Original Article

Study and ICH validation of a reverse-phase liquid chromatographic method for the quantification of the intact monoclonal antibody cetuximab ☆

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ABSTRACT

Cetuximab (CTX) is a potent chimeric mouse/human monoclonal antibody (mAb) approved worldwide for treatment of metastatic colorectal cancer. Among the various biological and physical analyses performed for full study on this biopharmaceutic, the determination of the concentration preparations throughout manufacturing and subsequent handling in hospital is particularly relevant. In the present work, the study and validation of a method for quantifying intact CTX by reverse-phase high-performance liquid chromatography with diode array detection ((RP)HPLC/DAD) is presented. With that end, we checked the performance of a chromatographic method for quantifying CTX and conducted a study to validate the method as stability-indicating in accordance with the International Conference on Harmonization guidelines (ICH) for biotechnological drugs; therefore, we evaluated linearity, accuracy, precision, detection and quantification limits, robustness and system suitability. The specificity of the method and the robustness of the mAb formulation against external stress factors were estimated by comprehensive chromatographic analysis by subjecting CTX to several informative stress conditions. As demonstrated, the method is rapid, accurate, and reproducible for CTX quantification. It was also successfully used to quantify CTX in a long-term stability study performed under hospital conditions.

1. Introduction

Cetuximab (CTX) is a potent chimeric mouse/human monoclonal antibody (mAb) approved worldwide for the treatment of colon and head and neck cancers. It is directed against the human epidermal growth factor receptor (EGFR) [1]. Of the five classes of antibodies in humans, CTX is an immunoglobulin G (IgG) from Subclass 1 (IgG1), the most commonly used subclass for pharmaceutical and biomedical purposes [2] (Fig. 1). Due to the increasing importance of this class of biopharmaceuticals, a dynamic field of research has arisen around the development of analytical methods for their detailed characterization.

MAbs including IgG1 are large glycoproteins (≈ 150 kDa),

composed of four peptide chains, two identical heavy chains (≈ 50 kDa) and two identical light chains (≈ 25 kDa), which are connected by disulfide bonds (Fig. 1) at their hinge region [3], giving them a specific Y shape. From the structural point of view, mass spectrometry (MS) is the most versatile technology for the in-depth structural analysis of mAbs [4,5]. MS can be used for the characterization of both small and large chemical changes in the mAbs, i.e., primary amino acid elucidation, glycosylation profiles and posttranslational modification such as deamidation and oxidation. In recent research, several structural approaches based on MS techniques were used to assess the primary amino acid sequence, the glyco-profile of CTX, and the mAb studied here [6]. Nevertheless, problems arise when intact mAbs analysis has to be performed coupled to a liquid chromatographic (LC) system. In this case, when performing liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) analysis, the uncommon solvent systems used for the elution of the mAbs from the latest generation of chromatographic columns complicate the ionization process in the
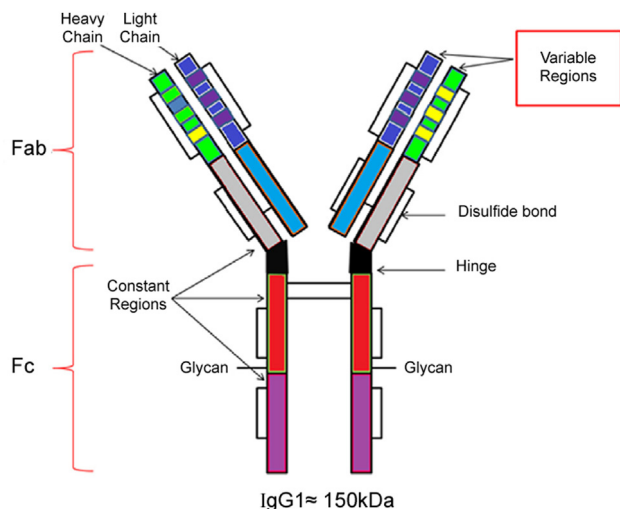

Fig. 1. General structure of IgG1.

electrospray. In fact, to date, very few published methods have been used for quantifying intact mAbs by LC–MS [7], and none of them has been validated following the International Conference for Harmonization (ICH) guidelines. The quantification of therapeutic mAbs was carried out by enzyme-linked immuno sorbent assay (ELISA) [8,9]. However, the lower precision (about 10% of relative standard deviation in repeatability) and robustness of the immunoassays compared with physicochemical-based techniques led ICH to recommend the latter for quantification purposes (Q6B) [10].

Among the different modes of LC, reverse-phase (RP)–LC appears to be one of the most promising analytical techniques for the study of peptides and intact protein, including mAbs [11–13]. RP–LC is generally more efficient than ion-exchange (IXC) and size-exclusion chromatography (SEC) [14], making it the best technique for quantification purposes [15]. Furthermore, RP–LC has a quicker separation time than IXC. However, taking into account that several methods are required to perform a rigorous study of a biopharmaceutical, IXC is the most suitable method for tracking charge profile [16] and SEC for detecting mAb aggregates [17].

The use of stationary phases with wider pores (300 Å) and long alkyl chains (C8 and C18) together with a high column temperature (60–75 °C) and a combination of non-traditional solvent systems with ion pairing agents [18,19] is gradually being consolidated as the established procedure for RP chromatographic analysis of intact mAbs. However, the small chemical differences produced by deamidation, cysteine oxidation or the heterogeneity in the glycans profile of the mAbs generate such small changes in the polarity of the molecules that LC columns currently available cannot separate them chromatographically [20]. Chromatographic separation of marketed mAbs is not successful for the same reason, i.e., the chemical differences between them are insufficient to bring about significant changes in the polarity that enable them to be separated chromatographically [15].

From the point of view of the analytical validation of biopharmaceuticals, the ICH Q6B guidelines [10] indicate that the validation of analytical procedures used in the characterization of biotechnological products (such as therapeutic mAbs) should be performed in compliance with the ICH Q2(R1) guidelines relating to the validation of analytical procedures [21]. Despite the fact that ICH Q2(R1) was originally developed for low-molecular-weight drugs, it can also be adapted for use with biotechnology-based pharmaceuticals. In a recently published work [15], we proposed that the quantification of intact rituximab (RTX), another marketed mAb IgG1, could be achieved using a reverse-phase high-performance liquid chromatography with diode array detection

((RP)HPLC/DAD) method validated according to the ICH Q2(R1) guidelines. We proposed that the method was validated as stability-indicating for quantification since it was qualified for detecting modification/degradation by means of the peak purity analysis proposed, even though total chromatographic separation was not achieved in the stress study we performed. In the present study, we analyzed the performance of (RP)HPLC/DAD and discussed the validation of the method for the quantification of the intact CTX.

2. Materials and methods

2.1. Chemicals and reagents

Erbitux® (Merck KGaA, Darmstad, Germany) was used as a representative CTX reference material. The daily surplus was kindly supplied for this study by the Pharmacy Unit of the University Hospital "San Cecilio" (Granada, Spain). All of the standard solutions of CTX were prepared from this medicine. The specifications for the medicine indicated that there were 100 mg/20 mL of CTX in each single-use vial along with sodium citrate, polysorbate 80, sodium chloride, glycine, citric monohydrate acid, sodium hydroxide and water for injections, giving a final concentration of 5 mg/mL of CTX [2]. The working standard solutions of CTX were prepared daily from marketed CTX (immediately after opening it) by appropriate dilution with 0.9% NaCl aqueous solution. Different batches were used randomly throughout the study.

All reagents were of analytical reagent grade unless otherwise stated. Reverse-osmosis-quality water purified (≥ 18 MΩ/cm) with a Milli-RO plus Milli-Q station from Millipore Corp. (Madrid, Spain) was used throughout the study. Methanol of HPLC grade, hydrochloric acid, sodium chloride and sodium hydroxide (pure for analysis) and propan-2-ol ($\geq 98.0\%$) were supplied by Panreac (Barcelona, Spain). Hydrogen peroxide (30%) was supplied by Technical (Fontenay-sous-Bois, France). Trifluoroacetic acid ($\geq 98.0\%$) (TFA) was from Merck KGaA (Darmstadt, Germany) and HPLC grade acetonitrile was from Poch S.A. (Gliwice, Poland). An isotonic solution of 0.9% NaCl was supplied by B. Braun Medical (Madrid, Spain).

2.2. CTX standard sample solutions

All of the standard solutions of CTX were prepared from the medicine Erbitux®. The working standard solutions of CTX were prepared daily from marketed CTX (immediately after opening it) by appropriate dilution with 0.9% NaCl aqueous solution. We intended to avoid any kind of change or degradation in the mAb due to its complex protein nature. Also, different batches were used randomly throughout the study.

2.3. Chromatographic system and software

All chromatographic analyses were performed using an Agilent 110 HPLC system equipped with a quaternary pump, a degasser, an autosampler, a column oven and a 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 conditions are summarized in Table 1.

The Statgraphics Plus 6.0 (Statistical Graphics System, 1992, Warrenton, VA, USA) statistical software package was used to deal with the chromatographic data.

2.4. Analytical method validation

2.4.1. Linearity, LOD, and LOQ

Linearity was evaluated up to 5.0 mg/mL. To this end, appropriate

Table 1
Chromatographic conditions.

Column	Zorbax 300 SB-C8 (150 mm × 2.1 mm, 5 μm, Agilent Technologies)
Eluent	A: Milli-Q water and 0.1% TFA B: iPrOH, ACN, Milli-Q water and TFA (70:20:9.9:0.1, v/v/v/v)
Elution	Gradient program (time, % B): 0–1 min, 10% B; 1–1.05 min, 20% B; 1.05–4 min, 20% B; 4–4.05 min, 30% B; 4.05–10 min, 60% B, 10–10.05 min, 80% B; 10.05–12 min, 80% B; post-time: 3 min
Flow rate	1 mL/min
Injection volume	1 μL
Column temperature	70 °C
Autosampler temperature	25 °C
Injection washing solution	Milli-Q water
Detection	214 nm 280 nm (reference)

CTX standard solutions of 0.25, 0.5, 0.75, 1.0, 2.5, and 5.0 mg/mL were prepared in quadruplicate and injected into the chromatographic system. A linear calibration function was fitted by least-squares regression, and linearity was accepted, if the statistical probability level in the corresponding lack-of-fit test (the P_{LOF}-value) was greater than 5% according to the Analytical Methods Committee [22].

The limit of detection (LOD) and the limit of quantification (LOQ) were estimated from the standard deviation (SD) of the lowest concentration tested. LOD and LOQ were calculated as $3 \times SD$ and $10 \times SD$, respectively.

2.4.2. Precision

The precision of the method was tested by estimating the intra-day precision (repeatability) and the intermediate precision (5 days, reproducibility), given as relative standard deviations (RSDs). The former was estimated from the results of the analysis of CTX standard solutions prepared at the same concentration on the same day. Thirty samples with high-concentration levels (10 samples of 5.0 mg/mL), medium-concentration levels (10 samples of 2.0 mg/mL), and low-concentration levels (10 samples of 0.5 mg/mL) were used for this purpose. The intermediate precision was estimated from the analysis of standard solutions at three concentration levels (0.5, 2.0 and 5.0 mg/mL) over five consecutive days. Two samples of each concentration were prepared and analyzed daily (ten samples of each concentration tested).

Because ICH Q2(R1) [21] does not establish any precision criteria for acceptance of the method, the US Food and Drug Administration (USFDA) criteria for HPLC methods for pharmaceutical analysis were followed [23,24], i.e., an intra-day precision of $\leq 2\%$.

2.4.3. Accuracy

The accuracy of the method was assessed from the average recovery value calculated by analyzing ten standard solution replicates at three concentration levels representing the highest value (5.0 mg/mL), the middle value (2.0 mg/mL), and the lowest value (0.5 mg/mL) of the calibration scale, in order to cover the stated linear range. Once again, ICH Q2(R1) [21] does not indicate criteria for acceptance of the accuracy of the method. We, therefore, decided to follow the USFDA accuracy criteria for HPLC methods for pharmaceutical analysis [23,24], i.e., an average recovery of $(100 \pm 0.1)\%$.

2.4.4. Specificity

Forced degradation studies were performed using diluted

Erbitux® sample solutions of 2.0 mg/mL of CTX in 0.9% NaCl. ICH Q5C [25] recommends that stability testing should be carried out at representative concentrations of the final product, which, in the case of typical hospital conditions for CTX, would be in the range of 2.0–5.0 mg/mL for injected solutions for perfusion [1]. The lowest concentration was used for the stress study. The stress conditions studied were high temperature, high ionic strength, light exposure, addition of an acid and a base, and addition of an oxidant. The chromatograms for the stressed samples were compared with those for a freshly prepared CTX standard solution that had not undergone degradation treatment. All samples were analyzed in triplicate.

Two levels of stress conditions were tested when the CTX sample was put under stress using acid (1 M HCl), alkaline (0.1 M NaOH), ionic (1.5 M NaCl) and oxidative media (1 M H_2O_2). For the lower level stress condition, the concentration of the stressed agent was 3.3% (v/v) (50 μL of the stressed agent was added to 1.5 mL of mAb standard solution), while for the higher level stress condition, the stressed agent concentration was 25% (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 the CTX standard solution in an oven (Heraeus S.A., Madrid, Spain) at the temperature of 50 °C for 24 h, which is considered the maximum temperature to which samples could be accidentally exposed in hospital. After the specified time had elapsed, the solutions were analyzed. The effect of light was investigated by placing aliquots of the CTX standard solution samples in an accelerated stress test chamber to simulate sun light (Solarbox 3000e RH, Cofomegra, Milan, Italy). The samples were light irradiated with a xenon lamp using a UV filter S208/S408 soda lime glass filter to simulate indoor exposure conditions with infrared rejection coating to reduce samples temperature. The temperature was controlled and maintained at 24 °C (considered as room temperature). Irradiance was set up at 250 W/m² between 300 and 800 nm.

Spectral peak purity was checked using the corresponding ChemStation software [26,27]. In brief, this assessment was based on a 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 regression coefficient (R^2). All of the spectra in the chromatographic peak were averaged to obtain a pooled spectrum which was compared with all the spectra in the peak to obtain the similarity function. The similarity factor was set to a value of $\geq 99.0\%$ for acceptance of the spectral peak purity.

2.4.5. System suitability

For system suitability testing, we used standard samples of 2.0 mg/mL prepared in 0.9% NaCl. ICH Q2(R1) [21] does not indicate criteria for the acceptance of system suitability either. We, therefore, decided to follow the USFDA criteria for HPLC methods of pharmaceutical analysis [23,24], although we used the symmetry factor instead of the asymmetry ratio of a peak. The selected system suitability parameters were N (acceptance criterion N > 2000), k′ (acceptance criterion k′ > 2.0), S (acceptance criterion S ≤ 2), and injection repeatability (acceptance criterion RSD ≤ 1%).

2.4.6. Robustness

Robustness was evaluated by making small changes in the mobile phase composition (B ± 5%), the mobile phase flow rate (± 0.1 mL) and the temperature (tested from 60 to 70 °C), and these parameters mostly affect the chromatographic process. Aliquots of the CTX standard solution of 2.0 mg/mL were analyzed at each modified condition to assess the impact of the condition on the assay results. The robustness of the method was then estimated in terms of the retention time, the symmetry factor (S), the capacity factor (k′), and the number of theoretical plates (N).
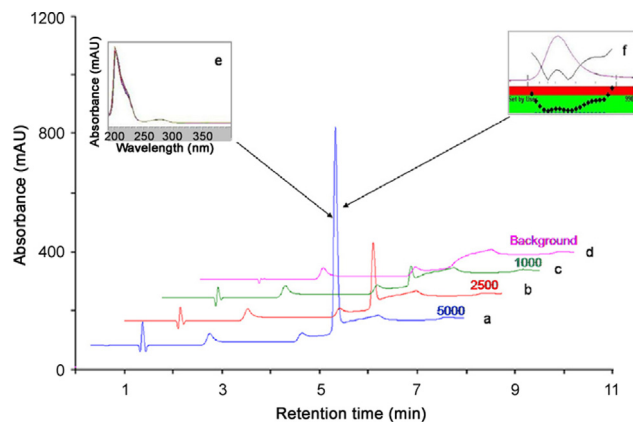
2.5. Long-term study of diluted CTX samples (hospital administration conditions)

A diluted CTX sample of 2.0 mg/mL in 0.9% NaCl was prepared from the medicine Erbitux® as indicated in the scientific technical report for CTX [1]. This CTX sample was divided into 1 mL of aliquots and placed in amber glass vials (protected from daylight). One aliquot was stored refrigerated at 4 °C, and several aliquots were stored frozen at −20 °C. In the long-term study, the concentration of CTX was expressed as the 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. The refrigerated sample was analyzed 1, 3, 7, 14 and 31 days after preparation. The frozen samples were analyzed after a month.

3. Results and discussion

3.1. Method development

The (RP)HPLC/DAD method previously developed for the analysis of intact RTX [15] was here checked for the analysis of CTX. In brief, we used a Zorbax 300 SB C_8 analytical column because of its reported excellent temperature stability at low pH [28]; the mobile phase was a combination of isopropanol (i-PrOH), acetonitrile (ACN), water and trifluoroacetic acid (TFA), a strong eleutropic solvent system required for the analysis of antibodies in order to reduce column interactions due to their high hydrophobicity [19]. The conditions used in the gradient allowed the elution of the intact CTX at a retention time between 7 and 8 min. This enables both a rapid result, which is important for routine analysis, and separation of CTX from degradation products, as occurs for example when subjected to oxidative stress conditions (discussed below). As in the previous work [15], two equilibration steps of the column were necessary to elute the mAb at the selected retention time (between 7 and 8 min), and without these steps, a small chromatographic peak from the system solvent overlapped with the chromatographic peak of the mAb (see Fig. 2, chromatographic peak at approximately 7 min). The peaks at 4 and 7 min were from the solvent system because they were detected when we injected water into samples (blank samples) (Fig. 2d). Despite using a post-time of 3 min with 10% phase B (the initial gradient conditions) to equilibrate the column, an extra minute was necessary under

these analytical conditions to avoid changes in the retention time of the chromatographic peak of the mAb. As described in the literature and later confirmed in a robustness study, slight changes in the mobile phase composition are critical in the elution of mAbs [18,19]. We also corroborated that column temperature was a critical chromatographic parameter. CTX was not eluted at temperatures below 50 °C; with the increase of the temperature, CTX was eluted with a narrower and well defined chromatographic peak. Then, we selected 70 °C, the maximum value specified for this column. The chromatograms were recorded at different wavelengths (λ=214 nm, 220 nm, 254 nm, and 280 nm), using λ= (360 ± 20) nm as the reference wavelength in all cases. The analytical parameters of the method were calculated using λ=214 nm, because it was the maximum absorption and the signal was less affected by the inherent heterogeneity of CTX.

In this way, we confirmed that the conditions selected for quantifying intact RTX are also suitable for the quantification of CTX. No appreciable differences were detected either in the chromatographic behavior or in the UV spectra of the two mAbs.

Fig. 2 shows the chromatograms for CTX standard solutions under the chromatographic conditions. The UV absorption spectra recorded at the peak are also displayed (Fig. 2e), together with the results from the peak purity analysis (Fig. 2f), both of which are discussed in the next section.

3.2. Analytical method validation

The medicine Erbitux was used throughout the study as reference standard material for CTX. It is the marketed form of CTX and none of the ingredients interfere with the chromatographic analysis. In addition, in order to avoid any kind of change or degradation in CTX, the working standard solutions were prepared daily from the medicine (immediately after opening it) by appropriate dilution with 0.9% NaCl aqueous solution.

3.2.1. Linearity, LOD, and LOQ

The linearity of the method was corroborated up to 5.0 mg/mL, considering 2.0 mg/mL as the CTX target concentration. These concentrations were high, so the volume injected was as small as possible, i.e., 1 μL. This was to avoid further dilution of the CTX samples when prepared at hospital conditions of use, i.e., between 2.0 and 5.0 mg/mL, so that they could be injected into the chromatographic system without the need for any previous manipulation that could bring about slight changes in the mAb. Higher CTX concentrations gave wider chromatographic peaks. Table 2 shows the results of the calibration function. The intercept (a) was

Fig. 2. Standard solutions of 5000 mg/L (a), 2500 mg/L (b), and 1000 mg/L (c) of CTX standard samples analyzed by (RP)HPLC/DAD. UV absorption spectra recorded at the chromatographic peak and the graphic results for the spectral peak purity analysis using 99.3% as similarity factor. Chromatogram of a blank sample (d). UV absorption spectra recorded at the peak (e). Results from peak purity analysis (f).

Table 2
Performance of the analytical methods.

Figure of merit	Value
Intercept (a)	−9.84
Slope (b)	1.050
s (a)[a]	20.6
P-value (%)[b]	0.64
s (b)[c]	0.007
R² [d]	0.999
P-value (%)[e]	0.194
Practical linear range (mg/mL)	0.5–5.00
Limit of quantification (LOQ)[f] (mg/mL)	0.21
Limit of detection (LOD)[f] (mg/mL)	0.06

[a] Standard deviation of the intercept.
[b] Probability of intercept significant.
[c] Standard deviation of the slope.
[d] Determination coefficient.
[e] Probability of lack-of-fit test.
[f] Estimated from the SD of the intercept (a).

not significant since the corresponding *P*-values were more than 5% whereas the slope (b) was significant with a *P*-value close to 0. The coefficient of determination (R^2), and the probability of a lack-of-fit (P_{LOF}-value, %) indicated a high degree of fit for the experimental data to the linear model proposed. All criteria were fulfilled, indicating that the linearity of the response of the detector under the conditions selected for determining CTX was successfully demonstrated.

The LOD and LOQ were estimated as indicated in the Experimental section. The working range was then established between 0.21 and 5.0 mg/mL, with the LOQ being the lowest concentration in this range.

A proper quantification strategy in routine analysis could be implemented with this method since the null intercept and the linearity of the calibration curve have been corroborated. Under these conditions, the response factor (RF), defined as the ratio between the measured absorbance of a CTX-based medicine sample and its CTX concentration, can be considered to remain constant throughout the working range. To this end, one representative CTX standard can be analyzed with each analytical batch, and quantification was carried out by applying the estimated RF value of the standard [29].

We also estimated the retention time of CTX. For this purpose, we used 40 chromatograms of CTX standard samples obtained at different concentrations and on different days, in order to gather all possible sources of variation in the measurement of the retention time. The average retention time calculated was 7.86 min with SD of 0.06 min, which means that, for a confidence level of 99.5% ($t = 2.704$, $n = 40$), the retention time interval of CTX was estimated as 7.86 ± 0.06 min. This retention time overlapped with the retention time of RTX (7.6 ± 0.2 min) [15].

3.2.2. Accuracy

The accuracy (trueness and precision) of the analytical method for quantifying intact CTX was verified across the linear range as stated in the ICH Q2(R1) guidelines [21]. As shown in Table 3, satisfactory results were obtained for the trueness and for both the intra-day and inter-day precisions of the method, expressed as recovery and relative standard deviation (RSD) values, respectively. The intra-day and inter-day RSDs were $\leq 2\%$ for all concentrations tested. The recovery values in all cases were close to 100% of the concentration checked, and the trueness fulfilled the acceptance criterion by falling within the range $100\% \pm 0.1\%$.

3.2.3. Specificity

Forced degradation studies were performed (according to the ICH Q2(R1) guidelines [21]) on Erbitux® sample solutions to evaluate the specificity of the proposed method. This stress study was also carried out to gather information about the degradation of the mAb under hospital conditions, as a means of evaluating the robustness of this CTX formulation against external factors. The stress factors we studied were those that could potentially affect

stability. Furthermore, whereas stability testing requirements were defined in regulatory guidelines, the particular procedures for forced degradation studies of therapeutic proteins have not yet been standardized [30]. We, therefore, carried out the forced degradation studies described in the Experimental section.

Chromatographic separation of the modified/degraded CTX was not always achieved. New chromatographic peaks that were clearly separated from the CTX peak were only observed in the ionic and oxidative stressed samples (Fig. 3). For these reasons, we proposed three different aspects to test CTX degradation/modification. Firstly, we looked for new chromatographic peaks; secondly, we evaluated CTX chromatographic peak deformation; and thirdly, we carried out the peak purity analysis referred to above [15]. Modification/degradation of CTX could be detected by gathering the information about these three aspects. The method is, therefore, qualified to detect degradation/modification, although it can only be used to quantify CTX until modification/degradation is detected. After that, the degradation can be evaluated but CTX cannot be quantified with the necessary scientific rigor.

Table 4 summarizes the results of the stress study conducted to evaluate the specificity of the method. The similarity factor (also peak purity factor) for CTX QC sample was set at 99.3%. Due to the inherent micro-heterogeneity of CTX [31], the spectra obtained from the chromatographic peaks of fresh standard samples were not identical (Fig. 2e), with the main dissimilarity observed between approximately $\lambda = 220$ and $\lambda = 235$ nm. As a result of these slight spectral variations, peak purity analysis indicated impurities, considering the reference value of the similarity factor, i.e., 99.3% the default value for the ChemStation software [27]. This analysis represents an attractive alternative to the use of a mass spectrometric detector for assessing peak purity, due to its cost/benefits and simplicity. These numerical methods for calculating similarity based on the correlation coefficient are also considered to be suitable for assessing the degree of similarity between protein absorption spectra [32]. In the stress study of CTX, spectral peak purity was only assumed, i.e., no significant changes were detected compared with the CTX standard, when the similarity function calculated for the corresponding chromatographic peak was higher than 99.3%.

Modification/degradation of CTX in medicine samples subjected to stress conditions, both weak and strong, was detected from the similarity factor value, i.e., below the fixed value of 99.3% as can be noted from results in Table 4. Fig. 3 shows the chromatograms of the stressed samples. Nevertheless, a new chromatographic peak clearly separated from the CTX peak was only observed when samples were subjected to strong ionic and oxidative stress. This new peak was observed at 1.7 min. Substances at this short retention time were not retained in the column (retention time of the front). In the case of the oxidative stress, this peak could be attributed to the oxidation of methionine (Met), the amino acid most susceptible to oxidation, the result of which is a polar sulfonic group not retained in the column [33]. Other minor modifications such as oxidation of aromatic amino acids (Trp, Tyr, and Phe residues) [34, 35] and deamidation of specific amino acids with the pH (mainly Asn and Gln residues) [33] produce slight differences in the hydrophobicity relative to that of the intact CTX that cannot be chromatographically detected by (RP)HPLC/DAD.

It is also worth highlighting that the formation of aggregates is likely when mAbs are subjected to high temperatures or to light due to unfolding or partial unfolding of the molecule [30,31]. The results for CTX indicated that if the mAb underwent such conformational changes, they were not reflected in the chromatograms as new peaks. This means that these changes did not affect the physicochemical chromatographic process, which indicated that aggregates could not be separated on the RP column. The

Table 3
Precision and accuracy of the method.

Concentration tested (mg/mL)	Recovery[a] (%)	Precision (RSD, %)[b]	
		Intra-day	Inter-day (5 days)
0.5	100	0.7	0.6
2.0	100.1	1.8	2.0
5.0	99.9	1.7	1.6

[a] Recovery value obtained from ten samples prepared from the standard.
[b] Relative standard deviation from ten standard samples.
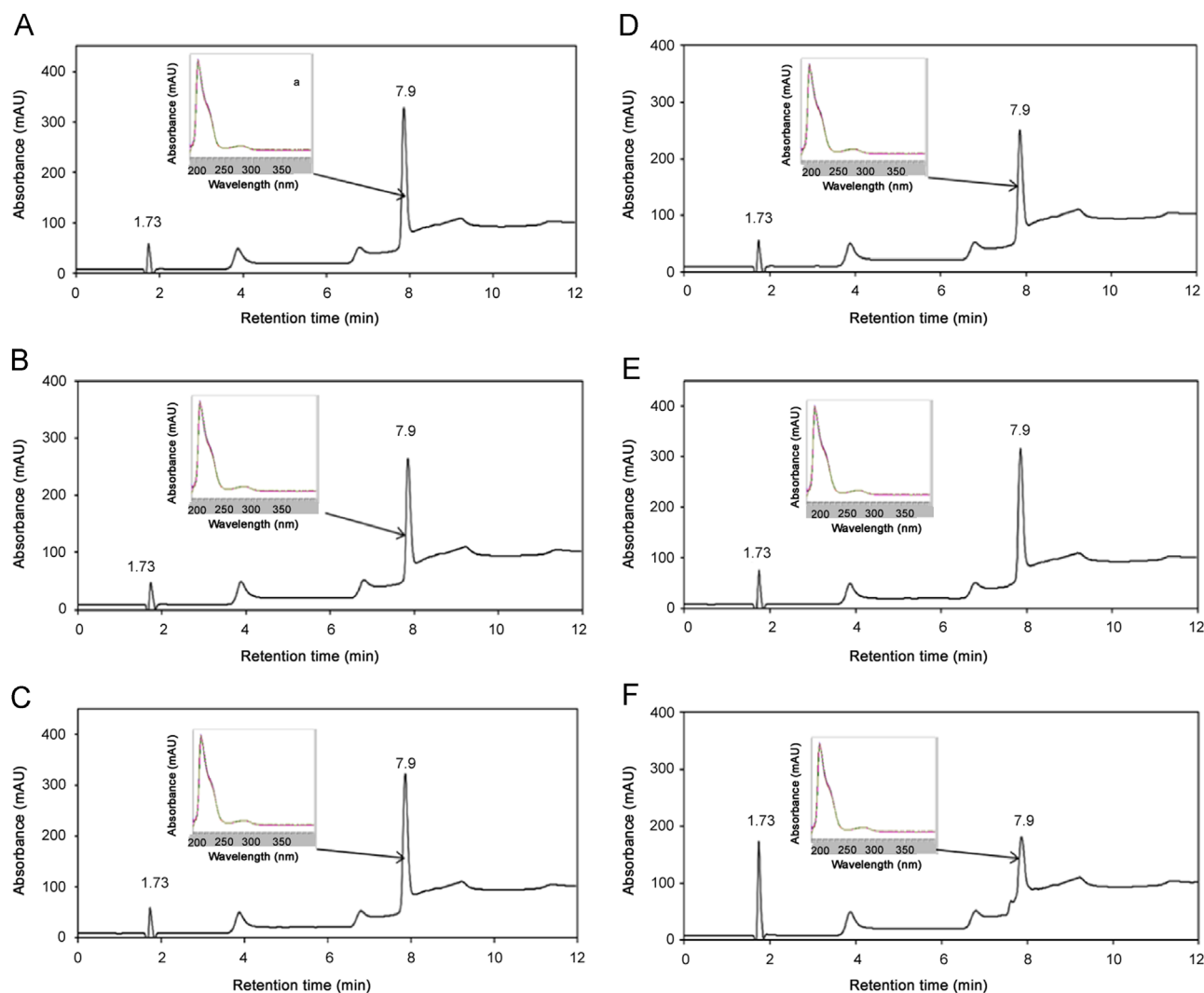
Fig. 3. Stress study of medicine sample solutions of 2000 mg/L of CTX prepared in 0.9% NaCl. Stronger stress condition studied: (A) Temperature, 50 °C; (B) light stress; (C) weak basic media; (D) strong basic media; (E) weak ionic stress; and (F) strong oxidative stress.

Table 4
Specificity study (stress study).

Stress type	Stress intensity	Similarity factor (%)	Degradation peaks	Decreased area under CTX peak (%)	CTX peak deformation
Quality control	–	99.3	No	–	No
Temperature	50 °C	99.0	No	3.8	No
Light stress	–	98.7	No	28.3	No
Ionic stress	weak	99.3	No	7.8	No
	strong	99.2	Yes	31.6	No
Oxidative media	weak	99.1	No	10.1	No
	strong	98.8	Yes	55.7	No
Acidic media	weak	99.2	No	8.6	No
	strong	99.1	No	31.1	No
Basic media	weak	99.1	No	6.6	No
	strong	98.9	No	33.2	No

slight differences in the similarity factor may be attributed to the formation of aggregates. More research is currently being conducted on this aspect using other forms of chromatography.

Therefore, from the point of view of the specificity of the proposed (RP)HPLC/DAD method, the results of the stress study indicated that the degraded or modified fraction of CTX could not be chromatographically separated from the non-degraded CTX. Efforts were made throughout the stress study to chromatographically separate these different forms by modifying the gradient of the mobile phase, testing both more and less polar phases, but the relatively slight differences in polarity of the degraded/modified forms of CTX prevented separate elution. Nevertheless, the procedure based on the three aspects cited above could be used to detect slight modifications in the intact CTX, so fulfilling in part the criterion for the specificity. For this reason, we propose that the method be considered stability-indicating for quantifying intact CTX, since it is qualified to detect degradation/modification in the mAb. Once these changes are detected, the

Table 5
Robustness study.

Variations	Retention time (min)	Symmetry factor	Theoretical plates	Capacity factor
Flow rate (mL/ min)				
1.0[a]	7.8	0.78	42333	3.51
0.9	8.2	0.68	35541	3.70
1.1	7.6	0.73	31626	3.38
Gradient varia- tion (B %)[b]				
30:60[a]	7.8	0.78	42333	3.51
25:55	8.8	0.72	41390	4.07
35:65	7.1	0.68	41089	3.08
Temperature (°C)				
70[a]	7.8	0.78	42333	3.51
68	7.5	0.72	39842	3.22
60	No peak detected			

[a] Selected chromatographic conditions for the HPLC method.
[b] Indicted as the modification of B (%) in the gradient of the mobile phase.

method cannot be used for quantification purposes because the different degraded forms of CTX cannot be chromatographically separated. This means that the method fulfills the ICH guidelines in part, although not completely.

### 3.2.4. System suitability

The system suitability study focused on the evaluation of parameters affected quantification. In this way, CTX standard samples at the targeted concentration (2 mg/mL) were used to evaluate various chromatographic parameters i.e. the retention time (7.8 min), symmetry factor (0.78), theoretical plates (42333) and the capacity factor (3.51). The suitability of the analytical system (instrument+method+operator) was demonstrated, as these chromatographic parameters coincided with previously established values (see Section 2). In addition, the reproducibility of the injection volume (0.05% RSD for 1 μL) was less than that established in the criteria (RSD ≤ 1%).

3.2.5. Robustness

The results of the study performed to evaluate the robustness of the proposed method are summarized in Table 5. As expected, the composition of the mobile phase and temperature were the most important influences on the robustness. Indeed, when the polarity of the mobile phase was increased, intact CTX was eluted at a shorter retention time and overlapped with a peak at 7.1 min from the base line. The same thing occurred when temperature decreased to 68 °C. This was why the number of theoretical plates was the highest when the mobile phase contained 35% phase B or the column temperature was set at 68 °C (Table 5). Column temperature < 60 °C caused notable deformation of the chromatographic peak of intact CTX and prevented it from being analyzed. At temperatures of > 65 °C, peak deformation was not detected, although the area under the peak decreased slightly, probably due to adsorption onto the column.

3.3. Assay of CTX in a long-term study (hospital conditions)

The proposed method was used to quantify and assess CTX samples under hospital conditions in an ongoing long-term stability study. The study was conducted at a target concentration of 2.0 mg/mL, considered representative of the final product when
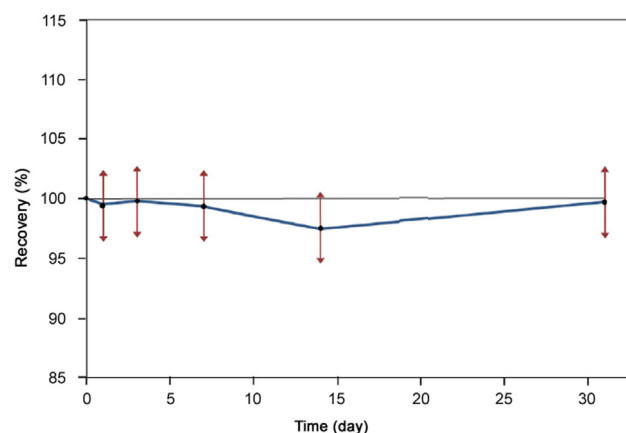
Fig. 4. Graphical results of the long-term study of CTX sample solutions of 2000 mg/L prepared from dilution of Erbitux® in 0.9% NaCl.

administered in hospital [25]. 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 being to extend, if possible, the shelf lives of these surplus biotechnological products used in hospital pharmacy departments.

Fig. 4 shows the evolution of the CTX concentration for the sample refrigerated at 4 °C. Table 6 summarizes the criteria to detect the modification/degradation of CTX to this study. These results show a surprising stability for this mAb, indicating no modification/degradation for a month when stored at 4 °C, in contrast to the results of studies we are currently performing on other mAbs (some of which have already been published), in which changes are detected after about a week [15]. We are currently conducting more studies using different liquid chromatographic modes and by ELISA.

4. Conclusions

This study contributes to demonstrating the suitability of RP–LC coupled to DAD for the rigorous quantification of therapeutic intact monoclonal antibodies despite the inherent complexity of this class of biotherapeutic drugs. In particular, we proposed the rigorous quantification of the intact CTX using a simple, accurate and validated (RP)HPLC/DAD method. All the aspects considered in the validation procedure following the ICH guidelines were successfully fulfilled except for those related to the chromatographic separation of the different forms of the intact CTX when degraded under specific stress conditions. This was because the differences in the polarity are insufficient to enable degraded form of CTX to be chromatographically separated by reverse-phase chromatography. Nevertheless, the method can be considered stability-indicating as it is qualified to detect the modifications in the intact CTX by applying the criteria proposed

Table 6
Long-term stability study results. Criteria to detect modification/degradation in CTX in solution at 2 mg/mL.

Day	Similarity factor (%)	Degradation peaks	Intensity lost (%)	Peak deformation
0	99.3	No	–	No
1	99.3	No	0.17	No
3	99.3	No	0.29	No
7	99.3	No	0.36	No
14	99.3	No	0.51	No
31	99.2	No	0.68	No

based on the analysis of the chromatograms and the spectral peak purity. The method can, therefore, be used to test mAb stability in both quality control processes and long-term stability studies, and we should always bear in mind that biopharmaceuticals such as mAbs should be analyzed and studied by various analytical methods simultaneously. The proposed method here is only focused on quantification aspects. Taking this into account, in addition to being simple, the method is also precise, accurate and robust and it is a good alternative for the analysis of intact therapeutic mAbs. The method also proved successful in quantifying intact CTX in a long-term stability study, demonstrating its usefulness for such purposes. This work is part of a wider project that aims to propose rigorous analytical methods and validated protocols for the study of marketed therapeutic mAbs. This method represents only one of the analyses needed to characterize mAbs.

Acknowledgments

References

[1] Scientific discussion cetuximabmab EMEA, 2004. ⟨http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000558/WC500029113.pdf⟩.

[2] D.S. Dimitrov, Therapeutic Proteins, in: V. Voynov, J.A. Caravella (Eds.), Springer, New York, New Jersey, 2012, pp. 1–26.

[3] D.R. Mould, K.R.D. Sweeney, The pharmacokinetics and pharmacodynamics of monoclonal antibodies, Curr. Opin. Drug. Discov. Dev. 10 (2007) 84–96.

[4] S. Rosati, N.J. Thompson, A.J.R. Heck, Tackling the increasing complexity of therapeutic monoclonal antibodies with mass spectrometry, TrAC-Trends Anal. Chem. 48 (2013) 72–80.

[5] A. Beck, E. Wagner-Rousset, D. Ayoub, et al., Characterization of therapeutic antibodies and related products, Anal. Chem. 85 (2013) 715–736.

[6] D. Ayou, W. Jabs, A. Resemann, et al., Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques, mAbs 5 (2013) 699–710.

[7] C.W. Damena, H. Rosing, J.H. Schellens, et al., Quantitative aspects of the analysis of the monoclonal antibody trastuzumab using high-performance liquid chromatography coupled with electrospray mass spectrometry, J. Pharm. Biomed. Anal. 46 (2008) 449–455.

[8] H.J. Stemmler, M. Schmitt, A. Willems, et al., Ratio of trastuzumab levels in serum and cerebrospinal fluid is altered in HER2-positive breast cancer patients with brain metastases and impairment of blood–brain barrier, Anticancer Drugs 18 (2007) 23–28.

[9] G. Hampson, T.H. Ward, J. Cummings, et al., Validation of an ELISA for the determination of rituximab pharmacokinetics in clinical trials subjects, J. Immunol. Methods 360 (2010) 30–38.

[10] International Conference on Harmonisation (ICH) guidelines ICH Q6B, Specifications: test procedures and acceptance criteria for biotechnological/biological products, 1999.

[11] D. Staub, D. Guillarme, J. Schappler, et al., Intact protein analysis in the biopharmaceutical field, J. Pharm. Biomed. Anal. 55 (2011) 810–822.

[12] S. Fekete, S. Rudaz, J. Fekete, et al., Analysis of recombinant monoclonal antibodies by RPLC: toward a generic method development approach, J. Pharm. Biomed. Anal. 70 (2012) 158–168.

[13] S. Grotefend, L. Kaminski, S. Wroblewitz, et al., Protein quantitation using various modes of high performance liquid chromatography, J. Pharm. Biomed. Anal. 71 (2012) 127–138.

[14] I. Molnar, C.S. Horvath, Separation of amino acids and peptides on non-polar stationary phases by HPLC, J. Chromatogr. A 142 (1977) 623–640.

[15] N. Navas, A. Herrera, A. Martínez-Ortega, et al., Quantification of an intact monoclonal antibody, rituximab, by (RP)HPLC/DAD in compliance with ICH guidelines, Anal. Bioanal. Chem. 405 (2013) 9351–9363.

[16] L. Zhang, T. Patapoff, D. Farnan, et al., Improving pH gradient cation-exchange chromatography of monoclonal antibodies by controlling ionic strength, J. Chromatogr. A 1272 (2013) 56–64.

[17] S. Guha, J.R. Wayment, M.J. Tarlov, et al., Electrospray – differential mobility analysis as an orthogonal tool to size-exclusion chromatography for characterization of protein aggregates, J. Pharm. Sci. 101 (2012) 1985–1994.

[18] T.M. Dillon, D.V. Bondarenko, M.S. Ricci, Development of an analytical reversed phase high-performance liquid chromatography–electrospray ionization mass spectrometry method for characterization of recombinant antibodies, J. Chromatogr. A 1053 (2004) 299–305.

[19] T.M. Dillon, P.V. Bondarenko, D.S. Rehder, et al., Optimization of a reversed-phase high-performance liquid chromatography/mass spectrometry method for characterizing recombinant antibody heterogeneity and stability, J. Chromatogr. A 1120 (2006) 112–120.

[20] J. Yang, S. Wang, J. Liu, et al., Determination of tryptophan oxidation of monoclonal anibody by reversed phase high performance liquid chromatography, J. Chromatogr. A 1156 (2007) 174–182.

[21] International Conference on Harmonization (ICH) guidelines ICH Q2(R1), Validation of analytical procedures: text and methodology, 2005.

[22] Analytical Methods Committee, Is my calibration linear? Analyst 9 (1994) 2363–2366.

[23] Reviewer guidance, Validation of Chromatographic Methods, Center for Drug Evaluation and Research, US Food and Drug Administration, USA, 1994.

[24] G.A. Shabir, Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, J. Chromatogr. A 987 (2003) 57–66.

[25] International Conference on Harmonizsation (ICH) guidelines Q5C, Quality of biotechnological products: stability testing of biotechnological/biological products, 1995.

[26] Agilent Technologies, Evaluating System Suitability CE, GC, LC and A/D ChemStation Revisions: A.03.0x- A.08.0x, ⟨http://www.chem.agilent.com/Library/Support/Documents/a10424.pdf⟩.

[27] Agilent Technologies, Peak Purity Analysis in HPLC and CE Using Diode-array Technology, Publication Number 5988-8647EN, Waldbronn, Germany, 2003.

[28] Y. Chen, C.T. Mant, R.S. Hodges, Temperature selectivity effects in reversed-phase liquid chromatography due to conformation differences between helical and non-helical peptides, J. Chromatogr. A 1010 (2003) 45–61.

[29] L. Cuadros-Rodríguez, M.G. Bagur-González, M. Sánchez-Viñas, et al., Principles of analytical calibration/quantification for the separation sciences, J. Chromatogr. A 1158 (2007) 33–46.

[30] A. Hawe, M. Wiggenhorn, M. Van de Weert, et al., Forced degradation of therapeutic proteins, J. Pharm. Sci. 101 (2012) 895–913.

[31] H. Liu, G. Gaza-Bulseco, D. Faldu, et al., Heterogeneity of monoclonal antibodie, J. Pharm. Sci. 97 (2008) 2426–2447.

[32] B.M. Teska, C. Li, B.C. Winn, et al., Comparison of quantitative spectral similarity analysis methods for protein higher-order structure confirmation, Anal. Biochem. 434 (2013) 153–165.

[33] M.C. Manning, D.K. Chou, B.M. Murphy, et al., Stability of protein pharmaceuticals: an update, Pharm. Res. 27 (2010) 544–574.

[34] P. Qi, D.B. Volkin, H. Zhao, et al., Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form, J. Pharm. Sci. 98 (2009) 3117–3130.

[35] J.F. Shen, Y.M. Kwong, G.R. Keck, et al., The application of tert-Butylhydroperoxide oxidization to study sites of potential methionine oxidization in a recombinant antibody, Tech. Protein Chem. 7 (1996) 275–284.