus, CE-MS/MS analysis revealed that N57 deamidation was occurring during IFX residence time. The residue N57 is located in the complement determining region (CDR) of IFX, which interacts directly with TNF-α [66]. Therefore, deamidation of this residue to aspartate could potentially alter the interaction of IFX with the corresponding antigen. Concerning Crohn's disease patients, the anteriority since the last IFX administration at the time of blood sampling was provided. To further understand the evolution of IFX, the concentration measured in the patient serums and the level of N57 modification were plotted against the anteriority of the administration. As illustrated in Fig. 9a, the remaining IFX concentration was not influenced by the anteriority of the injection. However, CE-MS/MS analysis demonstrated a gradual deamidation of the N57 residue to aspartate as the IFX residence time was increased (Fig. 9b). This effect could not be observed for the other asparagine residues suggesting a specific sensitivity of the amino acid N57. Also, the amount of N57 deamidation was not related to the concentration of IFX in the characterized sample.

Currently, the clinical follow-up of IFX-treated patients is performed by ELISA assay, which strictly focuses on the evolution of mAb concentration after administration. The CE-MS/MS analytical workflow demonstrated accurate quantification of IFX in patient serums, in addition to characterizing N-glycosylation and amino acid PTMs hotspots. The advanced clinical follow-up provided by CE-MS/MS demonstrated that monitoring IFX concentration may not be sufficient to fully understand the complex dynamics regarding IFX evolution post-administration. Indeed, even if the IFX concentration is successfully maintained within the therapeutic range over an extended period of time, PTMs potentially affecting the potency of the biomolecule could occur, especially since mAbs have a significantly prolonged serum half-life. Therefore, the CE-MS/MS methodology, which provides simultaneous absolute quantification and advanced characterization of PTMs, represents a relevant analytical methodology to study the outcome of mAbs at an unprecedented level.

4. Conclusion

In the present work, we developed a novel analytical strategy based on CE-MS/MS analysis in order to simultaneously perform absolute quantification of IFX, characterization of the 6 major N-glycosylations and 6 hotspots amino acid PTMs in serum samples in a single analysis. The validation of the CE-MS/MS method demonstrated relevant analytical performances for the analysis of serum samples from IFX-treated patients, especially regarding specificity and sensitivity. The results obtained proved the feasibility of absolute protein quantification using CE-MS/MS analysis. Thus, the developed analytical workflow provided an accurate quantification while avoiding matrix effects, that may potentially interfere in the case of ELISA assay. Consequently, the CE-MS/MS workflow was applied to the analysis of serum samples collected from patients treated for Crohn's disease using IFX. The experimental results provided a reliable quantification of IFX. Concerning the structural characterization, an original data treatment methodology was developed to confidently estimate the level of PTMs that occurred exclusively during IFX residence time in the organism of patients. Thus, CE-MS/MS data allowed the identification of structural modifications of IFX occurring following its administration to the patient. In particular, the analysis of patient serums using the CE-MS/MS method developed identified a correlation showing progressive deamidation of the residue N57 which had the particularity of being located in the CDR of IFX. However, the evolution of the IFX concentration exhibited an important variability from patient-to-patient and could not be correlated with the residence time of the protein in the system of the patient.

CE-MS/MS analytical strategy demonstrated the possibility to reach an additional dimension of characterization regarding the evolution of therapeutic proteins after administration. In addition, it is the first time that protein absolute quantification in biological samples could be realized concomitantly to structural characterization regarding PTMs. The analytical strategy described could represent a promising alternative in order to realize an advanced follow-up of patients treated with therapeutic mAbs. Nevertheless, the elaborated multi-characterization offered by this method would be particularly relevant for research, especially for a cohort study in order to better understand the outcome of mAbs after administration. Disparities in clinical response among patients are not yet fully understood and could be elucidated by a method that simultaneously links PTM levels of hotspots and infliximab levels. The opportunity to obtain such comprehensive information in a single analysis could significantly reduce the biases and errors associated with the need for multiple workflows to independently obtain quantification, N-glycosylation and amino acid PTMs characterization.

Finally, further research could enable to investigate the impact of the PTMs on the bioactivity of therapeutic mAbs in order to potentially interpreting the complex behavior of this type of treatment and

attributing the occurrence of unusual clinical responses. The CE-MS/MS analysis can also be transposed in a straightforward manner to other types of mAbs which in this case also illustrates the potential of mAbs comprehensive follow-up using this methodology.

Author statement

R.G, P.H and Y.F conceived the project. T.R and A.K performed the experiments and interpreted the data. T.R, R.G and Y.F wrote the manuscript with input from all of the authors. R.G. and Y.F are the scientific coordinator of the research project MethAmAbs (project no. ANR-19-CE29–0009).

CRediT authorship contribution statement

Tessa Reinert: Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Visualization. **Rabah Gahoual:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing, Funding acquisition. **Nathalie Mignet:** Investigation. **Alexandre Kulus:** Investigation. **Matthieu Allez:** Resources. **Pascal Houzé:** Resources, Validation, Writing – review & editing, Supervision. **Yannis-Nicolas François:** Methodology, Validation, Supervision, Writing – review & editing. Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115446.

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