Journal of Chromatography B, 955–956 (2014) 26–33

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Ion-pair reversed-phase high performance liquid chromatography method for the quantification of isoaspartic acid in a monoclonal antibody

Wolfram Kern [1], Robin Mende [1], Blandine Denefeld, Mirko Sackewitz, Dirk Chelius *

Biologics Process Research and Development, Novartis Pharma, Postfach, 4002 Basel, Switzerland

ARTICLE INFO

Article history:
Received 15 October 2013
Received in revised form 22 January 2014
Accepted 2 February 2014
Available online 18 February 2014

Keywords:
Isomerization
Monoclonal antibody
Mass spectrometry
Aspartic acid
Reversed-phase high performance liquid chromatography
Critical quality attributes
Quality by design
Developability

ABSTRACT

Isomerization of aspartic acid residues is one of the major causes of chemical degradation during the shelf life of biological pharmaceuticals. Monoclonal antibody biopharmaceuticals are typically stored at mildly acidic pH conditions, which can lead to the isomerization reaction. The mechanism of this non-enzymatic chemical reaction has been studied in great detail. However, the identification and quantification of the isomerization sites in a given protein still remains a challenge. We developed an ion-pair reversed-phase HPLC method for the separation of an intact monoclonal antibody variant containing a single isoaspartic acid residue from its native counterpart. We identified and characterized the isomerization site using ion-pair reversed-phase HPLC mass spectrometry methods of the reduced and alkylated antibody and the enzymatically cleaved antibody. Lys-C followed by Asp-N digestion of the antibody was used for the identification of the isomerization site. Electron transfer dissociation (ETD) mass spectrometry was used to confirm the isomerization site at a DY motif at an aspartic acid residue in the CDR-H3 region of the antibody. Tyrosine at the C-terminus of an aspartic acid residue is typically not regarded as a hot spot for isomerization. Our findings suggest that it is not possible to predict isomerization sites in proteins with confidence and all aspartic acid residues located in the CDR regions of antibodies must be considered as potential isomerization site due to the solvent exposure or the flexibility of these regions of the molecule. Additionally, the effect of the pH on the isomerization rate was evaluated using the ion-pair reversed-phase HPLC method, showing that at a lower pH the isomerization rate is faster. Storage at 25 °C for 6 months resulted in an increase of the amount of isoaspartic acid to 6.6% at pH 5.4, 6.0% at pH 5.8, and 5.6% at pH 6.2.

1. Introduction

The control strategy of monoclonal antibody biopharmaceuticals consists of a multitude of physico-chemical analytical methods, used for release analytics and to monitor the stability of the molecules during their shelf life. Methods need to be developed, addressing all critical quality attributes of a molecule, typically determined during intensive characterization of the molecule. Temperature stress, among other stress conditions, is used to determine the degradation profile of the molecule, and to evaluate which analytical methods can be used for the detection and quantification of the degradation products. Degradation products include chemical degradation like oxidation, deamidation, isomerization, cyclization, as well as physical degradation like the formation of aggregates or fragments.

Isomerization of aspartic acid residues is one of the causes of chemical degradation during the shelf life of biological pharmaceuticals. Monoclonal antibodies are typically formulated in neutral to mildly acidic buffers (pH range between 5.2 and 7.2) [1], which are conditions that can lead to the isomerization reaction. Isomerization of aspartic acid residue in proteins has been characterized in great detail. Aspartic acid isomerizes to isoaspartic acid through a cyclic imide intermediate [2]. The effect of the pH on the non-enzymatic isomerization has been studied as well, showing that the reaction is favored at low pH values [3]. The isomerization rate depends largely on the flexibility and solvent exposure of the isomerization site. Small residues like glycine following the

Abbreviations: DTT, Dithiothreitol; EDTA, Ethylendiamintetraacetate; ESI, Electrospray ionization; ETD, Electron transfer dissociation; IP-RP HPLC, Ion-pair reversed-phase high performance liquid chromatography; mAb, monoclonal antibody; TFA, Trifluoroacetic acid.

* Corresponding author. Tel.: +41 0 61 6964663; fax: +41 0 61 6963776.
E-mail address: dirk.chelius@novartis.com (D. Chelius).

[1] Both authors contributed equally to the work.

http://dx.doi.org/10.1016/j.jchromb.2014.02.017
1570-0232/

isomerization site show the fastest isomerization rate and the solvent exposure [2–7].

Identification of isoaspartic acid can be achieved by Edman sequencing [8], by using carboxylmethyltransferase (an enzyme that specifically methylates the isoAsp sites) [9], or by using Asp-N protease, which specifically cleaves at the N-termini of aspartic acids, but not isoaspartic acids [10]. Mass spectrometry techniques have also been successfully employed for the identification of isoaspartic acid residue [11–17].

For the separation of isoaspartic acid residue containing antibodies from their native counterparts, the use of ion exchange chromatography [18] and hydrophobic interaction chromatography [19] have been reported.

Ion-pair reversed-phase (IP-RP)-HPLC methods have been used successfully for the analysis of monoclonal antibodies in the biopharmaceutical industry [20–25]. Typically, cleavage products of the molecules can be separated and quantified using IP-RP-HPLC methods, but smaller molecular modifications like oxidation [26], the formation of pyroglutamic acid [21], and disulfide scrambling [27,28] have also been reported as well.

Isomerization of aspartic acid has been detected in monoclonal antibodies using IP-RP-HPLC methods as well. Rehder et al. [29] reported the separation and identification of a light chain isoform from the native light chain. However, the separation was achieved after reduction on the light chain of the antibody and not on the intact antibody.

Here we show for the first time an IP-RP-HPLC method routinely used for the separation and quantification of an intact monoclonal antibody variant containing a single isoaspartic acid residue. We show the identification of the isoaspartic acid residue in the CDR region of the heavy chain using IP-RP-HPLC mass spectrometry methods of the reduced, alkylated, and enzymatic digested antibody. The identified DY motif as isomerization site highlights the problems in predicting isomerization sites in proteins. The IP-RP-HPLC method was used to determine the isomerization rate and was used during formulation development.

2. Materials and methods

2.1. Materials

Recombinant monoclonal IgG1 antibody was produced and purified at Novartis. Accelerated degradation samples were incubated at 25 °C or 40 °C in formulation buffer for up to 6 months to enrich the structural variants. Endoproteinase Lys-C was purchased from Wako pure chemical (Osaka, Japan), Asp-N was obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade and purchased from Sigma Aldrich and Thermo Scientific.

2.2. Methods

2.2.1. IP-RP-HPLC mass spectrometry of the intact antibody

IP-RP-HPLC was performed on an Agilent 1290 Infinity HPLC system. A Zorbax 300SB-C8 column (2.1 × 150 mm, 3.5 μm particle size, narrow-bore) was used for separation. The column temperature was 64 °C and the solvent flow rate was 0.4 mL/min. The mobile phase consisted of solvent A (water containing 0.05% TFA) and solvent B (acetonitrile containing 0.05% TFA). Separation was achieved over 30 min using a linear gradient from 35% to 42% of solvent B. The UV absorbance was monitored at 280 nm.

The HPLC was directly coupled to a Q-TOF 6530 mass spectrometer (Agilent, Waldbronn, Germany). The HPLC flow of 0.4 mL/min was split to approximately 0.2 mL/min prior to the mass spectrometer ionization source to improve sensitivity. The ESI-TOF mass spectrometer was set to run in positive ion mode. The instrument was calibrated externally prior to the analyses.

The deconvolution of the averaged mass spectra was carried out using the MassHunter analysis software (Agilent, Waldbronn,Germany). The background subtract parameter was 7.00 for the baseline factor. The deconvolution parameters were the following: “peak signal-to-noise” ≥30; “maximum number of peaks”: limited to the largest 100; “calculate average mass using top” 25% of the peak height.

2.2.2. Fraction collection and buffer exchange

Fraction collection was performed on an Agilent 1200 system using the same chromatographic conditions as described above. Twenty chromatographic runs (250 μg injections each) were performed to generate enough material for further analyses. The provided vials were filled with 1 mL formulation buffer to avoid oxidation. Five injections were collected in one set of vials.

The collected fractions were buffer exchanged into formulation buffer five times by using centrifugal filter units 30 kDa (Millipore). Centrifugation with 13,000 rpm in two minutes steps were performed to get a pool of each fraction of about 120 μL. The determination of the concentration by UV at 280 nm was performed with the NanoDrop1000 instrument (Thermo Scientific, Waltham, MA).

2.2.3. Reduction and alkylation

Prior to reduction and alkylation, an exchange (four times) into a pH 8.0 buffer containing 6 M guanidine hydrochloride, 50 mM Tris–HCl, 5 mM Na_2EDTA was performed resulting in 50–100 μg of each sample in 150 μL denaturing solution. The sample was reduced by addition of 1 μL Tris–HCl (1 M, pH 7.5), 1.5 μL 1 M DTT and incubation at 37 °C for 1 h. Alkylation was performed by addition of 3.0 μL 1.0 M Iodoacetamide and incubation for 1 h at room temperature in the dark. The reaction was quenched by adding 1.0 μL 1 M DTT.

2.2.4. IP-RP-HPLC mass spectrometry of reduced samples

The reduced/alkylated samples were separated by IP-RP-HPLC using an Agilent1260 Infinity system. For separation a 2.1 × 150 mm PLRPS column packed with 5 μm particles, 300 Å pore size was used. Eluents were eluent A (water containing 0.1% TFA) and eluent B (0.09% TFA in 70% IPA, 20% ACN, 10% water). Separation was achieved over 37 min using a linear gradient from 32% to 47% of solvent B at a flow rate of 0.2 mL/min. The column was heated to 60 °C.

The HPLC was directly coupled with a Synapt G2S mass spectrometer (Waters, Manchester, UK). The ESI-Q-TOF mass spectrometer was set to run in positive ion mode with capillary voltage of 3000V, sample cone voltage of 80V, m/z range of 600–3000, mass resolution of 18,000 and was tuned for proteins and calibrated externally using sodium iodide.

The deconvolution of the averaged mass spectra was carried out using the MaxEnt algorithm, a part of the MassLynx analysis software (Waters, Manchester, UK). The deconvolution parameters were the following: “max numbers of iterations” are 20; resolution is 1.0 Da/channel; Uniform Gaussian width at half height is 0.5 Da, and the minimum intensity ratios are left 33% and right 33%.

The theoretical average mass of intact antibody and the corresponding mass of the light and heavy chain after reduction/alkylation were calculated using GPMAW 8.10 sr.1 software (Lighthouse Data, Odense, Denmark) based on the protein sequence.

2.2.5. Reduction, alkylation and Lys-C digestion

Prior to digestion, an exchange (four times) into a pH 8.0 buffer containing 6 M guanidine hydrochloride, 50 mM Tris–HCl, 5 mM Na_2EDTA was performed resulting in 50–100 μg of each sample in