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Degradation and in-use stability study of five marketed therapeutic monoclonal antibodies by generic weak cation exchange liquid chromatographic method ((WCX)HPLC/DAD)

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A B S T R A C T

Therapeutic monoclonal antibodies (mAbs) represent a very important class of the current biopharmaceutics. The great complexity of their structure made necessary the use of different analytical approaches for assessing different physico-chemical properties. In this work, weak cation exchange (WCX) high performance liquid chromatography with diode array detection ((WCX)HPLC/DAD) is used to assess the charge variant profile. The method here developed combined the effect of ionic strength and controlled pH gradient and allows for the charge variants analysis of the five mAbs studied, namely bevacizumab (BVZ), cetuximab (CTX) infliximab (INF), rituximab (RTX) and trastuzumab (TTZ), which are among the most used mAbs worldwide. The differences in the charge variants in the natural isoforms of the mAbs promoted characteristic WCX chromatograms for each of mAbs that can be also useful for identification purposes. These chromatograms have provided to be suitable for tracking changes in the charge variants of each mAb analyzed both in controlled degraded and in stabilities study along time of in-use samples solutions at 2 mg/mL in 0.9% NaCl stored refrigerated (at 4 °C) and frozen (−20 °C) for two months. The results obtained indicated different stabilities of these mAbs, all IgG1, against degradation by different stressed environmental conditions and in-use stability along two months.

1. Introduction

Monoclonal antibodies are currently one of the most important class of biotechnological drugs, leading the global pharmaceutical market nowadays. These kinds of glycoproteins, characterized by their specificity and affinity in the recognition of targets and their relatively long half-lives [1], have become the spearhead of new therapies for the treatment of diseases with an increasing incidence in the population such as cancer, autoimmune, inflammatory, infectious and degenerative diseases, consolidating at the top of the biopharmaceutical market [2–4]. Also, from the start of the pandemic of the COVID-19, mAbs has been explored as potential therapeutic [5].

Currently, it is well-known that of the five classes of antibodies in humans, the subclass 1 (IgG1), is the most commonly used for pharmaceutical and biomedical purposes [6,7].

Example of the IgG1 subclass are the therapeutic marketed mAbs bevacizumab (BVZ), cetuximab (CTX), infliximab (INF), rituximab (RTX) and trastuzumab (TTZ). BVZ (Avastin®) is a humanized antibody indicated mainly for the treatment of various types of cancer, such as metastatic colon carcinoma, metastatic breast cancer, lung cancer, etc. [8]. CTX (Erbitux®) is a potent chimeric mouse/human mAb approved for the treatment of colon cancer and other types of squamous cell cancer such as cancer of the mouth or larynx [9]. INF (Remicade®) is a murine-human chimeric antibody indicated for the treatment of many autoimmune diseases such as of Crohn's disease, rheumatoid arthritis, psoriasis, ankylosing spondylitis, psoriatic arthritis, and ulcerative colitis [10]. RTX (MabThera®) is a murine/human chimeric antibody intended for use in the treatment of non-Hodgkin's lymphoma [11], rheumatoid polyarthritis [12] and chronic lymphoid leukemia [13]. TTZ (Herceptin®) is a humanized antibody indicated for the treatment of

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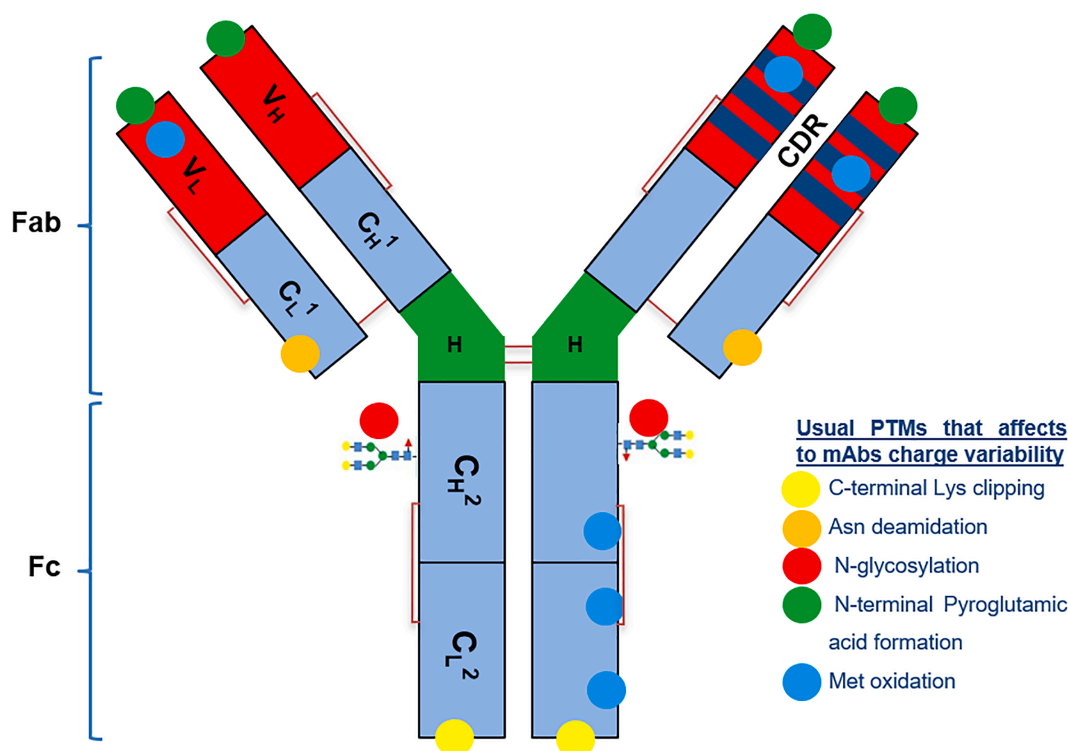

Fig. 1. IgG1 structure with indication of the most usual post-translational modifications (PTMs).

patients with metastatic breast cancer whose tumors over express HER2 (25% of the patients) [14]. As IgG1, the five mAbs share the same general structure varying mainly in the variable region where the complementary determining region (CDR) is located (Fig. 1) [15]. These 5 mAbs are among the biotechnological drugs most consumed worldwide [16].

MAbs characterization is a complex task, due to the characteristic heterogeneity of biotherapeutics. This feature start at the beginning of the production process originated by both chemical and enzymatic reactions called post-translational modifications (PTMs) that take place not only in upstream and downstream bioprocessing but also in storage and handling procedures before patient administration [17]. Numerous of these PTMs, such as deamidation of asparagine residues, N-glycosilation, C-terminal Lys clipping and N-terminal pyroglutamic formation, are involved in the charge heterogeneity of biotherapeutics by the modification of the charge distribution in the protein surface, altering its isoelectric point (pI) and generating different charge variants [17,18] in a same sample protein. Therefore, charge heterogeneity is a critical quality attribute (CQA) for therapeutic proteins [18], especially relevant for immunoglobulins subclass IgG1 and derivates, such as antibody drug conjugates (ADCs) [19] or single-domain antibodies also known as nanobodies [20] and also for Fc-fusion proteins. The charge heterogeneity is crucial issue related to the quality assessment of the final product, and therefore the ICH guidelines establish that variant profile has to be monitored and characterized [21].

Ion exchange chromatography (IEX) is considered the gold standard technique for the characterization and monitoring of charge heterogeneity in biopharmaceuticals and it is widely used also for the detection of degradation patterns, due to its simplicity and rapid set-up [22,23]. Cation exchange chromatography (CEX) is the most extended form of IEX technique for mAbs and related protein analysis, where differences in the surface charge of the proteins lead the separation based on the interactions between the positive charges on the proteins surface and the negative charges of the column resin [24–29]. These methods are already well-stablished for high-resolution monoclonal antibody charge variant separations as demonstrated in the last years [25–29]. The elution is commonly performed basing on the application of a simultaneous controlled pH gradient and an ionic strength gradient both in the mobile phase [25–30]. In this way, the charge variant profile obtained shows different acid and basic forms separated from the main peak. The elution times depend fundamentally on their isoelectric point (pI), lower in acidic forms (earlier elutions) and greater in basic forms (later elutions) with respect to the main peak [24,25]. Charge variants have been attributed to PTMs and are directly related to the stability of the protein since they are involved in numerous degradation processes [31–33]. The use of mobile phases which contains non-volatile high salt concentration restricts the detection by mass spectrometry (MS) due to their incompatibility with the ionization sources [28,32,33,34,35]. Bottom-up approaches are usually carried out for identification purposes, with peptide mapping analysis by LC-MS/MS after IEX peak collection step [18,36]. Despite its high sensitivity, the triptic digestion procedures together with the reduction of the disulfide bridges and the alkylation prior to the analysis may generate additional degradations [22]. This limitation has led to propose alternative strategies for the identification of charge variants, such as the combination of two-dimensional LC methods coupled to mass spectrometry (2D/LC-MS). In this strategy, the first step takes place the IEX column, which is not compatible with MS, and the second step, takes place in the reverse phase (RP) column, therefore, compatible with MS and allowing the identification of the charge variants previously separated by IEX [37]. However, this 2D/LC-MS technique is complex and expensive, which makes it exclusive to a few laboratories specialized mainly in proteomics. For this reason, the development of a simple, fast and generic method for monitoring the charge heterogeneity is required also. More recently, the use of volatile salts for non-denaturing chromatographic techniques, such as IEX, has allowed for coupling the LC effluent to the mass spectrometer detector (IEX-MS) to characterize the mass of the therapeutic proteins [32,38,39]. Although the use a MS detector for the characterization of the individual charge variants represents a great advantage, not always the volatile salts allow for obtaining good enough charge variants profiles [28,29].

In the present work, we developed and validated a generic fast weak

Table 1
Chromatographic conditions.

Column	Bio MAb NP10 (50 mm × 4.6 mm, 10 μm, Agilent Technologies)
Eluent	A: Na_2HPO_4 10 mM in Milli-Q water at pH 6.0B: Na_2HPO_4 10 mM and NaCl 1 M in Milli-Q water at pH 8.1
Elution	Gradient program: 0 min –10% B; 25 min – 100% B; PostTime: 3 min
Flow rate	0.8 ml/min
Injection volume	1 μL
Column temperature	25 °C
Autosampler temperature	5 °C
Injection washing solution	Milli-Q water
Detection	214 nm 220 nm 235 nm 280 nm

cation exchange high performance liquid chromatographic method with diode array detection (WCX)HPLC/DAD to detect changes in the charge heterogeneity -or charge variants- in five of the most used worldwide therapeutic mAbs, i.e. BVZ, CTX, INF, RTX and TTZ. This method was based on the simultaneous application of a controlled pH gradient in the mobile phase and an ionic strength gradient (salt-mediated pH-IEX methods) [25–29]. The method was developed as a single method for the analysis of the 5 mAbs studied here, without the need of any specific modification or adjustment in the experimental parameters for the analysis of each individual mAb. Although they share the same IgG1 structure, different charge profiles were exhibited for each mAb allowing the identification of each one. Forced degradation studies were carried out in order to validate the method as stability-indicating demonstrating its capability to identify degradation by detecting changes in the corresponding charge variants profiles [40]. Furthermore, and once validated the method for detecting modification on the charges profile, a stability study along 2 months was performed on the 5 mAbs under their typical therapeutic condition of use, i.e. clinical concentration and recommended storage temperature when used in hospital [8,9,10,13,14].

2. Material and methods

2.1. Chemicals and reagents

All reagents were of analytical reagent grade unless otherwise stated. Reverse-osmosis quality water (purified with a Milli-RO plus Milli-Q station from Millipore Corp., Madrid, Spain) was used throughout hydrochloric acid, sodium hydroxide, disodium phosphate, sodium dihydrogen phosphate and sodium chloride were supplied by Panreac (Barcelona, Spain). Hydrogen peroxide was supplied by Technical Fontenay (Sous-Bois, France).

An isotonic solution of 0.9% NaCl was supplied by B. Braun Medical (Madrid, Spain).

2.2. Therapeutic monoclonal antibodies (mAbs)

The monoclonal antibodies BVZ, CTX, INF, RTX and TTZ were purchased as Avastin®, Erbitux® (Merck KGaA,Darmstadt, Germany), Remicade® (Janssen Biotech, Inc.Horsham, Pennsylvania, USA), MabThera® (Roche Pharma AG, Grenzach-Wyhlen, Germany) and Herceptin® (Roche Pharma AG, Grenzach-Wyhlen, Germany) respectively. Avastin®, MabThera® and Herceptin® respectively (RochePharma AG, Grenzach-Wyhlen, Germany). CTX was obtained as Erbitux® (Merck KGaA,Darmstadt, Germany) and INF as Remicade® (Janssen Biotech, Inc.Horsham, Pennsylvania, USA). Units of these medicines were kindly supplied for this study by the Pharmacy Unit of the University Hospital "San Cecilio" (Granada, Spain). The medicines indicated a quantitative composition of 25 mg/ml of BVZ [8], 5 mg/ml of CTX [9], 100 mg/vial of INF [10], 10 mg/ml of RTX [13] and 150 mg/vial of TTZ [14]. The working standard solutions of each mAb were prepared daily from the corresponding medicine immediately after opening it by appropriate dilution with 0.9% NaCl isotonic solution to avoid any kind of degradation in the drugs. All the experiments were conducted with fresh mAb medicine samples to ensure full molecular and structural integrity of the mAbs during the whole study.

2.3. Chromatographic system and software

Chromatography was performed using an Agilent 1100 liquid chromatograph equipped with a quaternary pump, degasser, autosampler, column oven and photodiode array detector (Agilent Technologies, Madrid, Spain). The instrument was connected to a personal computer fitted with an HPLC ChemStation workstation for LC 3D systems (rev. A.0903, Agilent Technologies). Chromatographic analyses were carried out in a BIO MAb NP10 250 mm × 4.6 mm i.d., 10 μm particle size, weak cation exchange analytical column (Agilent Technologies, USA). A Bio MAb NP10 50 mm × 4.6 mm i.d., 10 μm particle size, weak cation exchange guard column (Agilent Technologies, USA) was also used. The eluent flow rate was 0.8 ml/min and consisted of a gradient of Na_2HPO_4 10 mM in Milli-Q water at pH 6.0 adjusted with HCl 2 M (eluent A) and a mixture of Na_2HPO_4 10 mM and NaCl 1 M in Milli-Q water at pH 8.1 (eluent B). The gradient started at 10% of eluent B and was increased to 100% in 25 min. A post time of 3 min was used keeping 10% of eluent B. The temperature of the column was maintained at 25 °C, and the injection volume was 1 μL. UV spectra were recorded between 200 nm and 399 nm, with a data point every 0.5 nm. Chromatograms were registered at 214 nm, 220 nm, 235 nm and 280 nm using 360 ± 20 nm as the reference wavelength. The chromatograms of this work were studied at 214 nm due to their higher peak intensity. The chromatographic conditions are summarized in Table 1.

The STATGRAPHICS Plus 6.0 (Statistical Graphics System, 1992, Warrenton, VA) statistical software package was used for processing the chromatographic data.

2.4. Forced degradation studies of the mAbs

Forced degradation studies were performed using solutions of 2.0 mg/ml of each mAb prepared in NaCl 0.9% and placed in glass vials to perform the stress. This concentration was selected as representative of the lowest concentration of the final product used in hospital. This study was conducted to validate the method for its intended propose, that is, to detect and track changes in the charge variant isoforms chromatographic profile and also to get knowledge about degradation when the mAbs solutions are handled and administered in hospital conditions. We therefore studied factors such as high temperature, high ionic strength and UV light exposure, in addition to subject the mAbs solution to acidic, basic and oxidant media in order to degrade the mAbs. The effect of agitation in mAbs was not evaluated in this work. The chromatograms of the stressed samples were compared with those of the fresh mAbs samples to detect the changes in the isoform profiles. Samples were analyzed in triplicate and the most representative chromatogram for each mAbs is showed in the corresponding Figures.

The effects of acid and alkaline media, ionic strength and oxidation medium were evaluated using solutions of HCl 1.0 M, NaOH 0.1 M, NaCl 1.5 M and H_2O_2 1% respectively. The stressed agent concentration was 25.0%*(v/v)* (500 μL of the stressing agent was added to 1.5 ml of mAb standard solution). The samples were analyzed 24 h after preparation. The effect of high temperature was evaluated by placing aliquots of each mAb solution in an oven (Heraeus S.A., Madrid, Spain) at 50 °C for 24 h. All stress conditions, except temperature stress, were carried out at room temperature (22 °C aprox). The effect of UV light was investigated by exposing aliquots in a UV chamber (SUNTEST CPS, Heraeus, Hanau,

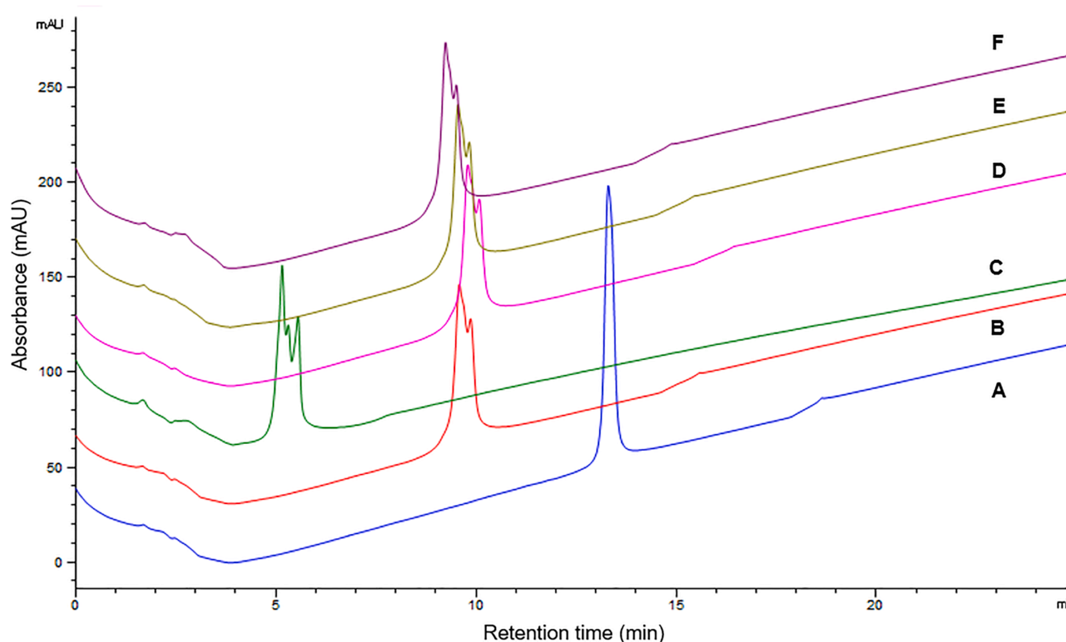

Fig. 2. WCX method optimization tests based on the pH gradient windows in the mobile phase using INF 2.0 mg/mL as model samples: 6.0 to 8.1 (A), 4.0 to 8.1 (B), 7.0 to 8.1 (C), 6.0 to 7.0 (D), 6.0 to 9.0 (E) and 6.0 to 10.0 (F).

Germany) with the walls covered in mirrors and equipped with a Xenon lamp. Irradiation at 250 nm was established at 765 W/m^2 and chromatograms were recorded after 24 h of irradiation.

2.5. Two-months stability studies of the mAbs clinical solutions

Samples of BVZ, CTX, INF, RTX and TTZ, (2.0 mg/mL) with 0.9% NaCl, were prepared from their innovative medicines Avastin®, Erbitux®, Remicade®, MabThera® and Herceptin®, respectively. The specification included in their scientific technical reports were followed for preparing all these solutions [8,9,10,13,14], including working in aseptic conditions using a laminar flow cabinet (Aura Vertical S.D.4, Bio Air Instruments, Italy). Since this study was focused in the evaluation of the charge variants by the proposed method and not in a wider stability study of the studied proteins, the clinical samples solutions were prepared in glass devices, then aliquoted, placed in amber glass vials (protected from daylight) and stored refrigerated at 4 °C. Likewise, several aliquots were stored frozen at −20 °C. The refrigerated samples were analyzed at 1, 4, 7, 14, 30, 45 and 60 days after preparation. The frozen samples were analyzed after 7 (only for CTX and BVZ) 30, 45 and 60 days of storage, performing in the samples only one frozen/thawed cycle. In the two-months stability study, % of area and the retention time (RT) of the main isoforms, the disappearance of initial isoforms and the detection of new degradation peaks of the 5 mAbs were evaluated. This evaluation was conducted by comparing the charge variant profile of the analyzed samples along time with the initial chromatographic profiles recorded in the fresh samples, i.e. the day when the samples were prepared (Day 0). Samples were analyzed in triplicate and the most representative chromatogram for each mAbs is showed in the corresponding Figures.

3. Results and discussion

3.1. (WCX)HPLC/DAD method performance for mAbs characteristic charge variant profile

The preliminary analyses were performed using the mobile phase composition indicated he manufacturer of the column which was selected from the market [42], and corresponds to salt gradient conditions. As indicated in the technical report of the WCX column, the followed salt gradient was initially checked in the mobile phase: constant pH value of 6 -which was adjusted with NaH_2PO_4 10 mM- and a ionic strength gradient from 0 to 100% (NaCl 0.1 M). Solutions of 2.0 mg/mL of each mAb were prepared by appropriated dilution of the corresponding medicine in NaCl 0.9%. No chromatographic peaks were obtained for any of the five mAbs with these salt gradient conditions, indicating strong binding to the cation-exchanger. This was expected since the *pI* values of the five therapeutic mAbs are very close and higher than 8 since they all are representative of IgG1 structure. The theoretical *pI* values estimated by their amino acid sequences were: 8.4 for BVZ [43], 8.48 for CTX [44], 8.25 for INF [45], 8.68 for RTX [46] and 8.45 for TTZ [47]; Then, pH 6 is 2 unit below the theoretical *pI* and it justifies mAbs were strong retained into the column.

Therefore, salt-mediated pH-gradients conditions were selected to obtain the chromatographic charge variant profiles. The first step was the selection of the nature of the buffer. A stronger ionic strength buffer was checked using the same pH value (6) in the mobile phase, i.e. Na_2HPO_4 with a concentration of 6 mM, 8 mM and 10 mM. Results demonstrated that higher concentration gave shorter retention times for all the mAbs, not affecting the particular profile of the chromatographic charge variants. This was attributed to the increase of the eluting power with the increase of the ionic strength of the mobile phase.

Once selected the buffer (Na_2HPO_4 10 mM), several experiments were carried out testing different pH windows looking for high-resolution on the chromatographic charge variants separation, since this factor is crucial for that aim. For this optimization, a unique mAb was selected as a representative sample, i.e. INF at 2.0 mg/mL since it is one of the most structurally complex of the 5 studied (high glycosylated and not N-terminal lysine completely removed) [48]. The lower values tested for the pH window were 4.0, 6.0 and 7.0, and the higher values were 7.0, 8.1, 9.0 and 10.0. Each pH window was checked at least with two different gradient times, i.e. 15 min and 30 min. Fig. 2 shows results of this experiment 30 min of analysis. As it was expected, the elution time increased with the decrease of the starting pH value in the gradient (see Fig. 2 A, B and C) and from pH > 7 INF was not retained into the column, eluting in the front (chromatogram not shown) in a single chromatographic peak. Again, as expected, the increase of the final pH value in the gradient promoted the increase of the eluting time, as the

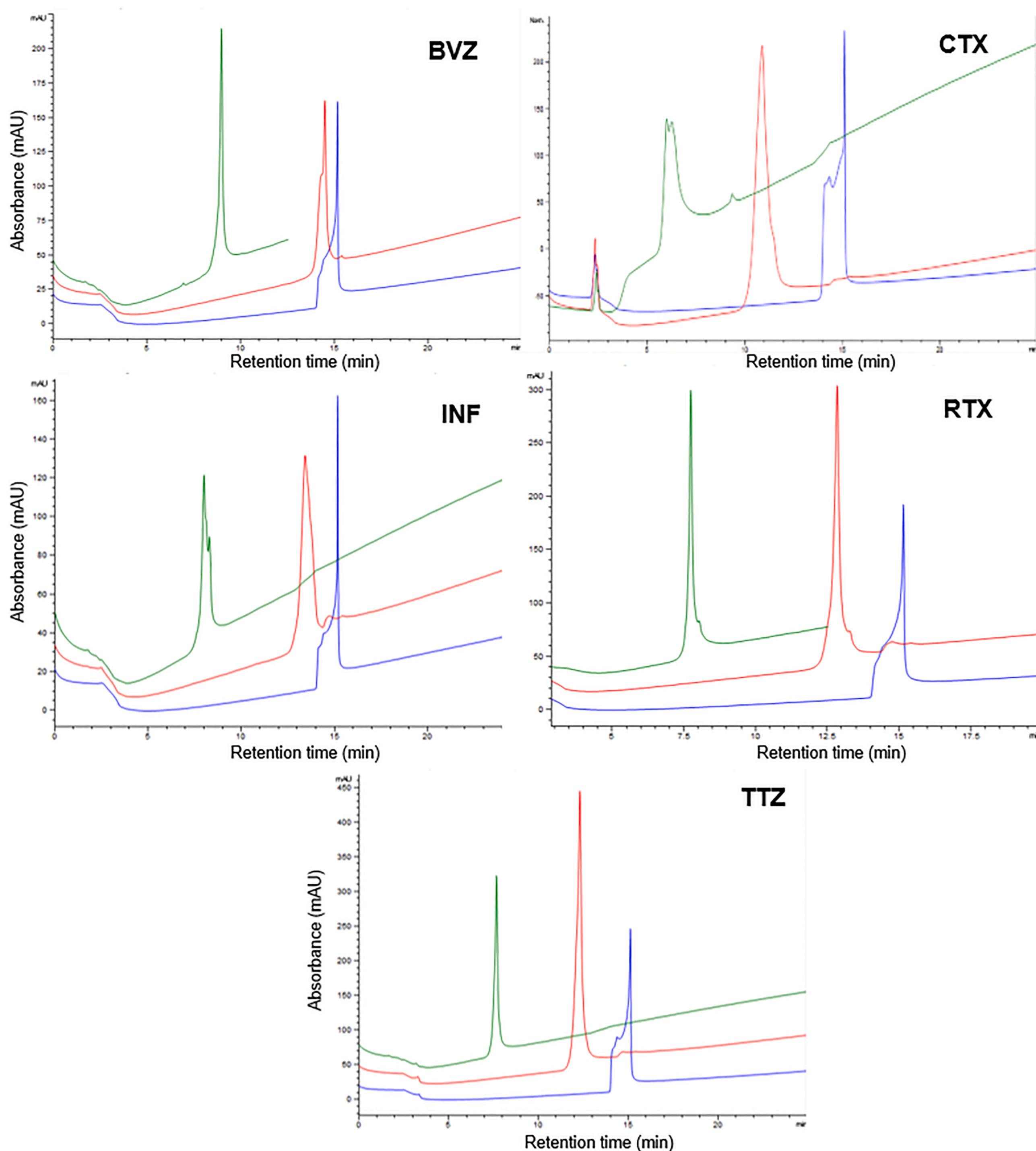

Fig. 3. WCX method optimization tests based on salt gradient in the mobile phase. 2 mg/mL solutions of BVZ, CTX, INF, RTX and TTZ. 0.25 M NaCl (blue trace), 0.5 M NaCl (red trace) and 1.0 M NaCl (green trace), using the optimized pH window from pH 6.0 to pH 8.1.

INF was retained into the column with no improvements observed in the chromatographic profiles since they tended to show a more defined INF chromatographic peak overlaying the variants (Fig. 2 D, E and F). Two peaks were clearly detected (that not chromatographically resolved) in the INF chromatographic charge variants profile for these pH windows tested. Finally, pH 6.0 to pH 8.1 was selected since allowed for the suitable detection of the INF charge variants in a reasonable time of analysis considering the aim of propose a single method for the charge variants of 5 deferent IgG1.

The effect of the ionic strength was evaluated in the 5 mAbs by decreasing the concentration of NaCl (1.0 M) in the mobile phase using the above selected pH window. Experiments were carried out using 0.25 M, 0.5 M and 1.0 M of NaCl, with optimized pH window from pH 6.0 to pH 8.1, and they are summarized in Fig. 3. The decrease of the ionic strength produced an increment in the elution time of all the mAbs. The charge variant profiles were very similar for all the mAbs, both in shape and retention time, when using the lower NaCl concentration tested (0.25 M), this was attributed to the low ionic strength power of the mobile phase at this NaCl concentration, which affect the resolving power of the mobile phase on the charge variants separation. As the

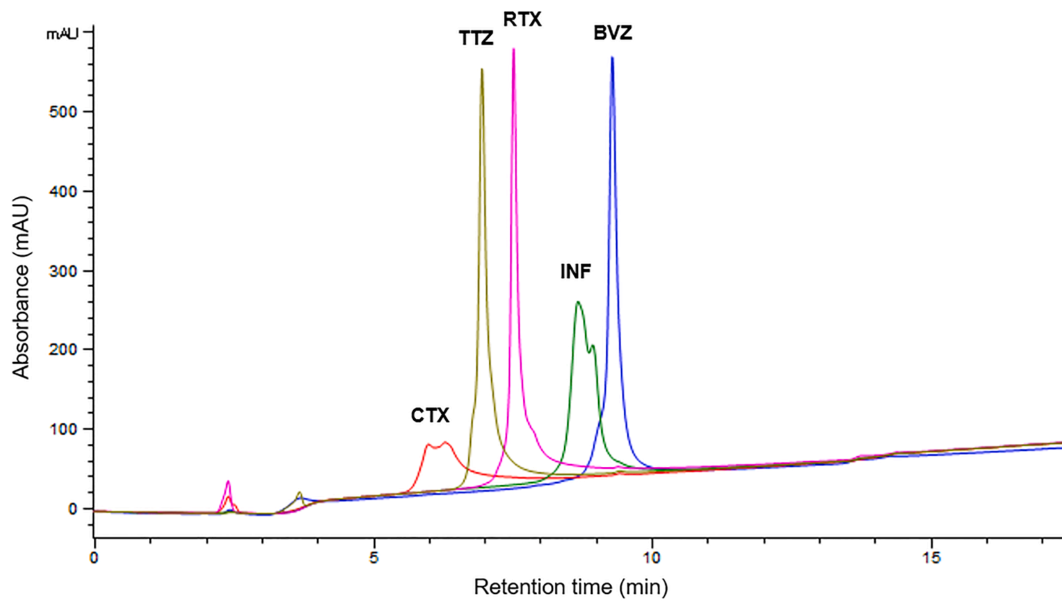

Fig. 4. Representative individual charge variant profiles obtained with the WCX optimized method conditions at 2.0 mg/mL: BVZ, CTX, INF, RTX and TTZ.

Fig. 5. Stress study of BVZ, CTX, INF, RTX and TTZ, 2 mg/mL in NaCl 0.9%. Stress conditions: acid stress (red trace), basic stress (green trace), high ionic strength (pink trace), oxidative stress (olive green), high temperature stress (purple trace), light exposure stress (dark green) and no-degraded –control- (blue trace) mAbs samples.

Table 2
Stress study results.

Stress type	BBZ		CTX		IFX		RTX		TTX	
	% Loss area[1]	Peak change/ new peaks	% Loss area[1]	Peak change/ new peaks	% Loss area[1]	Peak change/ new peaks	% Loss area[1]	Peak change/ new peaks	% Loss area[1]	Peak change/ new peaks
Temperature	4.9	No	0.0	No	4.1	No	3.8	No	0.0	No
UV Stress	No	No	24.6	No	12.6	No	70.6	No	0.0	No
Ionic Stress	34.9	No	39.0	No	50.1	No	35.4	No	35.5	No
Oxidative Media	46.2	Yes	43.4	Yes	47.8	Yes	39.5	Yes	42.0	Yes
Acidic Media	79.9	No	37.5	No	100	n.d*	38.9	No	100	n.d*
Basic Media	100	n.d*	37.3	No	100	n.d*	100	n.d*	100	n.d*

[1] [1] With respect to the control sample * No chromatographic peak detection.

concentration of the salt increased, the charge variant profiles were more defined and a greater number of peaks were detected in all the chromatograms. Consequently, the use of a concentration 1.0 M of NaCl in the mobile phase was confirmed since the decrease of the ionic strength prevented the detection of charge variants in the 5 mAbs analyzed.

In Fig. 4 the cation exchange chromatographic elution profiles obtained for the mAbs using the above optimized conditions are shown. Each one has a clearly different charge variant profile that could be used even with identification purposes. The chromatograms showed a main peak with different variant pattern for each protein. All the main chromatographic peaks in the profiles showed shoulders and unresolved peaks demonstrating the presence of charge variants in the final medicine product (dilutions at 2.0 mg/ml). In the case on TTZ and BVZ (Fig. 4, TTZ and BVZ respectively), acid variants (those early eluted) were noted although not resolved, being most evident for BVZ. It was preferred to develop a single method for the 5 mAbs instead of adjusting conditions for a complete separation of the variants of each one. For RTX (Fig. 4, RTX), clearly were noted basic variants (those lately eluted regarding the main peak). For CTX and INF, (Fig. 4, CTX and INF respectively) charge variant profiles were the most different from the other profiles. CTX showed an important acid variant eluting close to the main peak but not completely resolved, and the INF profile was characterized by a main peak eluting early and two important basic variants, one eluting close to the main peak although not completely resolved and the other eluting in a separated chromatographic basic peak. It is interesting to highlight the different eluting time observed for each mAb despite their similar *pI* (between 8.25 and 8.68): CTX eluted at the lower pH value in the gradients (therefore, at the lower retention times), followed by TTZ, RTX, INF and BVZ that eluted the last one at the highest pH (Fig. 4). Therefore, the mAb with the smallest pI (INF) eluted in fourth position, and the one with greatest pI (RTX) eluted in third position. These results indicated that the behavior of the elution of the mAbs in salt-mediated pH-gradients conditions in weak cation exchange chromatography not only depends on the charged surface, because in the case of high molecular weight multi-domain proteins the surface charge is not the only factor that influence the chromatographic separation processes, which avoid predicting the elution order based on the pI of a mixture of mAbs [33,49].

3.2. *Charge variants chromatographic profile of the mAbs after controlled degradation study.*

Forced degradation experiments were performed on the 5 medicines: Erbitux®, Avastin®, Remicade®, MabThera® and Herceptin®, to validate the method for detecting changes in the optimized charge variant profile of CTX, BVZ, INF, RTX and TTZ respectively. Also, this stress study was carried out with the aim of obtaining information of the degradation patterns of these 5 mAbs when hospital handle, for a deeper knowledge of their robustness to modification by ambient conditions during storage or administration. This is important because these kinds of therapeutic protein are exposed to various types of stress during administration and could suffer more serious changes or degradation than chemical drugs [50], so this study was also focused on the factors that may affect their stability. Furthermore, whereas stability testing requirements are defined in regulatory guidelines, the specific procedures for forced degradation studies of therapeutic proteins have not yet been standardized [51]. We therefore carried out the forced degradation studies described below.

In the forced degradation experiments, the evaluation of the degradation of each of the antibodies was carried out by comparing the chromatographic profiles with a reference profile (quality control) of each mAbs. The quality control profiles were the charge variants profile of the medicine without submitting to any stressful condition and prepared fresh prior to its analysis.

As a general behavior, it could be highlighted that when subjected to the accelerated degradation conditions, different results were obtained for the 5 mAbs (Fig. 5). Although, all of them showed a similar tendency to elute at earlier retention time, thus indicating acidification as a common pattern of the degradation processes in all of them.

Temperature stress was carried out at 50 °C for 24 h (Fig. 5). Most of the mAbs showed no differences in their chromatographic profile compared to the reference sample (undegraded mAbs) and only CTX showed important change on its profile, with an increase of the acidic peak showed by a decrease in the retention times (Fig. 5, CTX). In the case of BVZ, chromatogram also exhibited a slightly decrease in its retention times (Fig. 5, BVZ).

The force-oxidative conditions promoted asymmetric chromatographic peaks with a drastic decrease in resolution compared with the undegraded samples and also higher elution times in the chromatograms of the 5 mAbs (Fig. 5, all cases). This behavior could be explained by conformational modification in the structure of mAbs that modify the exposition of the ionic residues of the protein [49]. In this case, in which the oxidative medium is relatively strong, the modification affects to important domine of the proteins, resulting in important modification of the charge variant profiles. These results agree with our previous works where reverse phase technique [1,52,53] was used, in which the behavior of the oxidized variants was attributed to the oxidation of the Met residues from the sulfhydryl to the sulfoxide group affecting the polarity of the mAb. This modification is one of the most common degradations known to occur in mAbs during the manufacturing, formulation, and/or storage process [49].

The acidic and basic stresses showed very similar results, except in the case of CTX. In any case, with the acidic stress, a high rate of degradation was observed in the 5 mAbs. An important decrease in the area of the chromatographic peaks was observed in the case of BVZ (80% lost) and RTX (39% lost) (Fig. 5, BVZ and RTX respectively). Total degradation was induced in INF and TTZ samples, with no chromatographic peak detection (Fig. 5, INF and TTZ respectively). This could be attributed to the strong stress conditions used (HCl 1 M) that could have promoted acid total hydrolyzation of the proteins. In the CTX stressed samples, the chromatographic peaks eluted at shorter retention time than the control samples (undegraded sample), indicating an increase in the acidity of the charge variants, which were also revealed with a

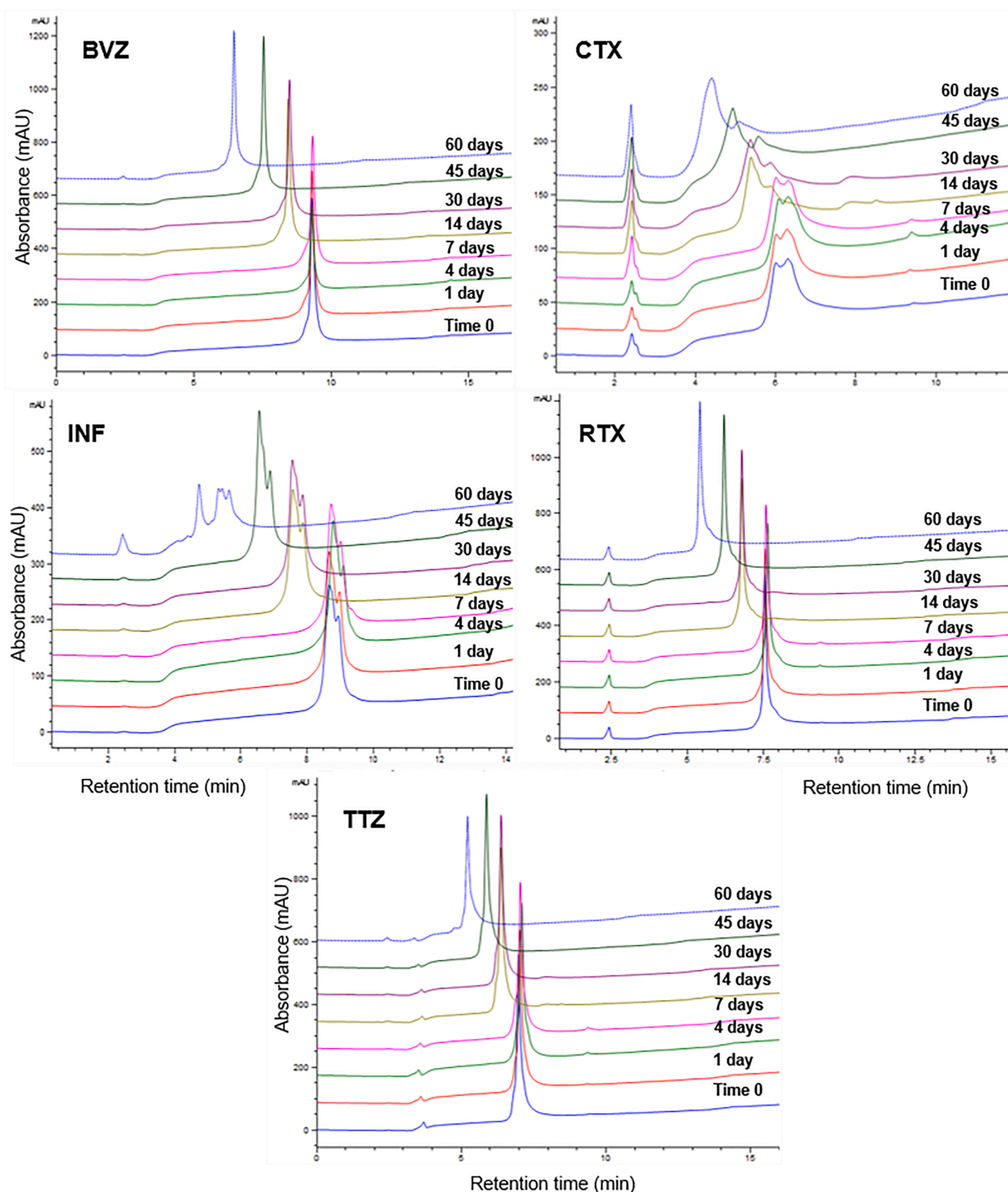

Fig. 6. Long-term stability study (two months) of mAbs clinical samples solutions 2.0 mg/mL in 0.9% NaCl stored refrigerated at 4 °C. Representative chromatographic charge variant profiles from BVZ, CTX, INF, RTX and TTZ.

different pattern profile. The basic stress results were very similar to those obtained in the acid stress, but even more drastic, including a total degradation of the mAbs except in the case of the CTX, that was again the most robust. CTX results were similar to those obtained in the acidic stress, with a decrease in the retention time of the charge variants to a more acidic behavior. Also, the charge variant profile was similar to those obtained for the acidic stress and different from the undegraded samples (Fig. 5, CTX). The total degradation of the others mAbs, was again attributed to the strong stress conditions applied that could have promoted basic protein hydrolyzation.

The results of the ionic strength stress indicated no modification in the charge variant profiles of the mAbs, except for CTX samples, in which a shift of the profile to shorter retention time was observed. Regarding the charge profile pattern, an increase of the acidic peak was observed, very similar to the profiles obtained in acid and basic stress, which demonstrates the sensitivity of CTX to change in aqueous medium

Table 3
Charge variants long-term stability study results: 2.0 mg/mL mAbs clinical solutions stored refrigerated at 4 °C.

Day	BBZ		CTX		IFX		RTX		TTX	
	RT to acid (min)[1]	Degradation Peaks/Mod peaks	RT to acid (min)[1]	Degradation Peaks/Mod peaks	RT to acid (min)[1]	Degradation Peaks/Mod peaks	RT to acid (min)[1]	Degradation Peaks/Mod peaks	RT to acid (min)[1]	Degradation Peaks/Mod peaks
1	*0.0*	No	*0.0*	No	*0.0*	No	*0.0*	No	*0.0*	No
4	*0.1*	No	*0.1*	No	*0.1*	No	*0.1*	No	*0.1*	No
7	*0.0*	No	*0.0*	No	*0.1*	No	*0.0*	No	*0.0*	No
14	*0.9*	No	*0.6*	Yes	*1.1*	No	*0.8*	No	*0.7*	No
21	*0.8*	No	*0.6*	Yes	*1.1*	No	*0.8*	No	*0.6*	No
30	*1.7*	No	*1.1*	Yes	*2.1*	Yes	*1.4*	No	*1.1*	No
60	*2.8*	Yes	*1.6*	Yes	*3.3*	Yes	*2.1*	No	*1.8*	Yes

[1] Shift measured in min referred to the control sample registered at day 0 (day of the sample preparation).

Table 4
CTX and INF: acidic and basic charge variants modification along time.

Day	Cetuximab (CTX)			Infliximab (IFX)		
	Acid forms (%)	Basic forms (%)	Acidic Peak Retention time	Acid forms (%)	Basic forms (%)	Acidic Peak Retention time
0	27.7	72.3	6.0	66.1	33.9	8.6
1	27.7	72.3	6.0	66.2	33.8	8.6
4	31.6	68.4	6.0	69.5	30.5	8.7
7	36.7	63.3	6.0	66.7	33.3	8.7
14	60.5	39.5	5.3	61.3	38.7	7.5
21	56.4	43.6	5.3	62.7	37.3	7.5
30	66.0	34.0	4.9	66.5	33.5	6.5
60	75.1	24.9	4.3	66.1	33.9	5.3

(Fig. 5, CTX). Nevertheless, all mAbs except CTX seem to be robust against degradation promoted by the increase of the ionic strength of the aqueous medium.

Light exposure is considered a critical condition in therapeutic proteins that affect directly to the mAbs quality during all product steps, including storage and patient's administration [51]. It is well known that light promote aggregation in therapeutic proteins [39,54] likely related with the oxidation of the aromatic amino acids (Trp, Tyr and Phe) [51]. Regarding charge variants, the resulting chromatograms here showed that light promoted different degradation behavior. INF and TTZ results indicated no modification respect the controls samples since no changes were observed neither in the area nor in the shape of the chromatograms (Fig. 5, INF and TTZ respectively); on the contrary, BVZ and RTX did suffer a significant decrease in the area of the main peaks (Fig. 5, BVZ and RTX respectively). CTX degradation pattern was very similar to the other stress conditions applied except the oxidation stress, therefore indicating a main path-way of degradation which two more acidic charge variants (Fig. 5, CTX).

The results of these stress study, in addition to show patter of degradation of the 5 mAbs, validated the method for detecting charge variants modifications. Gathering all the results, the main degradation pattern indicated the increase of the acidic forms since there was detected a shift to shorter retention times of the chromatographic profiles to a more acidic pH with respect to the control samples (fresh mAbs). The decrease of the area under the main chromatographic peaks, the detection of new chromatographic peaks at different retention times or changes in the shape of the profiles were degradation patterns shared by the 5 mAbs with more or less extension (Table 2). Nevertheless, these modifications were specific for each mAb and also for each stress applied, in the way that the profile could be useful to identify a particular mAbs and also their environmental conditions. The degradation conditions here tested also have demonstrated to promote change of concern by the modification of the chromatographic size exclusion profile, detecting no-natural aggregation increased among others [55,54].

3.3. Two-months stability charge variants chromatographic profile study of mAbs clinical solutions

Once the (WCX)HPLC-DAD method was validated to detect modifications on the charge variants profile in these 5 marketed mAbs, we checked its suitability for tracking changes in this critical quality attribute when stored (refrigerated at 4 °C and frozen at −20 °C) in-use samples in hospital. As in the previous stress study, we analyzed mAbs clinical solutions at 2.0 mg/mL, the lowest concentration used when administered in hospital.

The results of this two-months stability study from the refrigerated stored samples are showed in Fig. 6 and Table 3. The first evident result was the common chromatographic behavior of the 5 mAbs, i.e. the decrease on the retention time in all the profiles which was detected from the 7th day of the storage at 4 °C until the end of the study (60 days) in all the tested mAbs, (Fig. 6 and Table 3). This change could be attributed to a progressive loos of ion exchange capacity of the stationary phase functional groups (carboxylic acids), due to a decrease of specific surface area which leads in an earlier elution of the mAb isoforms and therefore a decrease in the retention time [56]. This behavior could be produced by an incomplete regeneration of the column charges, which resulted in a decrease in the number of positive charges and, consequently, in a lower chromatographic retention capacity [57]. Despite this, this phenomenon is very common in this kind of WCX columns and had no impact on the chromatographic performance, that is, on the selectivity and resolution of the isoforms, since only the retention time was affected.

Regarding the individual mAbs, it was observed for BVZ that the retention time was unchanged up to the 7th day of the study, eluting around 9.2 min; however, from 14th day, the retention time progressively decreased until the end of the study, eluting at 6.4 min after 2 months of refrigerated storage.. No changes in the profile shape were observed in any of the recorded chromatograms (Fig. 6, BVZ).

In CTX samples, the most evident modification was the change in the proportion of the charged variants profile from day 7 until the end of the study (Table 4). At the beginning of the study, the charged profile showed two peaks, one main basic peak eluting at 6.2 min with a % area of 72.3%, and other at retention times corresponding to a more acidic compound, eluting at 6.0 min representing the 27.7% of the chromatographic area. These percentages and retention times remained almost unaltered until the 14th day of the study. A noticeable change in the proportion of the acidic/basic charge variants started at the 14th day, increasing the acidic form even to show higher percentage (60.5%) than the basic form (39.5%) arising to 75.1% the last checked day (see Table 4) probably related with deamidation of Asn and Gln residues, which leads to more mAbs acidic forms [58,59]. In addition, it was observed an advance of 1.6 min in the retention time of the acid variant regarding the fresh sample checked the day of preparation (Time 0) (Fig. 6, CTX).

In INF samples, unaltered profiles were observed up to 7th day of the study. At the beginning of the study (Time 0), the INF showed a basic

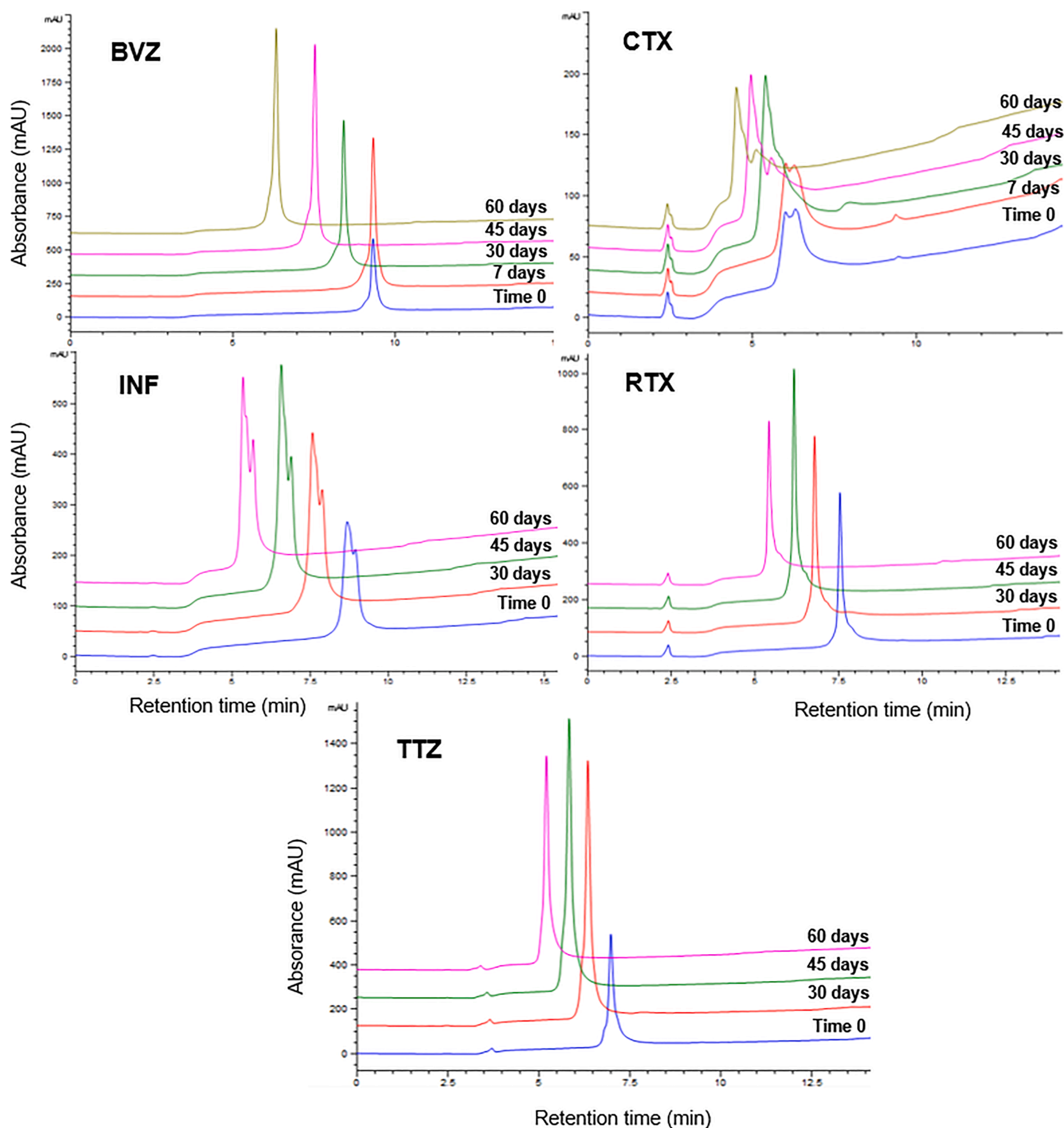

Fig. 7. Long-term stability study (two months) of mAbs clinical samples solutions 2.0 mg/mL in 0.9% NaCl stored frozen at −20 °C. Representative chromatographic charge variant profiles from BVZ, CTX, INF, RTX and TTZ.

peak eluting at 8.9 min with 33.9% of the area and a main acidic peak eluting at 8.6 min with 66.1% of the area (Table 4). Some slight modifications were observed in these percentages until the important modification in the shape of the profile from the 45th day and showing a different charge variant profile at the end of the study (Fig. 6, INF). In terms of retention time, a progressive forward shift of the chromatogram was observed up to the end of the study, with a forward shift of 3.3 min regarding the initial value, i.e first peak detected at 5.3 min (Time 60) instead of at 8.6 min (Time 0).

RTX charge variant chromatographic profile were invariant up to the 7th day of the study; from this checked day the forward shift occurred. Although the observed proportion of the main peak and its basic charge variants was kept practically invariant in terms of percentage until the end of the study, with no charge variant modifications throughout the study (Fig. 6, RTX, 60 days). Results regarding the retention time were similar to the previous mAbs, i.e. the retention time of the main variant was unaltered up to the 14th day (around 7.4 min), where was already observed a slight decrease of 0.8 min(Fig. 6, RTX).

Finally, TTZ results showed an invariant shape of the charge variant profile with a forward shift in the elution detected from the 14th checked day (Fig. 6, TTZ). This profile was characterized by just one peak at around 7.0 min, which eluted progressively to shorter retention time from the 14th days to the end of the study (5.2 min). Also, the last checked day was observed an important degradation of TTZ evidence from acharge variants modification, with the detection of new charge variants (Fig. 6, TTZ, 60 days).

Therefore, based on these results, it can be proposed that BVZ, RTZ and TTZ samples were the most stable mAbs along time regarding the charge variant profile modifications, with CTX and INF the most unstable when stored refrigerated at 4 °C (Table 3).

Regarding the frozen stored samples, results were close to the refrigerated samples (Fig. 7), with BVZ, RTZ and TTZ suffering no modification in the shape of the profile but just the forward shift to shorter retention times. Only for BVZ and CTX, samples were checked at the first week of the frozen storage, no detecting modification, neither in the shape nor in the elution time. For all the five mAbs, after one month of storage, the forward shift of the profile to shorter retention times was detected which was progressive to the end of the study (two months). Similarly to the refrigerated samples, CTX and INF suffered modifications in their charge variants as to promote changes in their chromatographic shape of the profiles (Fig. 7). These results indicated that the frozen storage at −20 °C of these mAbs samples is not adequate to prevent modification on mAbs structure.

4. Conclusions

A generic analytical weak cation exchange chromatographic method which allows for the detection and characterization of the charge variants profile of BVZ, CTX, INF, RTX and TTZ is here described. This method uses a salt-mediated pH gradient mode and classical non-volatile, high ionic strength buffers which were compatible with ultraviolet detection. The method enables the recording of particular and very specific charge variant chromatographic profiles for each studied mAb, which demonstrated to be very useful to further track modifications, both in the stress studied conducted and in the stability study along two months of diluted clinical solutions of the 5 mAbs here studied. Also, the characteristic of each profile could be useful for identification purposes. The stress study conducted, in addition to provide information about stability of the mAbs solutions against environmental factor degradation, was used to validate the method for the detection modification in the charge variant profiles. It has been proved that these modifications were very specific for each mAb and for each stress applied, in the way that the profile could be useful for detecting environmental conditions to which the mAbs would have been submitted to.

This method is simple and offers a simple strategy for 5 mAbs characterization on the natural intact level without any need of sample preparation. No changes in the chromatographic parameters neither in the mobile phases had to be applied to enable the characterization of the charge variant profiles of these 5 commercial mAbs widely used worldwide. This feature makes this method very useful and applicable to be used in those situations in which fast and easy analyses are required, as for example in hospital control laboratories, when a quick response is required.

Regarding the results obtained on the particular mAbs, they indicated different stability both against degradation and in the two-months stability study. The modifications on the profiles when submitted to stress were specific for each mAb and for each stress applied, in that way that the profile could be useful to identify the particular environmental conditions a mAbs has been submitted to. The charge variants chromatographic profiles observed during the stability study along time indicated that BVZ, RTX and TTZ 2.0 mg/mL clinical samples solution were most stable than CTX and INF in the two storage conditions checked. To highlight that, all of them were very stable during the first 7th days when stored refrigerated at 4 °C, also for those samples checked after one week of frozen storage, i.e. BVZ and CTX, with no modification on the charge variants observed, therefore indicating stability regarding this critical quality attribute. Therefore, frozen storage at −20 °C is not enough to prevent modification in these mAbs solutions. Furthermore, a progressive decrease on the retention time of the profiles was detected in all the samples from the second week of refrigerated storage and from the first months of frozen storage, this fact could be attributed to a column efficiency decrease with no impact in the charge variant profile of the proteins.

CRediT authorship contribution statement

Antonio Martínez-Ortega: Investigation, Formal analysis. **Agustín Herrera:** Investigation. **Antonio Salmerón-García:** Conceptualization, Funding acquisition, Project administration, Methodology. **José Cabeza:** Conceptualization, Funding acquisition, Project administration, Methodology. **Raquel Perez-Robles:** Formal analysis. **Natalia Navas:** Conceptualization, Funding acquisition, Project administration, Supervision, Methodology, Resources.

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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