Validated reverse phase HPLC diode array method for the quantification of intact bevacizumab, infliximab and trastuzumab for long-term stability study

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1. Introduction

Therapeutic monoclonal antibodies (mAbs) represent nowadays a very important class of biotechnological drugs characterized by remarkable specificity and affinity in target recognition. These properties, combined with their relatively long half-lives, have contributed to their success in the treatment of significant diseases such as cancer and immunological illness. Nowadays they are of great and growing interest for the biopharmaceutical industry [1–3] representing the major class of biopharmaceutical products worldwide [4].

The most used immunoglobulin for pharmaceutical purposes is the subclass 1, i.e. IgG1 (Fig. 1) due mainly to its relatively long half-lives compared to the others IgG subclass [5,6]. The marketed mAbs bevacizumab (BVZ), infliximab (INF) and trastuzumab (TTZ) are representatives of this subclass, therefore they are glycoprotein with high molecular weight (approximately 150,000 Da). BVZ (Avastin®) is indicated for the treatment of several kind of cancer (metastatic carcinoma of the colon or rectum, breast cancer, lung cancer, etc) [7]. INF (Remicade®) is used for the treatment of psoriasis, Crohn’s disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis and ulcerative colitis [8]. TTZ (Herceptin®) is indicated for the treatment of patients with metastatic breast cancer whose tumors over express HER2 (25% of the patients) [9]. As IgG1, the three mAbs share the same structure varying mainly in the variable region where is located the complementary determining region (CDR) through which are specifically bonded to their antigens (Fig. 1).

Because of the complex nature of therapeutic mAbs, a variety of orthogonal analytical methods must be used to achieve appropriate characterization. In addition, as they are for therapeutic use, each analytical method applied to characterize mAbs must fulfill as much as possible the requirements of the international drug regulatory organizations. The International Conference on Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use gathers the recommendations to ensure the quality of the biotechnological products in Q5A-Q5E guidelines [10]; particular specification upon the test procedures and acceptance criteria for biotechnological/biological products are established in Q6B guideline [11]. This last document indicates that
the validation of analytical procedures used in the characterization of biotechnological products should be made in compliance with the ICH Q2(R1) guideline, relating to the validation of analytical procedures [12]. In any case and to support research data, validated methods for their intended purposes should be always required [13,14].

Although size exclusion (SEC) and ion exchange (IEC) chromatography are well-established techniques for the study of intact protein, the reverse phase (RP) chromatographic stationary phases developed with large pore size 300 Å or fused core particles with short alkyl chain make this mode of liquid chromatography a very effective technique for the analysis of intact proteins [15,16] by conventional HPLC systems. In these cases, high column temperature (60–75 °C) and a combination of non-traditional solvent systems with ion pairing agents are needed [17,18]. Also monoliths silica standard- and narrow-bore columns provide high reproducibility and efficiency in LC/MS analysis of biomolecules [19,20]. Nevertheless, SEC is the standard method for the analysis of mAb aggregates [21,22] while IEC is used to profile the charge heterogeneity [23]. Both modes of chromatography can therefore be used to track changes in mAbs and could be validated for these purposes [24]. On the other hand, quantification of mAbs has been achieved traditionally by ELISA [25,26]. However the lower level of precision (about 10% of relative standard deviation in repeatability) and of robustness of the immunoassays compared with physicochemical-based techniques led ICH to recommend the latter for quantification purposes [11]. It is evident that accurate determination of protein concentration is nowadays essential in many studies involving characterization, functional assays, and during quantitative assessments such as for example protein–protein and protein–ligand interaction process [27].

Mass spectrometry (MS) is an excellent analytical tool for studying the properties and behavior of proteins such as mAbs [28,29]. MS-based methods are particularly useful for studying structural aspects such as primary sequence characterization [30], modifications (e.g. post-translational changes [31], C-terminal modification [32], degradation patterns (e.g. deamidation, [33], tryptophan oxidation [34]) etc. Primary structure assessment and extensive glycol-profiling of mAbs by a combination of intact, middle-up middle-down and bottom-up ESI and MALDI mass spectrometric techniques was demonstrated for the marketed mAb cetuximab (CTX) [35]. Nevertheless, in most of these studies, a previous step is required involving either enzymatic digestion or the fragmentation of the mAbs into their light and heavy chains. This is because mAbs are very large proteins (150 kDa molecular weight) and the quality of the results depends on the resolution and mass accuracy of the mass spectrometers. However the continuous advances in tandem mass spectrometric instrumentation (tQ, quadrupole-time of flight (qTOF), high resolution Orbitrap, and Fourier transform-ion cyclotron resonance (FT-ICR) MS equipment) have made this a powerful technique for the structural analysis of intact mAbs [36,37]. Nevertheless these are all complex, highly expensive strategies which are not always available for quality control or investigation purposes.

Two previous published works have demonstrated that two marketed mAbs, rituximab (RTX) and CTX, could be successfully quantified using an (RP)HPLC/DAD method that could be validated as stability-indicating in accordance with the ICH guidelines [38,39]. These two methods are therefore useful in the context of the quality control of the final products. In the present work we studied the quantification of BVZ, INF and TTZ by (RP)HPLC/DAD. With that aim, the method previously developed has been checked and validated here for the quantification of BVZ, INF and TTZ in compliance with the ICH guidelines for biotechnological products, including a wider stress study to check the feasibility of using it in the presence of modified or degraded products. It is proposed the use of spectral peak purity analysis which proved to be a simple way of detecting modifications in the intact mAbs. Therefore, in terms of analytical quality assurance, the method has been validated for quantification purposes and qualified for the detection of modification/degradation of the mAbs. We also present a comparative study of the method for each mAb, which shows that there were no significant differences between them in terms of the Figs. of merit of the method or in their chromatographic behavior. Even when subjected to particular accelerate degradation conditions, they do not exhibit different degradation patterns that could be tracked by RP chromatography. All the experiments were conducted with fresh mAb medicine samples to ensure full molecular and structural integrity of the mAbs during the study.
2. Experimental

2.1. Standards, substances and solvents

Avastin® (RochePharma AG, Grenzach-Wyhlen, Germany) was used as a representative BVZ reference material. Remicade® (Janssen Biotech, Inc. Horsham, Pennsylvania, USA) was used as a representative INF reference material. Herceptin® (Roche Pharma AG, Grenzach-Wyhlen, Germany) was used as a representative TTZ reference material. These were kindly supplied for this study by the Pharmacy Unit of the University Hospital “San Cecilio” (Granada, Spain) out of their daily surplus. All the standard solutions of the mAbs were prepared from these medicines. This was an essential preliminary step to enable us to conduct the study because it was impossible to obtain a proper standard of the mAbs. The medicines indicate a quantitative composition of 25 mg/mL of BVZ [7], 100 mg/vial of INF [8] and 150 mg/vial of TTZ [9]. 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 aqueous solution. Our aim here was to avoid any kind of change or degradation in the mAbs, given that the stability indicated by the manufacturer once the vials have been opened is up to 48 h for BVZ [7], 24 h for INF [8] and 48 h for TTZ [9].

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. Methanol, hydrochloric acid, sodium hydroxide, propan-2-ol and sodium chloride were supplied by Panreac (Barcelona, Spain). Hydrogen peroxide was supplied by Technical Fontenay-sous-Bois, France). Trifluoroacetic acid (TFA) was from Merck KGaA (Darmstadt, Germany) and acetonitrile from Poch S.A. (Gliwice, Poland). An isotonic solution of 0.9% NaCl was supplied by B. Braun Medical (Madrid, Spain).

2.2. Chromatographic instrumentation and method

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).

(RP)Chromatographic analyses were carried out in a 150 mm × 2.1 mm i.d., 5 μm particle size, wide pore 300 Å C8 analytical column (300SB-C8 Zorbax, Agilent Technologies, USA). A 2.1 mm × 12.5 mm i.d., 5 μm particle size, C8 guard column (SBC8 Zorbax, Agilent Technologies, USA) was also used. The eluent flow rate was 1.0 mL/min and consisted of a gradient of 0.1% TFA in Milli-Q water (eluent A) and a mixture of isopropanol, acetonitrile, Milli-Q water and TFA (70/20/9.9/0.1, v/v/v/v) (eluent B). The gradient started at 10% of eluent B for 1 min. It was then increased to 20% of B in 0.1 min and kept constant for 3 min; then eluent B was raised again to 30% B in 0.1 min, and a linear gradient was applied from 4.1 to 10 min; from 10 to 10.1 min eluent B was increased to 80% and this percentage was maintained until 12 min. A post time of 3 min was required at the initial conditions of the gradient, i.e. 10% of B eluent. The temperature of the column was maintained at 70 °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 using 360 ± 20 nm as the reference wavelength.

2.3. Software

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

2.4. Validation procedure

The validation procedure was performed according to the guidelines issued by the International Conference Guideline [11,12] and, when needed, criteria from the Food and Drug Administration (FDA) were also followed.

2.4.1. Linearity, LOD, and LOQ

Linearity was evaluated up to 10.0 mg/mL. To this end, appropriate individual mAbs standard solutions of 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/mL were prepared in quadruplicate and injected into the chromatograph. Linear calibration functions were fitted by least-squares regression, and linearity was accepted if the statistical probability level of the corresponding lack-of-fit test (PLOF-value) was >5% according to the Analytical Methods Committee [40].

The detection limit (LOD) and quantification limit (LOQ) for each mAb were estimated from the standard deviation of the lowest concentration tested (SDlow-conc) which is estimated as it is describe in the section bellow. LOD and LOQ were calculated as $3 \cdot (SD_{low-conc})$ and $10 \cdot (SD_{low-conc})$ respectively.

2.4.2. Precision

The precision of the method for each mAb was determined as intra-day precision (repeatability) and intermediate precision and given as relative standard deviation (RSD %). The former was estimated from the results of the analysis of the corresponding mAb standard solution prepared at the same concentration on the same day. Thirty samples with high concentration levels (10 samples), medium concentration levels (10 samples) and low concentration levels (10 samples) were used for this purpose and for each mAb. The intermediate precision was estimated from the analysis of standard solutions at three concentration levels (high, medium and low) over five consecutive days. Two samples of each concentration were prepared and analyzed daily.

Because ICH Q2(R1) [12] does not establish any precision criteria for acceptance of the method, the U.S. Food and Drug Administration (FDA) criteria for HPLC methods for pharmaceutical analysis were followed [41], i.e. an intra-day precision ≤2%.

2.4.3. Accuracy (trueness)

The accuracy of the method for each mAb was assessed from the average recovery value calculated on ten standard solution replicates at three concentration levels representing the top end, middle value and bottom end of the calibration, in order to cover the stated linear range. Again, ICH Q2 (R1) [12] does not indicate accuracy criteria for acceptance; we therefore decided to follow the FDA accuracy criteria for HPLC methods for pharmaceutical analysis [41], i.e. an average recovery within 100 ± 2%.

2.4.4. Specificity by forced degraded studies

Forced degradation studies were performed using different mAb concentrations in NaCl 0.9%. For BVZ and INF, specificity was studied at 0.5 mg/mL, 2.0 mg/mL and 10 mg/mL. For TTZ, it was studied at 0.5 mg/mL and 2.5 mg/mL. All these concentrations are representative of the final product when used in typical hospital conditions.

We recorded the chromatograms of sample solutions submitted to degradation for two reasons: firstly to evaluate the specificity of the method, and secondly to detect degradation when the mAb is handled and administered in hospital conditions. We therefore studied the stress conditions of high temperature, high ionic strength, UV light exposure, addition of an acid and a base, and addition of an oxidant. The chromatograms of the stressed samples were compared with those for the corresponding mAb standard solution that had been freshly prepared and had not undergone degradation treatment. All samples were analyzed in triplicate.

The spectral peak purity was checked using the ChemStation software tools [42]. This assessment was based on the comparison of all
the spectra recorded during the elution of the peak, one every 0.2 min, and on the calculation of a similarity function based on the determination coefficient (R², in percent). All the spectra in the chromatographic peak were averaged to obtain a pooled spectrum. This was compared with all the spectra in the peak to obtain the similarity function. The similarity factor was set to a value ±99% for acceptance of the spectral peak purity.

In the studies of the effects of acid and alkaline media, ionic strength and oxidation medium, two studies were conducted applying weak and strong conditions. HCl 1 M, NaOH 0.1 M, NaCl 1.5 M and H₂O₂ 1% were used to degraded mAbs samples. For the weak condition, the stressed agent concentration was 3.3% (v/v) (50 μL of the stressed agent was added to 1.5 ml of mAb standard solution), while for the strong condition the stressed agent concentration was 33.3% (v/v) (500 μL of the stressed 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 sample in an oven (Heraeus S.A., Madrid, Spain) at a temperature of 50 °C for 24 h.

The effect of the light was investigated following the guidelines issued by the International Conference on Harmonization (ICH Q1B) for photostability testing [43]. To this aim, the mAb samples were placed in an accelerated stress test chamber to simulate sunlight (Solarbox 3000e RH, Cofomegra, Milan, Italy). The samples were light irradiated with a xenon lamp (according to ICH Q1B) using an S208/S408 UV filter made of soda lime glass to simulate indoor exposure conditions with infrared rejection coating to reduce the temperature of the samples. The temperature was controlled and maintained at 24 °C. Irradiance level was set at 250 W/m², between 320 and 800 nm.

2.4.5. Robustness

Robustness of the method for each mAb was evaluated by small variations in those chromatographic conditions affecting more the results, such as temperature of the column, mobile phase composition and flow rate. Several aliquots of each mAb solution of 5.0 mg/mL were analyzed at each modified condition to assess any impact on assay results. The robustness of the method was then estimated by means of the retention time, symmetry factor (S), capacity factor (k′), and the number of theoretical plates (N).

2.4.6. System suitability

For system suitability testing, we used mAb samples of 1.0, 2.5 and 5.0 mg/mL prepared in NaCl 0.9%. ICH Q2(R1) [12] does not indicate system suitability criteria for acceptance either; we therefore decided to follow the FDA criteria for HPLC methods of pharmaceutical analysis [41], although we used the symmetry factor instead of the asymmetry ratio of a peak. The selected system suitability parameters were therefore N (acceptance criterion N > 2000), k′ (acceptance criterion k′ > 2.0), S (acceptance criterion S ≤ 2), and injection repeatability (acceptance criterion RSD ≤ 1%).

2.5. Long-term quantification study of BVZ, INF and TTZ in pharmaceutical preparation samples (hospital administration conditions)

Diluted samples of BVZ (0.5 mg/mL, 2.0 mg/mL and 10 mg/mL), INF (0.5 mg/mL, 2.0 mg/mL and 10 mg/mL) and TTZ (0.5 mg/mL and 2.5 mg/mL) in 0.9% NaCl were prepared from their medicines Avastin®, Remicade® and Herceptin® respectively. The specification included in their scientific technical reports were followed for the preparation of all these solutions [7,8,9]. These samples were 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, 3, 7, 14 and 31 days after preparation. The frozen samples were analyzed after a month and considering only un cicle frozen/thawed. In the long-term quantification study, the concentrations of BVZ, INF and TTZ were expressed as percentage of the initial mAb concentration remaining at each checked time, where the initial concentration was that on the day when the samples were prepared (Day 0). In all cases, the reported concentration is the average of three replicates.

3. Results and discussion

3.1. (RP) HPLC/DAD method performance for the quantification of BVZ, INF and TTZ

The chromatographic conditions optimized in the previous work for the quantification of the two previous marketed mAbs [38,39] were here checked for the quantification of BVZ, INF and TTZ. The chromatographic analysis was based on the use of a C8 stationary phase and a combination of isopropanol (i-PrOH), acetonitrile (ACN), water and trifluoroacetic acid (TFA) as mobile phase. This mobile phase composed by stronger eluotropic strength solvents is required for the analysis of antibodies to reduce column interactions [21].

Again, it was corroborated that a temperature of 70 °C was mandatory to perform the analysis, being this parameter critical for obtaining suitable chromatographic peaks as afterward highlighted in the robustness study. Another similar results in the chromatographic analysis of all the mAbs studies up to now was the chromatographic profile, with mAbs retention time at around 7.5 min well separated from peaks of the solvent system that were detected at around 4 min and 7 min. Fig. 2 shows the chromatograms registered at 214 nm using 360 ± 20 nm as the reference wavelength for standard samples of 5000 mg/L of BVZ, INF and TTZ. The UV absorption spectra recorded at the peaks are also shown together with the graphic results for the peak purity analysis in this Fig. 2. As can be seen, there were no appreciable differences in the RP-chromatographic profile, with very similar retention times for the three mAbs, and also very similar to those previously obtained for RTX [38] and for Ctx [39]. These results indicated again that the chemical differences among all these therapeutic marketed mAbs are not sufficient to cause differences in their polarities that would enable separation in chromatographic column in reverse phase method using classical stationary phases despite their wider pores (300 Å) and long alkyl chains (C8 and C18).

This last is corroborated by the characteristic retention time established for each mAb. It was estimated from 40 chromatograms obtained from standard samples with different concentrations and registered on different days; this was in order to cover all possible sources of variation in the measurement. The average retention time estimated in this way was 7.5 ± 0.2 min for BVZ, 7.6 ± 0.2 min for INF and 7.5 ± 0.3 for TTZ (confidence level of 99.5%).

In addition, the UV absorption spectra of the three mAbs are similar both in shape and in sensitivity (and similar to the UV absorption spectra of RTX [38] and Ctx [39]).

Therefore, the therapeutic mAbs studied (IgG1 all of them) are indistinguishable by (RP)HPLC/DAD. Nevertheless, using these chromatographic conditions, an ICH validated method for quantification purposes can be proposed for each of them as shown next.

3.2. (RP) HPLC/DAD method validation for the quantification of BVZ, INF and TTZ

Once the method performance was assessed for each mAb, validation in compliance with the ICH guidelines was carried out. As already indicated, we followed the ICH Q6B [11] guidelines, which indicate that the quantity of a biotechnological product, measured as protein content, should be determined using an appropriate assay, usually physiochemical in nature. The analytical procedures developed for such purposes should be validated in compliance with the ICH Q2(R1) [12]. We therefore validated the (RP)HPLC/DAD whole analytical method for the determination of BVZ, INF and TTZ in terms of linearity,
limits of detection and quantification, precision, accuracy, specificity, robustness and system suitability.

3.2.1. Linearity

Linearity was studied bearing in mind further application of the method, i.e., BVZ, INF and TTZ in diluted solutions, those used in hospital. The interval of the concentration to be tested was also adjusted to avoid further dilution of these diluted solutions of the mAb, in order to inject them into the chromatogram equipment without previous manipulation that could bring about slight changes in their composition. In brief, the clinical target concentrations were 1.0 mg/mL, 5.0 mg/mL and 10.0 mg/mL, therefore linearity was studied up to 10.0 mg/mL. These represent high concentration values, so the volume we injected was as small as possible, i.e. 1 μL. Nine concentration levels with four independent replicates for each one were used to determine the calibration function, as described above in the Experimental section. Nevertheless linearity was established to 5000 mg/L because above this value a deviation of the linearity of the response of the detector was observed by studying the residual; therefore linearity was assumed only up to 5000 mg/mL. Once the LOD and the LOQ were calculated, the calibration working range was established, as can be seen in Table 1 with LOQ being the lowest concentration in this range. The results for the intercept (a), slope (b) and determination coefficient ($R^2$), used to check the linearity are summarized in Table 1. The $R^2$ value we obtained was evidence of the acceptable fit of the data to the estimated regression line and it could therefore be concluded that the linearity of the response of the detector in the selected conditions for determining BVZ, INF and TTZ was successfully demonstrated up to 5000 mg/L. In addition, the intercept was not significant since the corresponding P-values were >5% (see Table 1).

Both facts, the null intercept and the linearity of the calibration curve, are crucial for selecting a proper quantification strategy in routine analysis. In these conditions, the response factor (RF), defined as the ratio between the measured absorbance of a BVZ-, INF- and TTZ-based medicine sample and its concentrations could be considered to remain constant throughout the working range. Then, it can be proposed that one representative standard sample of each be analyzed with each

![Fig. 2. Standard solutions of 5000 mg/L of (a) BVZ, (b) INF and (c) TTZ analyzed by (RP)HPLC/DAD optimized conditions. UV absorption spectra recorded at the chromatographic peak and the graphic results for the spectral peak purity analysis using 99.5% as similarity factor for BVZ, INF and TTZ.](image)

### Table 1

<table>
<thead>
<tr>
<th>Feature</th>
<th>Bevacizumab</th>
<th>Infliximab</th>
<th>Trastuzumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (a)</td>
<td>−92.6</td>
<td>−95.7</td>
<td>−97.4</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>1.16</td>
<td>1.20</td>
<td>1.29</td>
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<tr>
<td>$s(a)^a$</td>
<td>47.00</td>
<td>48.37</td>
<td>46.58</td>
</tr>
<tr>
<td>P-value (%), for a = 0$^b$</td>
<td>0.06</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>$s(b)^c$</td>
<td>0.018</td>
<td>0.018</td>
<td>0.018</td>
</tr>
<tr>
<td>$R^2d$</td>
<td>0.9977</td>
<td>0.9978</td>
<td>0.9982</td>
</tr>
<tr>
<td>P$_{corr}$-value [%]$e$</td>
<td>0.028</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>Linear range (mg/L)</td>
<td>45–5000</td>
<td>88–5000</td>
<td>21–5000</td>
</tr>
<tr>
<td>Limit of detection (LOD) (mg/L)$f$</td>
<td>13</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>Limit of quantification (LOQ) (mg/L)$f$</td>
<td>45</td>
<td>88</td>
<td>21</td>
</tr>
</tbody>
</table>

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*a* Standard deviation of the intercept.  
*b* Statistical probability of intercept test (a = 0).  
*c* Standard deviation of the slope.  
*d* Determination coefficient.  
*e* Statistical probability of lack-of-fit test.  
*f* Calculated from the standard deviation of the mAb concentration, estimated at low concentration ($n = 10$).
analytical batch and quantification be carried out by applying the estimated RF value of the standard [44].

3.2.2. Limit of detection (LOD) and limit of quantitation (LOQ)

Although the LOD and LOQ are not critical in this study since the target concentrations of the mAbs are very high, they were estimated using the standard deviation of the lowest concentration tested. The values obtained are shown in Table 1. Using this criterion, the lowest LOD and LOQ were obtained for TTZ, followed for BVZ and the highest were for INF.

3.2.3. Accuracy and precision

The accuracy and precision of the analytical method for the therapeutic mAbs were established across their linear range as stated in the ICH Q2(R1) guidelines [12]. As shown in Table 2, satisfactory results were obtained for the accuracy and for both the intraday and interday precision of the method, expressed as recovery and RSD values, respectively. These results were very similar for the three mAbs (see Table 2). The intraday and interday RSD was ≤2% for all concentrations tested, with the highest values for the target concentration of 1.0 mg/mL (the lowest concentration assayed). In all cases the recovery values were close to 100% of the checked concentrations, so fulfilling the acceptance criterion for recovery by falling within the range 100 ± 3%.

3.2.4. Specificity by means of forced degraded studies (stress studies)

Forced degradation studies were performed, following the ICH Q2 (R1) guidelines [12], on Avastin®, Remicade® and Herceptin® to evaluate the specificity of the method for BVZ, INF and TTZ respectively. This stress study was also carried out to gather information about the degradation of these mAbs under hospital conditions, as a means of evaluating the robustness of the mAbs formulation against external factors. 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. Of the various stress factors that can be studied, we decided to focus on those that may affect 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 [45]. We therefore carried out the forced degradation studies described below.

In all the experiment, the assessing of the mAb degradation was performed by spectral peak purity analysis in addition to the detection of mAb peak deformation and/or new chromatographic peaks in the chromatograms. By comparing the absorption spectra from the chromatographic peak corresponding to the fresh mAb samples and the stressed samples, we were able to detect degradation/modification in the mAb. This strategy therefore offers an attractive alternative to using a mass spectrometric detector for peak purity, due to its cost-benefits and simplicity in routine quality control. On the other hand, these numerical methods for calculating similarity based on the correlation coefficient are common procedures to assess the degree of similarity between protein absorption spectra [46]. In the particular case of the mAbs, their inherent heterogeneities [47] promote that the recorded UV spectra in the chromatographic peak of a fresh standard sample were not identical (Fig. 2), with the main dissimilarity observed approximately between 220 and 235 nm. As a result of these slight spectral variations, the peak purity analysis indicated 99.5% for the similarity factor, and it was used as the reference value for accepting peak purity in the degradation studies [42]. This value was obtained from the analysis of the chromatograms of their fresh standard samples used to establish the calibration functions of each mAbs.

When subjected to the accelerated degradation conditions the three mAbs again exhibited similar behavior, and similar results to those obtained for RTX [38] and CTX [39]. Fig. 3 shows the results of the stress study, the only difference being when the samples were heated, when BVZ showed different degradation patterns.

Thermal stress was evaluated at temperatures of 30 °C and 50 °C (considered the maximum value for accidental exposure to high temperatures in hospital). When medicine samples were subjected to these temperatures for 24 h, the RP-chromatographic profiles of the three mAbs were similar to those for the reference samples. No unknown chromatographic peaks were found and the recovery values were close to 100%. As an example of these results, Fig. 3 shows the chromatographic results for TTZ submitted to 50 °C. In addition, the similarity factor indicated a high degree of spectral peak purity (99.5%) in all the analysis. It is described in the bibliography [47] that antibodies could undergo conformational changes such as unfolding or partial unfolding at these temperatures. These changes seem did not occur for these three marketed mAbs here studied.

For the rest of the stress conditions studied, the results were also similar for the three mAbs. When the medicine samples were subjected to weak stress conditions, the loss of concentration was <10% for all the samples analyzed, the spectral peak purity remained at the reference value of 99.5%, modifications in the shape of the chromatograms and new peaks were not detected in the chromatograms, except in the case of the samples subjected to oxidative conditions. In this last case, a peak appeared in the chromatograms at 1.7 min (as example, see Fig. 3, INF strong oxidative stress). It is well known that the amino acid methionine (Met) is highly susceptible to oxidation. As described in the previous paper for the oxidation of RTX, this chromatographic peak could be attributed to the oxidation of the Met residues that yielded a sulfoxide group that makes the side chain of the Met more polar. The fact that these peaks eluted earlier could be attributed to the degraded mAbs containing oxidized Met. The oxidation of Met has been reported for human IgG1 (like the mAbs studied here) when incubated with tert-butyl hydroperoxide and exposed to intense light or high temperature [48,49].

As expected, when the stress conditions were intensified, the modification/degradation of the mAbs increased (loss between 29% and 60%). The degradation patterns detected by RP chromatography were again similar for the three mAbs, and they had almost exactly the same chromatographic shape. When medicine samples were subjected to basic stress the chromatographic peaks of the mAbs were deformed in similar ways, and a new peak at shorter retention times (1.7 min) appeared only when the samples were subjected to oxidation, as explained above (see Fig. 3). Although the spectral peak purity factor remained at the same levels as for the fresh sample in most of the stress conditions (99.5%), we can assume that modifications have taken place in the mAbs because of the important decrease in the area under the peak. One explanation could be that the modifications affect all the isoforms of the mAb in the same way. Possible modifications include the well-documented deamidation of Asn and Gln residues by direct hydrolysis catalyzed at pH of ~4. In neutral to basic solutions (pH 6 and above) the reaction changes to an intramolecular cyclization reaction, more so in the case of Asn than Gln. The deamination of Asn is more marked,

### Table 2

**Accuracy (trueeness) and precision.**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Checked concentration (mg/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Intraday</th>
<th>Interday (5 days)</th>
</tr>
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<tbody>
<tr>
<td>Bevacizumab</td>
<td>1</td>
<td>98.5</td>
<td>0.92</td>
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<tr>
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<sup>a</sup> Mean of three determinations.

<sup>b</sup> Relative standard deviation of three determinations.
because a ring of five members (known as succinimide or Asu) is formed in the mechanism, which is more stable than the ring of six members formed during Gln deamination [50]. Nevertheless, although these (or other) modifications occurred, they were not detected individually by shifts in the retention times, by new chromatographic peaks or changes in the shape of the UV absorption spectra when samples submitted to acidic/basic conditions. Even when samples were submitted to high ionic strength (a stress condition studied because the medicine samples are diluted in NaCl 0.9% for clinical administration) the area under the chromatographic peak of each mAb decreased by around 33%, while the spectral peak purity factor remained at 99.5%, with UV absorption spectra unchanged with respect to the fresh control sample analyzed simultaneously.

Exposure to light was also considered in the stress study because therapeutic proteins are exposed to light in various situations, including administration of the drug to the patient [50]. In the chromatograms no new or deformed chromatographic peaks appeared (Fig. 3). Although ~4% of the area was lost, the spectral peak purity was slightly affected for BVZ and INF, in which the similarity factor dropped to 99.3% and 98.8% respectively. Light-induced oxidation of three aromatic amino acids –Trp, Tyr and Phe– is described in the bibliography as usual for light-induced stress of protein [51]. Discoloration due to Trp oxidation has also been described in high concentration formulation, particularly for IgG [52]. However the color of the three medicine solutions remained unchanged after UV light stress.

Therefore modifications in the mAbs could be detected in the forced degradation study of the three mAbs by means of the decrease of the area under the chromatographic peak, and/or the spectral peak purity analysis, and/or the presence of new chromatographic peaks at different retention times. Nevertheless, except in the case of detecting new chromatographic peaks at shorter retention times (around 1.5 min for the oxidative stress, Fig. 3), it was impossible to achieve a complete chromatographic separation of the degraded/modified mAbs. Despite the efforts made throughout the stress study to chromatographically separate different forms mainly by modifying the polarity of the mobile phase, we obtained single chromatographic peaks and at similar retention time as those of the standards. Hence it could be inferred from these results that the differences in polarity between the possible degraded/modified forms are not sufficient to enable them to be eluted separately, as occurred in our previous research on RTX. Thus, bearing in mind the specificity of the proposed (RP)HPLC/DAD method for the mAbs we analyzed, the results indicated that the degraded/modified mAbs could not be chromatographically separated, and that the different forms that eluted must have overlaid each other at a similar retention time. In consequence, the method is not suitable for quantifying the loss of the intact mAbs if the spectral peak purity analysis is not accepted, but it can be used for estimation purposes. The methods can therefore be considered stability-indicating as modification in the structure can be detected. As already occurred with RTX, this is the only aspect of the ICH guidelines that is not completely fulfilled by the proposed methods, although it is partially fulfilled. It is clear that other techniques will be needed to address the particular structural changes in the mAbs and that this methodology is a preliminary indicator that something has changed.

3.2.5. Robustness

As indicated in the ICH guidelines [12], the robustness of the methods for the analysis of each mAbs was also evaluated. Again the chromatographic behavior of the three mAbs was similar as can be seen from the results presented in Fig. 4. This Fig. illustrates the chromatograms recorded for each mAb when the experimental variables were changed slightly and it highlights the similarity between the chromatograms for the three mAbs, all of which were affected in exactly the same way by the different changes in the variables. The results of this study are also summarized in Table 3. The composition of the mobile phase was the most critical parameter affecting robustness. Similarly
to the RTX study [39], an increase in the polarity of the mobile phase caused the mAb peak to overlay the peak at 6.8 min from the baseline, yielding the highest values of the theoretical plates for a composition of 35% of phase A (see Table 2). Considering the column temperature, the method is robust at temperatures of over 65 °C, and below 60 °C it causes important deformations in the chromatographic mAb peaks preventing their analysis.

3.2.6. System suitability

System suitability testing was conducted using the mAbs standard samples at the targeted concentrations, i.e., 1.0, 2.5 and 5.0 mg/mL. Table 4 summarizes the results of this study. The suitability of the analytical system (instrument + method + operator) was demonstrated because the chromatographic parameters agreed with previously established values (see Experimental section). In addition, the reproducibility of the injection volume (RSD = 0.05%, for 1 μL) tested for the three mAbs was less than that established in the criteria (RSD ≤ 1%).

### 3.3. Long-term quantification study of BVZ, INF and TTZ in hospital conditions of use

The proposed method was used to quantify BVZ, INF and TTZ samples prepared under hospital conditions in a long-term stability study. The study was therefore conducted at three concentrations, i.e. 0.5 mg/mL, 2 mg/mL and 10 mg/mL for BVZ and INF, and 0.5 mg/mL and 2.5 mg/mL for TTZ; these concentrations cover the range of the clinical use when administered in hospital. Indicated here that this study was performed in the context of a wider project that seeks to implement and validate analytical methods and protocols to characterize mAbs, with an additional objective of extending, when possible, the shelf lives of the surplus of these expensive biotechnological medicines prepared in hospital pharmacy departments.

Fig. 5 shows the evolution of the BVZ (3A), INF (3B) and TTZ (3C) concentrations along the time checked for samples stored refrigerated at 4 °C. The RSD (%) of the concentration estimated for each checked time was lesser than 2%. Tables 5 (BVZ), 6 (INF), and 7 (TTZ) summarizes the criteria applied to detect the modification/degradation of the three mAbs during this study. The results of the peak purity spectra (not included in Table 5) were in all the cases similar to the values of the controls (those obtained at day 0 for INF, BVZ and TTZ). Therefore, these results show that the mAbs concentrations in the diluted samples at the targeted concentrations did not change significantly during the first month. Regarding only concentration, the results indicated great

---

**Table 3**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Gradient (%)</th>
<th>T (°C)</th>
<th>Flow (mL/min)</th>
<th>tR (min)</th>
<th>Sb</th>
<th>Sc</th>
<th>k'</th>
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- a Retention time.
- b Symmetry factor.
- c Theoretical plates.
- d Capacity factor.

---

**Table 4**

<table>
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<tr>
<th>mAb</th>
<th>Sample concentration (mg/mL)</th>
<th>tR (min)</th>
<th>Sb</th>
<th>Sc</th>
<th>k'</th>
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</table>

- a Retention time.
- b Symmetry factor.
- c Theoretical plates.
- d Capacity factor.
The stability of the three mAbs stored in dark vials refrigerated at 4 °C, that is, the results were all ±4% of the initial concentrations of each mAb: ±3.9% in case of BVZ, ±2.3% in case of INF and ±0.2% in case of TTZ. These results contrast to those obtained for example for RTX in which changes were detected after about a week of the storage in similar conditions [38].

4. Conclusions

Reliable and accurate quantification of intact marketed monoclonal antibodies BVZ, INF and TTZ by (RP)HPLC/DAD was demonstrated in this research despite their inherent complexity (glycoproteins of high molecular weight). The results of the method performance and validation for the three mAbs showed similar or identical chromatographic behavior, such that they were indistinguishable from many of the analytical results. This is an interesting result of this investigation, i.e., the evidence that despite their chemical differences the marketed mAbs show similar RP chromatographic behavior and also similar to others mAbs previously study (RTX and CTX).

As drug substances, the method was validated as stability-indicating for the quantification of each intact mAb in accordance with the ICH guidelines for biotechnological products. Although slight modifications in the intact mAbs could not be chromatographically separated in the stress study we conducted, the modifications could be detected by applying the proposed spectral peak purity analysis strategy. The method was therefore validated for quantification purposes since it was qualified to detect degradation/modification of the mAbs. This means that the method can be used for quantification purposes of BVZ, INF and TTZ.

---

**Table 5**

<table>
<thead>
<tr>
<th>mAb</th>
<th>Concentration (mg/mL)</th>
<th>Day</th>
<th>Degradation peaks</th>
<th>Intensity lost (%)</th>
<th>Peak deformation</th>
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**Table 6**

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<th>Degradation peaks</th>
<th>Intensity lost (%)</th>
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**Table 7**

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</table>
TIZ in the quality control process when manufacturing the mAbs in addition to long-term stability studies.

The particular long-term stability study tracking BVZ, INF and TIZ concentrations in clinical solution that covers the range of the clinical use when administered in hospital indicated great stability in the three mAbs, in contrast with results previously obtained for RTX and CTX, two other similar IgG1. Neither degradations nor modifications were detected in any of the three mAbs in the month of study.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

Acknowledgments

This project was entirely funded by Project FIS:PI10/00201 (Instituto Carlos III, Ministerio de Economía y Competitividad, Spain); therefore acknowledgments of interest.

Conclusions

We conclude that the three mAbs, in contrast with results previously obtained for RTX and CTX, were detected in any of the three mAbs in the month of study.

References


