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Article

Quantitation of certolizumab pegol by validated liquid chromatography methods

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Certolizumab pegol (CZP) is a Fab' fragment of the humanized antibody with anti-TNF-α activity that is indicated as therapy for Crohn's disease and rheumatoid arthritis. Using a BioSep-SEC-S3000 column (300 x 4.6 mm i.d., 5 µm particle size), a size exclusion liquid chromatography (SEC) method was developed. Mobile phase A consisted of 100 mM monobasic sodium phosphate and 200 mM sodium chloride (pH 7.0), while mobile phase B was ethanol (95:5, v/v), and the analysis was performed using a diode array detector (DAD) set to 214 nm and a flow rate of 0.5 ml min^{-1}. In addition, a reversed-phase liquid chromatography (RP-LC) method based on gradient elution was developed on a Zorbax 300 SB C18 column (150 mm x 4.6 mm i.d., 3.5 µm particle size) kept at 80 °C. Mobile phase A was 0.1% (v/v) TFA in ultrapure water, and mobile phase B was a mixture of propanol, acetonitrile, ultrapure water and TFA (70 + 20 + 9.9 + 0.1, v/v) operated at a flow rate of 1.0 ml min^{-1}, and DAD was applied at 214 nm. CZP elution was achieved with retention times of 5.6 min and 9.0 min for SEC and RP-LC, respectively.

Keywords: Certolizumab pegol. Monoclonal antibody. Size exclusion liquid chromatography. Reversed-phase liquid chromatography. Validation.

INTRODUCTION

Monoclonal antibodies (mAbs) have become very relevant in the pharmaceutical market. Due to their high specificity, these biomolecules have advantages such as high efficacy, wide therapeutic range, and few side effects (Fekete, Veuthey, Guillarme, 2017; Shepard *et al*., 2017).

Certolizumab pegol (CZP) is a polyethylene glycol (PEG)-attached recombinant humanized antibody Fab' fragment with affinity for human tumour necrosis factor alpha (TNF-α). It is expressed in *Escherichia coli* and has a molecular mass of approximately 47.8 kDa. CZP has 214 amino acids in the light chain and 229 amino acids in the heavy chain. The pegylated biomolecule has a total molecular mass of approximately 91 kDa, with an increased plasma half-life compared to that of the unmodified molecule (Horton, Walsh, Emery, 2012; Ueda, 2014).

Clinically, this biopharmaceutical is approved for the treatment of a variety of chronic inflammatory illnesses, including axial spondyloarthritis (axSpA), Crohn's disease (CD), psoriatic arthritis (PsA), and rheumatoid arthritis (RA). CZP can be administered as monotherapy or in combination with disease-modifying anti-rheumatic medicines, such as methotrexate (Launois *et al*., 2011; EMA, 2014).

The mechanism of action of CZP is based on immune system suppression by reducing the activity of TNF-α, a significant inducer of inflammation. Specifically, CZP binds with strong affinity and selectivity to TNF-α, neutralizing both soluble and membrane-bound TNF-α and inhibiting the signalling of the proinflammatory

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cascade; thus, CZP is considered an anti-TNF-α agent (Deeks, 2016; Mitoma *et al*., 2016).

The characterization of mAbs presents many challenges in comparison to conventional chemical drugs. According to the complex nature of the protein and significant inherent heterogeneity, these biological factors must be assessed and characterized using different analytical techniques (Navas *et al*., 2013; Fekete, Veuthey, Guillarme, 2017). Chromatographic techniques are widely employed to investigate the purity, concentration, and stability of mAbs, which can also easily form aggregates during their purification, formulation, packaging, and storage processes. One of the most challenging problems in pharmaceutical monoclonal antibody formulations is protein aggregation, which is directly associated with the quality of the final product, wherein high-molecular-weight aggregates form and can induce immunogenicity (Hernández-Jiménez *et al*., 2018). Size exclusion liquid chromatography (SEC) is commonly used and acknowledged as the best analytical methodology for the quantitation of the native protein and to monitor potential aggregation because it allows for characterization with minimal impact on the conformational structure (Stamm *et al*., 2013; Shahbazi *et al*., 2017; Perobelli *et al.,* 2018).

Moreover, reversed-phase liquid chromatography (RP-LC) using stationary phases with a large pore size (300 Å), long alkyl chains (C8 and C18), elevated column temperatures, and a solvent system with ion pairing agents is the most appropriate technique to quantify mAbs and a very efficient method for analysing intact proteins. With a shorter retention time, RP-LC is more advantageous than ion-exchange chromatography (IXC) (Martínez-Ortega *et al*., 2016). Chromatographic methods have been applied for the analysis of mAbs such as bevacizumab, cetuximab, and denosumab (Paul *et al*., 2012; Navas *et al*., 2013; Martínez-Ortega *et al*., 2016; Hernández-Jiménez *et al*., 2018; Perobelli *et al*., 2018). ELISA is a technique also widely used for the assessment of mAbs; however, these immunoassays demonstrated low levels of precision and robustness when compared to physicochemical methods (Navas *et al*., 2013).

It is very well known that after the development of novel analytical techniques, validation is necessary to ensure the reproducibility and reliability of the outcomes, demonstrating that the method is suitable for the planned purpose, as suggested by the official guides (ICH 2005; Shabir *et al*., 2007).

This study's objective was to design and validate size exclusion and reversed-phase liquid chromatography techniques for quantifying the monoclonal antibody certolizumab pegol in biopharmaceutical formulations and to assess the high-molecular-weight (HMW) forms and fragments. Thus, provide new analytical tools that can be applied to assess the quality and ensure the bioactivity and safety of this monoclonal antibody.

MATERIAL AND METHODS

Chemicals and reagents

Commercially available batches of biopharmaceutical formulations of Cimzia® AstraZeneca (Cotia – São Paulo, Brazil), comprising a 200 mg mL^{-1} solution in a prefilled syringe, were purchased from commercial sources during their shelf-life period, stored at 2–8 °C, and shielded from light. Monobasic sodium phosphate, sodium chloride, trifluoroacetic acid (TFA) of HPLC-grade, and 30% v/v hydrogen peroxide (H_2O_2) were purchased from Merck (Darmstadt, Germany). Ethanol, acetonitrile, and propanol (HPLC-grade) were acquired from J.T. Baker (Phillipsburg, NJ, USA). Other chemicals were of HPLC grade or analytical grade. Analyses were performed using ultrapure water obtained in an Elix 3 attached to a Milli-Q Gradient A10 system from Millipore (Bedford, MA, USA). The solutions were filtered using a 0.22 µm Millex filter (Millipore) and sonicated by a Tecnal ultrasound water bath (São Paulo, Brazil).

Apparatus

A Shimadzu LC system (Kyoto, Japan) with a CBM-20A system controller, an LC-20 AD pump, a DGU-20As degasser, an SIL-20ACHT autosampler, a CTO-20A column oven, and an SPD-M20A diode array detector (DAD) was used to carry out analyses. The LC Solution Version 1.22 SP1 program was used to automatically integrate peak areas. A Thermo Orion model 420 pH meter (Beverly, MA, USA) was used to measure the pH of the solutions.

Standard and sample solutions

As an official reference substance was not available, samples of three commercial batches of Cimzia® were combined for quantifying samples during method development. This material was then designated as the representative CZP biological reference substance (BS–CZP). Solutions were prepared daily by diluting BS-CZP in ultrapure water to a final concentration of 20 mg mL^{-1}. In addition, the samples of biopharmaceutical products (Cimzia® 200 mg mL^{-1}) were prepared immediately before analyses through dilution (1:10, *v/v*) in ultrapure water to 20 mg ml^{-1} (working solution concentration).

Procedures

SEC Method

SEC experiments were performed on a size-exclusion Phenomenex (Torrance, USA) BioSep SEC-s3000 column (300 mm x 4.6 mm i.d., with a pore size of 290 Å and particle size of 5 µm) maintained at 35 °C. To protect the analytical column, a security guard holder was used. Mobile phase A consisted of 100 mmol L^{-1} monobasic sodium phosphate and 200 mmol L^{-1} sodium chloride at pH 7.0. Mobile phase B was ethanol (95:05, v/v). Elution was performed in isocratic mode at a flow rate of 0.5 ml min^{-1} using DAD detection at 214 nm. For both the BS-CZP and the samples, an injection volume of 1 µL of a solution containing 20 mg mL^{-1} was utilized.

RP-LC Method

A reversed-phase Zorbax 300 SB C18 column (150 mm x 4.6 mm i.d., with a pore size of 300 Å and particle size of 3.5 µm) from Agilent (Santa Clara, CA, USA) was used for the RP-LC method and was kept at 80 °C in a column oven. A guard column was used. The mobile phase flow rate was 1.0 ml min^{-1} and consisted of a gradient of 0.1% (v/v) TFA in ultrapure water (mobile phase A) and a mixture of propanol, acetonitrile, ultrapure water and TFA (70 + 20 + 9.9 + 0.1, v/v) (mobile phase B). The chromatographic run started at 30% mobile phase B. A linear gradient was applied from 0.01 to 7.00 minutes, then increased to 40% mobile phase B at minute 7.00, and remained constant for 3.50 min. From 10.50 to 11.00 min, the concentration of mobile phase B was decreased to 30%. At the end of the analysis, initial run conditions were applied for an additional 4 min, achieving a final run time of 15 minutes. The injection volume was 1 µL of a solution containing CZP at a concentration of 20 mg mL^{-1} for both BS-CZP and samples, and chromatograms were obtained at 214 nm using DAD detection.

Validation of methods

The validation of the chromatographic methods was performed according to the official International guidelines ICH Q2(R1). In these studies, samples of medicinal products with a label claim of 200 mg mL^{-1} were used. The following validation parameters were determined according to the guidelines: specificity, linearity, precision, accuracy, detection limit (DL), quantitation limit (QL), robustness, and system suitability test (ICH, 2005).

Specificity

For SEC, a BS-CZP solution (20 mg mL^{-1}) was submitted to stress conditions. The thermal conditions were adjusted by subjecting the analyte to neutral hydrolysis at 50 °C for 24 h. In addition, sample solutions were subjected to stress using acid (0.1 M HCl), alkaline (0.1 M NaOH), and oxidative media (10% H_2O_2). For RP-LC, BS-CZP solutions (20 mg mL^{-1}) were stressed in acidic (1 M HCl), basic (0.1 M NaOH), oxidative (10% H_2O_2), and photolytic media. The effect of UV light was investigated by exposure to 200 W h/m^2 near ultraviolet light in a photostability chamber. The stress agents were applied at a concentration of 10% (v/v), and the samples were analysed 24 h after the addition (Navas *et al.*, 2013; Hernández-Jiménez *et al.*, 2018). Then, aliquots were assessed without further dilutions to avoid changes. The chromatograms of stressed samples were compared with those of the nondegraded BS-CZP. Moreover, a placebo containing an in-house combination of the formulation excipients (sodium acetate, sodium chloride, and water at pH 4.7) was analysed to identify any potential interference from the formulation (EMA, 2014). The specificity of the methods was also established

by determining the peaks purity (CZP and degraded forms) by overlaying the spectra captured with a DAD detector using LC Solution Version 1.22 SPI software.

Linearity

For both chromatographic methods, this parameter was investigated by constructing three independent analytical curves, each with seven dilutions of the BS-CZP solution (1, 5, 10, 15, 20, 30 and 40 mg mL^{-1}), injected in triplicate to verify the repeatability of the responses. Peak areas with relative standard deviation (RSD) < 2% were utilized with respective concentrations to generate a plot and subjected to regression analysis by the least-squares method to calculate the calibration equation and determination coefficient (r^2) > 0.999. The chromatographic data were processed using the statistical software program STATGRAPHICS Centurion XVII Version 17.2.05 (Statistical Graphics System, 2017, Warrenton, VA, USA).

Precision and accuracy

Repeatability (intraday) and intermediate precision (between-analysts and inter-days) were used to determine the precision. The repeatability was examined by six evaluations of BS-CZP under the same experimental conditions on the same day. The between-analysts precision was measured by analysing sample data collected by different analysts in the same laboratory. The inter-days precision was determined by the analysis of two samples of the biopharmaceutical formulation on three different days. Accuracy was tested by analysis of the in-house mixture of excipients spiked with a known quantity of BS-CZP to produce solutions at concentrations of 16, 20, and 24 mg mL^{-1}, equivalent to 80, 100, and 120% of the working solution concentrations, respectively. Accuracy was calculated as the percentage of drug recovered.

Detection and quantitation limits

The detection limit (DL) and the quantitation limit (QL) were calculated as defined by the International Council for Harmonisation (ICH) based on the standard deviation of the response and the slope, estimated from the three independent analytical curves of CZP determined by a linear-regression model. The DL and QL factors, 3.3 and 10, were multiplied by the standard deviation of the y-intercept and then divided by the slope (ICH, 2005; ANVISA, 2017).

Robustness

Robustness is a parameter typically used in the development of analytical methods to reflect their capacity to resist minor and intentional alterations in the analytical conditions. Therefore, the methods were evaluated by analysing identical samples containing 20 mg mL^{-1} CZP under one-variable-at-a-time (OVAT) conditions, as observed in Tables II and III. Furthermore, the robustness parameters were also assessed using an experimental design by the multi-variable-at-a-time (MVAT) methodology. The approach chosen was a two-level (i.e., 2^{4-1} or eight experiments), fractional factorial design carried out with four factors. The factors selected for SEC and RP-LC and their high and low levels are presented in Table IV. To assess the significance of the effects, the obtained results were processed using the Minitab 18.1 statistical program and analysed by Pareto charts, which consist of bars with lengths proportionate to the absolute value of the estimated effect divided by the pseudo standard error (Lenth, 1989). The chart contains a vertical line at the critical t value ($\alpha = 0.05$), and effects below this line are considered non-significant. The stability of the CZP samples was examined during storage, and any changes in the chromatographic pattern were compared to newly prepared samples.

System suitability test

Five replicate injections of 1 µL of BS-CZP solutions containing 20 mg mL^{-1} were used in the system suitability test to evaluate the suggested parameters.

Analysis of CZP in biopharmaceutical formulations

To quantify CZP and detect HMW forms and fragments in biopharmaceutical products by chromatographic methods, five batches of Cimzia® (commercially available for clinical use) were labelled

from 1 to 5 and diluted to the required concentrations in ultrapure water. Analyses were performed in triplicate, and the percentages were calculated against BS–CZP.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

Experiments were carried out to define the ideal conditions, including the column, mobile phase composition and pH, oven temperature, flow rate, elution mode, and wavelength, to develop sensitive, selective, robust, and reproducible chromatographic methods.

For the SEC method, mobile phases composed of potassium phosphate, phosphoric acid, and sodium phosphate were evaluated. Furthermore, the use of sodium chloride as an additive was investigated. The mobile phase containing monobasic sodium phosphate and sodium chloride demonstrated greater sensitivity and a shorter retention time with adequate resolution. The effect of the mobile phase salt concentration was investigated, and optimal conditions were obtained with 100 mmol L^{-1} monobasic sodium phosphate and 200 mmol L^{-1} sodium chloride. The mobile phase pH was investigated from 6.0 to 7.5, with the optimal outcomes obtained at pH 7.0. The influences of organic modifiers – ethanol, methanol, and isopropanol – were also evaluated at concentrations ranging from 5 to 15% v/v, and an optimal chromatographic profile was observed by the addition of ethanol at 5% v/v. In addition, Yarra SEC–2000, BioSep SEC–s2000, and BioSep SEC–s3000 columns were tested, and the BioSep SEC–s3000 column was selected. Using a DAD, the ideal wavelength was determined as 214 nm. Figure 1a shows a typical chromatogram exhibiting the symmetrical peak related to CZP.

To reduce possible electrostatic interactions, the analysis of biopharmaceutical proteins is usually performed by adding a large amount of salt to the mobile phase. Furthermore, analysis of pegylated proteins can induce rapid degradation of SEC columns, peak tailing and loss of resolution, observed after approximately 20 injections. Salts such as sodium chloride have been shown to be effective in reducing unwanted ionic interactions between the column packing surface and the protein. Other additives may also be needed, and it is well known that polar organic compounds, such as ethanol, can reduce protein packaging interactions (Ratto *et al.*, 1997; Goyon *et al.*, 2017; Shahbazi *et al.*, 2017).

For RP-LC, mobile phases containing 0.1% TFA in water (v/v) and 0.1% TFA in acetonitrile (v/v), TRIS buffer, monobasic sodium phosphate, sodium sulphate anhydrous and a mixture of isopropanol, acetonitrile, ultrapure water and TFA (70 + 20 + 9.9 + 0.1) (v/v) were tested. Moreover, other solvents, such as methanol and propanol, with high eluotropic strength coefficients were evaluated. The mobile phase consisting of a mixture of propanol, acetonitrile, ultrapure water and TFA (70 + 20 + 9.9 + 0.1) (v/v) led to improved sensitivity and resolution and a shorter retention time. The effect of the column temperature was evaluated between 30 and 80 °C, and the chromatographic profiles began to improve from 60 °C. At 80 °C, the chromatographic profile showed higher resolution between the peaks and greater symmetry. The isocratic and gradient elution modes were also evaluated, and better symmetry and peak resolution were observed using a gradient of mobile phase concentrations. In addition, Vydac 214TP C4, Jupiter C4, Zorbax 300 SB C8 and Zorbax 300 SB C18 columns were tested based on their separation potential, and the Zorbax 300 SB C18 column was selected. The optimal 214 nm wavelength was chosen for the DAD. A typical chromatogram exhibiting the symmetrical peak related to CZP is illustrated in Figure 2a.

The conditions of the RP-LC method selected with long alkyl chains in the stationary phase; a high column temperature combined with a high eluotropic strength coefficient of organic solvents; and ion pairing agents, such as trifluoroacetic acid (TFA), were selected according to the chromatographic procedures suggested for monoclonal antibodies (Navas *et al.*, 2013; Martínez-Ortega *et al.*, 2016).

Validation of LC methods

Specificity

The degradation conditions of the SEC method were evaluated, and a 7.6% reduction in the peak area

was observed under acidic conditions; additionally, one extra peak was identified at 4.9 min (Figure 1b). Under basic conditions, there was a 21.6% loss of the area, with four other peaks observed at 4.9, 6.2, 7.2, and 7.6 min (Figure 1c). Oxidative degradation revealed a reduction of 10.5% of the CZP peak area, with a peak for H_2O_2 appearing at 8.8 min and extra peaks at 4.9 and 7.6 min (Figure 1d). Heated conditions showed a decrease of 13.7% of the main peak area, and three extra peaks were identified at 4.9, 7.2, and 7.6 min (Figure 1e). Moreover, the placebo injection showed only the baseline of the chromatogram, with no evident peaks (Figure 1f), demonstrating that there was no interference from the excipients. In addition, investigations were performed by the RP-LC method, demonstrating a 12.8% decrease in the area and one additional peak at 2.5 min under acidic conditions (Figure 2b). Under basic conditions, a 20.6% reduction in the peak area was observed, and three other peaks were detected at 2.5, 3.2, and 7.6 min (Figure 2c). Oxidative degradation showed a reduction of 12.1% of the CZP peak area, with one peak attributed to peroxide at 1.7 min and additional peaks at 2.5 and 9.6 min (Figure 2d). Photolytic conditions demonstrated a reduction of 12.6% of the nonaltered peak area, with three additional peaks at 3.1, 3.3, and 8.4 min (Figure 2e). No evident peaks were detected by the injection of the placebo (Figure 2f), demonstrating that there was no interference from the formulation compounds. Moreover, the peak purity was determined, indicating that the peaks were spectrally pure, and there was no chromatographic or spectroscopic evidence that impurities were present. The peak purity index stayed in the 0.9999-1 range, showing that there were no coeluting peaks and that the formulation excipients did not affect the analysis of the CZP, which attested to the specificity of the techniques.

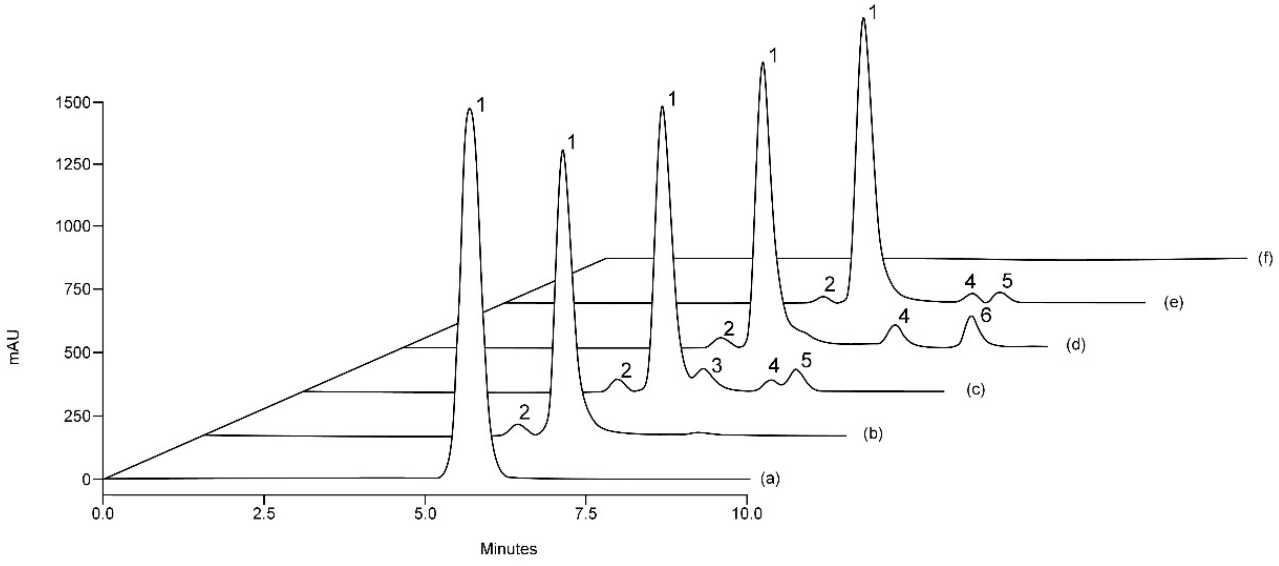

FIGURE 1 - SEC chromatograms of degradation studies. (a) CZP 20 mg mL^{-1} (b)acid; (c)basic; (d)oxidative; (e)thermal; (f) placebo. Peak 1 = CZP; peak 2 = HMW; peaks 3,4 and 5 = LMW; peak 6 = hydrogen peroxide.

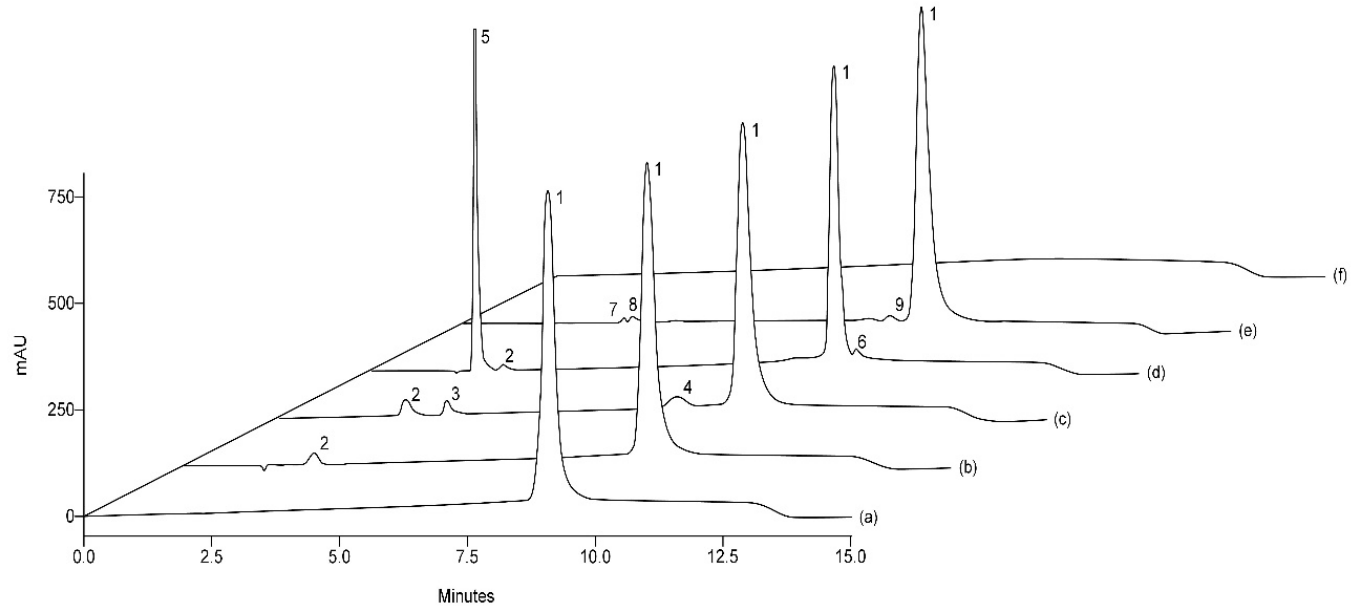

FIGURE 2 - RP-LC chromatograms of degradation studies. (a) CZP 20 mg mL^{-1} (b)acid; (c)basic; (d)oxidative; (e)photolytic; (f) placebo. Peak 1 = CZP; peaks 2, 3, 4, 6, 7, 8 and 9 = fragments; peak 5 = hydrogen peroxide.

Linearity

For the SEC technique, the analytical curves were found to be linear over a concentration range of 1 to 40 mg mL^{-1}. The calculation of the determination coefficient resulted in $r^2 = 0.9993$, y = 1237158,739x - 201746,749, where x represents the concentration and y represents the peak absolute area. Similar to the RP-LC method, the analytical curves were linear over a concentration range of 1 to 40 mg mL^{-1}. The determination coefficient obtained was $r^2 = 0.9997$, y = 581209,0009x + 204086,2547, where x represents the concentration and y represents the peak absolute area.

Precision and accuracy

The relative standard deviation (RSD%) was used to assess the precision of the LC techniques. For repeatability, the obtained RSD value was 1.13% for the SEC method and 1.78% for the RP-LC method. The inter-days precision showed RSD values of 0.51 and 0.31% and 0.70 and 0.99% for the SEC and RP-LC methods, respectively; the RSD values for between-analysts were 0.25 and 0.17% and 0.49 and 0.69% for the SEC and RP-LC methods, respectively. The accuracy of the methods was studied, and the absolute mean was 100.50% and 99.80% for the SEC and RP-LC methods, respectively, which demonstrated results according to the recommendations, as observed in Table I.

TABLE I - Accuracy of SEC and RP-LC methods for CZP

Method	Nominal concentration (mg mL^{-1})	Mean concentration measured[a] (mg mL^{-1})	RSD[b] (%)	Accuracy (%)	Bias[c] (%)
SEC	16	16.14	0.50	100.85	0.85
	20	20.05	0.30	100.27	0.27
	24	24.09	0.07	100.39	0.39

TABLE I - Accuracy of SEC and RP-LC methods for CZP

Method	Nominal concentration (mg mL^{-1})	Mean concentration measured[a] (mg mL^{-1})	RSD[b] (%)	Accuracy (%)	Bias[c] (%)
RP-LC	16	15.87	1.85	99.18	-0.82
	20	20.06	1.46	100.31	0.31
	24	23.98	0.54	99.90	-0.10

[a]Mean of three replicates

[b]RSD = Relative standard deviation

[c]Bias = [(Measured concentration - Nominal concentration)/Nominal concentration] x 100

Detection and quantitation limits

For the SEC and RP-LC methods, the DL and QL were estimated as 0.14 and 0.41 mg mL^{-1} and 0.06 and 0.17 mg mL^{-1}, respectively.

Robustness

Tables II and III show the outcomes and experimental range of the selected variables examined for robustness using the OVAT methodology, indicating that they stayed within the accepted deviation (RSD < 2%), with nonsignificant differences ($p > 0.05$), as determined through analysis of variance. Furthermore, the robustness was evaluated using the MVAT methodology, and the magnitude of the influences was assessed by employing Pareto charts. The parameters are listed in Table IV. The bars were generated and graded based on the strength of the effects, with the strongest at the top, indicating that they were not statistically significant ($\alpha > 0.05$), as illustrated in Figure 3 and Figure 4. In addition, the samples were analysed, and the data revealed that CZP was stable for 24 hours in the autosampler and 48 hours when kept at 2-8 °C.

TABLE II - Chromatographic conditions and range investigated during robustness testing using the one–variable–at–a–time (OVAT) procedure for the SEC method

Variable	Range investigated	CZP[a] (%)	RSD[b] (%)	Optimized value
Monobasic sodium phosphate concentration	90 mmol L^{-1}	99.11	0.21	100 mmol L^{-1}
	100 mmol L^{-1}	99.93	0.10	
	110 mmol L^{-1}	99.37	0.45	
Flow rate (mL/min)	0.4 mL min^{-1}	100.83	0.06	0.5 mL min^{-1}
	0.5 mL min^{-1}	99.30	0.20	
	0.6 mL min^{-1}	100.49	0.48	
Mobile phase pH	6.8	100.16	0.26	7.0
	7.0	99.93	0.10	
	7.2	99.85	0.14	

TABLE II - Chromatographic conditions and range investigated during robustness testing using the one–variable–at–a–time (OVAT) procedure for the SEC method

Variable	Range investigated	CZP[a] (%)	RSD[b] (%)	Optimized value
Ethanol (%)	4	99.69	0.55	5
	5	98.12	0.16	
	6	98.30	0.13	
Temperature (ºC)	33	100.10	0.32	35
	35	99.59	0.14	
	37	99.10	0.08	
Solution stability	Autosampler 24 h	101.45	0.28	-
	2 – 8 ºC 24 h	101.01	1.27	-
	2 – 8 ºC 48 h	99.18	0.55	-
Wavelength (nm)	190 – 320			214

[a]Mean of three replicates.

[b]RSD = Relative standard deviation.

TABLE III - Chromatographic conditions and range investigated during robustness testing using the one–variable–at–a–time (OVAT) procedure for the RP-LC method

Variable	Range investigated	CZP[a] (%)	RSD[b] (%)	Optimized value
Gradient: maximum percentage of mobile phase B (%B)	39 (% B)	100.61	1.45	40 (% B)
	40 (% B)	100.45	0.99	
	41(% B)	100.27	1.08	
Flow rate (mL/min)	0.9 mL min^{-1}	101.16	1.33	1.0 mL min^{-1}
	1.0 mL min^{-1}	100.94	0.47	
	1.1 mL min^{-1}	101.72	0.96	
TFA (%)	0.09	100.12	1.33	0.10
	0.10	100.85	0.44	
	0.11	101.08	0.62	
Temperature (ºC)	79	101.03	1.34	80
	80	100.76	0.87	
	81	100.84	1.68	
Solution stability	Autosampler 24 h	99.83	1.13	-
	2 – 8 ºC 24 h	99.12	0.41	-
	2 – 8 ºC 48 h	98.64	0.96	-
Wavelength (nm)	190 – 320			214

[a]Mean of three replicates.

[b]RSD = Relative standard deviation.

System suitability test

The system suitability was evaluated for the SEC, and the RSD values measured for retention time, peak symmetry, and peak area were 0.09, 1.77, and 1.18%, respectively, and the number of theoretical plates was 33102.91, with an RSD of 1.47%. The suitability of the system for RP-LC was tested, and the RSD results determined for the retention time, peak symmetry and peak area were 0.08, 0.28, and 1.50%, respectively. The number of theoretical plates was 6047.54, with an RSD of 1.93%. The parameters examined were deemed adequate (RSD < 2%).

TABLE IV - Variables selected as factors and levels investigated during the robustness testing using the multi–variable–at–a–time (MVAT) procedure for the chromatographic methods

Method	Factor	Optimal	Low level	High level
SEC	Mobile phase pH	7.0	6.8	7.2
	Flow rate (mL min^{-1})	0.5	0.4	0.6
	Column temperature (°C)	35	33	37
	Wavelength (nm)	214	212	216
RP-LC	Flow rate (mL min^{-1})	1.0	0.9	1.1
	Column temperature (°C)	80	79	81
	Gradient: mobile phase B (%)	40	39	41
	Wavelength (nm)	214	212	216

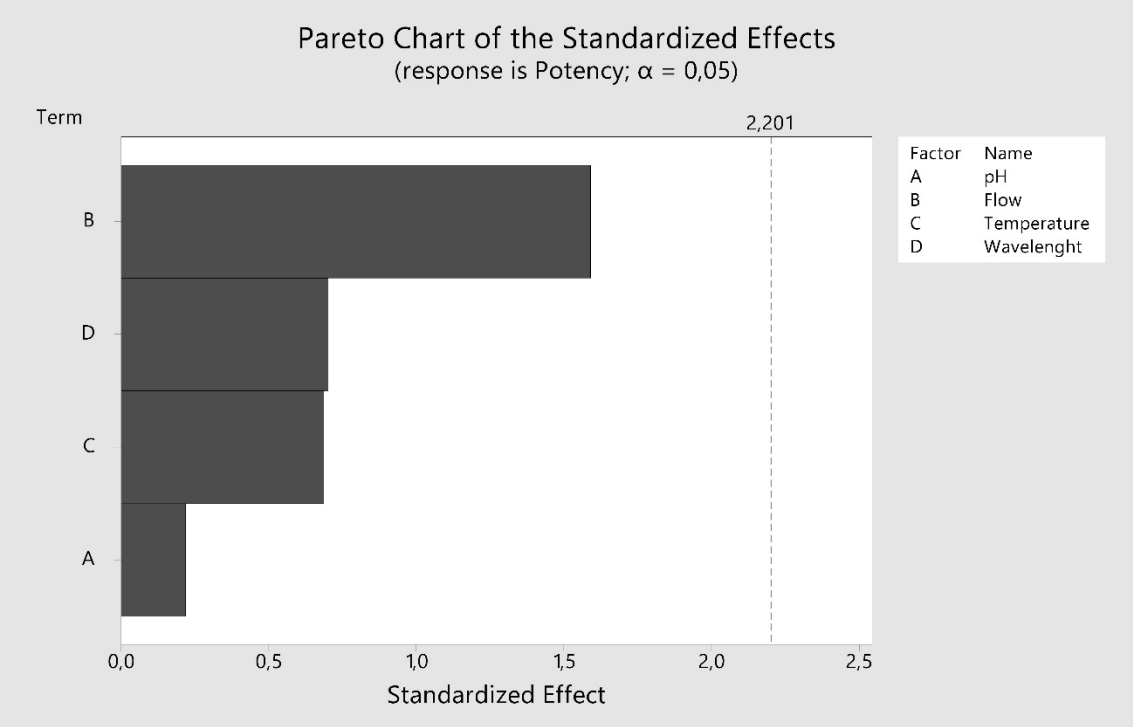

FIGURE 3 - Pareto graph showing the impact of the factors and their interactions studied during the SEC method's robustness testing MVAT.

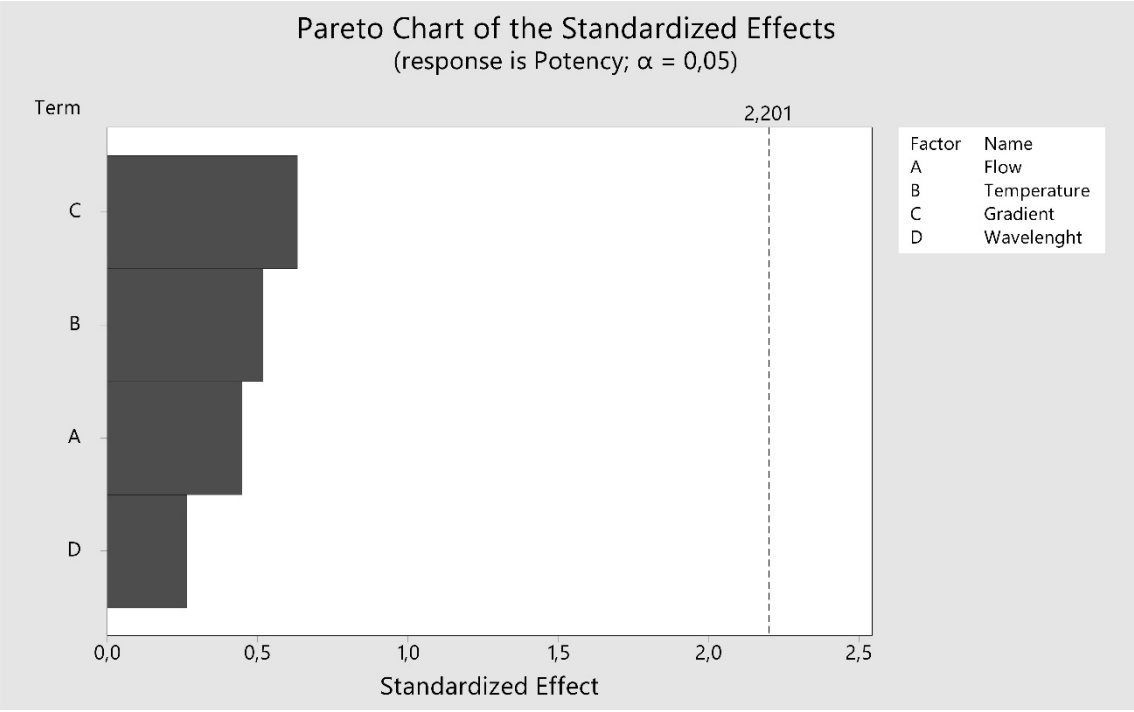

FIGURE 4 - Pareto graph showing the impact of the factors and their interactions studied during the RP-LC method's robustness testing MVAT.

Application of the methods

The chromatographic methods developed were used for the analyses of CZP in five different batches of commercially available biopharmaceutical products, as demonstrated in Table V, which will be helpful for the regulatory authorities when performing the studies to establish the reference substance. For the SEC method, the results were found in the range of 99.18-101.47% with HMW forms between 0.77 and 1.83%. For the RP-LC method, the concentration results ranged from 98.72-100.24% related to the label claimed with fragments constituting between 0.87 - 1.51%. In addition, as indicated by the specificity, biopharmaceutical samples were intentionally subjected to degradation conditions, and the SEC and RP-LC methods were successfully applied to assess the percentages of the high-molecular-weight forms and fragment fractions of CZP. It is worth mentioning that the degraded forms caused by stressors affect the quality of the product and can produce side human effects; thus, the technologies represent an improvement for the characterization of the biomolecule.

TABLE V - Quantification of CZP, HMW forms and fragments in biopharmaceutical formulations by the LC methods

Sample	Label claim (mg mL^{-1})	SEC		RP-LC	
		HMW[a] (%)	Content[a] (%)	Fragments[a] (%)	Main Peak[a] (%)
1	200	1.12	99.18	0.94	98.93
2	200	0.77	100.23	1.02	99.25
3	200	1.83	99.17	0.87	98.72

TABLE V - Quantification of CZP, HMW forms and fragments in biopharmaceutical formulations by the LC methods

Sample	Label claim (mg mL^{-1})	SEC		RP-LC	
		HMW[a] (%)	Content[a] (%)	Fragments[a] (%)	Main Peak[a] (%)
4	200	0.84	101.47	1.33	100.24
5	200	0.92	99.22	1.51	99.09
Mean	-	1.10	100.03	1.13	99.25
SD[b]	-	0.43	1.08	0.27	0.59

[a]Mean of three replicates.

[b]SD standard deviation.

CONCLUSION

The results of the validated SEC and RP-LC chromatographic methods show that the methods are specific, sensitive, accurate and robust. Thus, these stability-indicating methods can be applied for the quantitation of CZP, its aggregated forms and its fragments in biopharmaceutical formulations. In addition, they can be used to assess the biomolecule during biotechnology procedures and successive purification stages, as well as to monitor the stability and batch-to-batch consistency of bulk and final biotechnology-derived products. In the near future, the procedures could be applied to enable studies of biosimilar CZP.

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