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

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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.

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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

150 μL denaturing solution. The sample was reduced by addition of 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 2.0 μL 1 M DTT.

The reduced and alkylated samples were diluted with 750 μL of 50 mM Tris–HCl, pH 8.0 digestion buffer. The digest was performed by adding 4 μL Lys-C solution (1 mg/mL) and incubating at 37 °C for 1 h. Afterwards another 4 μL Lys-C solution (1 mg/mL) were added to get a sample to enzyme ratio of 1:25 (w:w). Incubation was continued for another 3 h at 37 °C. The enzymatic reaction was stopped by freezing samples at −20 °C.

2.2.6. Asp-N digestion

Lys-C digested samples were directly treated with endoproteinase Asp-N. Therefore 2 μg enzyme were added to 200 μL of Lys-C digested solution and incubated at 37 °C for 17 h.

2.2.7. IP-RP-HPLC mass spectrometry of enzymatic digestions

The peptides were separated by IP-RP-HPLC using an Agilent 1200 HPLC system. Separation was performed on a 2.1 × 150 mm Vydac C18 column packed with 5 μm particles, 300 Å pore size. The mobile phase consisted of eluent A (water containing 0.1% TFA) and B (90% acetonitrile containing 0.09% TFA). The column temperature was set to 40 °C and the flow rate was 0.2 mL/min. The gradient used for the separation of the peptides was as following: 5 min 2% B, increase to 50% B over 45 min, increase to 60% over 2 min, increase to 88% B over 12 min followed by washing of the column at 98% B over 17 min. The UV absorption was monitored at 214 nm.

The HPLC was directly coupled to an LTQ XL Orbitrap mass spectrometer (Thermo Scientific, San Jose CA). The ESI voltage was set to 4 kV in positive ion mode. The capillary temperature was 275 °C. One full scan mass spectrum was followed by MS/MS scans of the three most intense ions. MS/MS data were scanned in enhanced mode and the isolation width was 3.0 Da, the collision energy was 35 V, and the activation time was 10 ms. ETD was performed with an isolation width of 4.0 Da, activation time of 250 ms and the supplemental activation was selected.

Peptides were identified manually comparing the observed mass with the theoretical mass generated from the known amino acid sequence using the GPMAW software (Lighthouse data, Odense, Denmark).

3. Results

3.1. IP-RP-HPLC of stressed sample

A IP-RP-HPLC method was developed and validated for the determination of purity of a monoclonal IgG1 antibody. The method is routinely used for release analytics and during stability analyses. The analyses of temperature stress samples showed an increase of several peaks and the appearance of new peaks, demonstrating that this method is stability indicating for this molecule. Fig. 1 shows the UV chromatogram of a control sample and a sample stressed at 25 °C for 6 months. Mass spectrometry, directly coupled to HPLC system was used for the identification of the peaks. Most peaks were identified directly based on the accurate mass information as chemical clipping products like mAb molecules missing one Fab arm or individual Fab fragments. However, one peak eluting at 24 min could not be identified, which had a similar mass compared to the main peak. The deconvoluted mass spectra of the main peak and the peak eluting at 24 min are shown in Fig. 2. The observed mass of the main peak was 5 Da larger compared to the theoretical molecular weight of the intact antibody and the observed mass of the isomer peak was 4 Da larger compared to the theoretical molecular weight of the intact antibody. Both observed masses were within the expected mass of the intact antibody (The mass accuracy of the instrument is ±20 Da for the intact antibody). The different glycan-forms of the antibody containing either zero terminal galactose (G0/G0), one terminal galactose (G0/G1), or two terminal galactose (G0/G2 or G1/G1) are resolved using the Agilent ESI-TOF mass spectrometer. Based on the mass information of the species eluting at 24 min (24 min species), the modification of the isomer needed to be within a few Dalton of the native molecule. But differentiation between modifications resulting in small changes of mass for example deamidation of asparagine (change of 1 Da), isomerization of aspartic acid (no change in mass), or other modifications which result in small mass differences, was not possible.

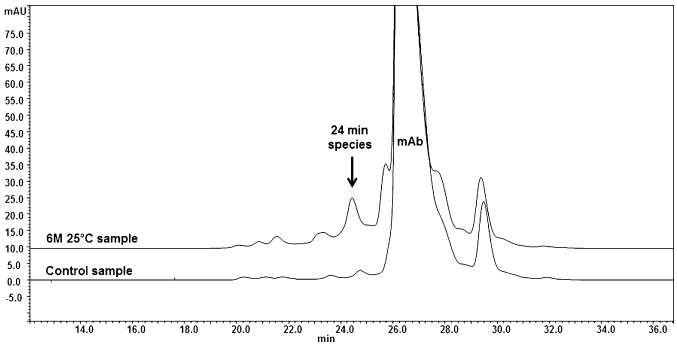

Fig. 1. UV chromatograms overlay recorded at 214 nm of the IP-RP-HPLC analyses of a control and temperature stress (6 month 25 °C) antibody samples.

3.2. Fraction collection

For identification of the modification, the 24 min species and the main peaks were collected. The fraction collection was performed with the same analytical IP-RP-HPLC method on an analytical column to avoid up-scaling issues. A sample stored at 40 °C for 3 months was used for the fractionation, showing higher amounts of the 24 min species peak. The injection volume was increased from 10 μL (100 μg) to 25 μL (250 μg) without losing resolution to increase the amount of protein per fraction. In total, 20 runs were fractionated. Fractions were collected in vials filled with 1 mL buffer solution containing 20 mM methionine, to prevent further chemical modifications. Fig. 3 demonstrates that the 24 min species peak was successfully collected by fractionation. The shift of the UV chromatogram retention times of approximately 3 min compared to the UV chromatogram shown in Fig. 1 (the 24 min

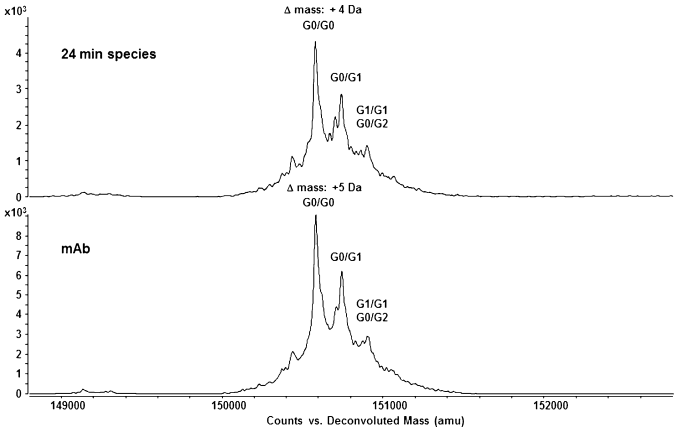

Fig. 2. Deconvoluted mass spectra of the antibody peak eluting at 24.5 min (top) and the main peak eluting at 27.0 min (bottom). The difference (Δ mass) from the theoretical mass of the mAb is shown.

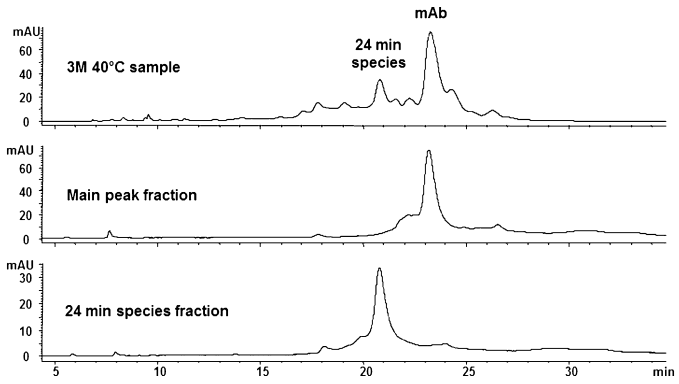

Fig. 3. UV chromatograms recorded at 214 nm of the IP-RP-HPLC analyses of a control (top), the main peak fraction (middle) and the 24 min species peak fraction (bottom).

species eluted at 21 min) was due to a different HPLC system (Agilent 1200 compared to Agilent 1290 Infinity system used for the LC–MS analysis).

3.3. Analyses of the fractions after reduction and alkylation

Sample material from the 24 min species peak and the main peak fractions were reduced, alkylated, and analyzed by LC–UV–MS. The resulting UV chromatogram overlay is shown in Fig. 4. The peaks were identified by mass spectrometry directly coupled to the LC system. The 24 min species fraction was highly enriched (almost 50%) in a heavy chain variant eluting prior to the heavy chain, whereas the main peak fraction contained less of the heavy chain variant. This indicates that the majority of modification was on one heavy chain of the antibody and the ratio was approximately 1:1 (modified: non-modified). The deconvoluted mass spectra of the heavy chain peak and the heavy chain isoform peak are shown in Fig. 5. The observed masses of the heavy chain and heavy chain variant were within 1 Da compared to the theoretical mass of the heavy chain. Both observed masses were within the expected mass of the intact heavy chain. Based on the mass accuracy of the ESI-TOF mass spectrometer (±1 Da for the heavy chain), it was concluded, that the modification of the heavy chain must be within 1 Dalton compared to the main peak. The most likely modification is deamidation of an asparagine residue (increase of 1 Da compared to the native molecule), or isomerization of an aspartic acid residue (identical mass compared to the native molecule). For identification of the modification, the collected HPLC fractions were further analyzed after enzymatic digestion of the fractions. The main peak showed high levels of oxidized heavy chain, which were comparable to oxidized heavy chain levels observed in the

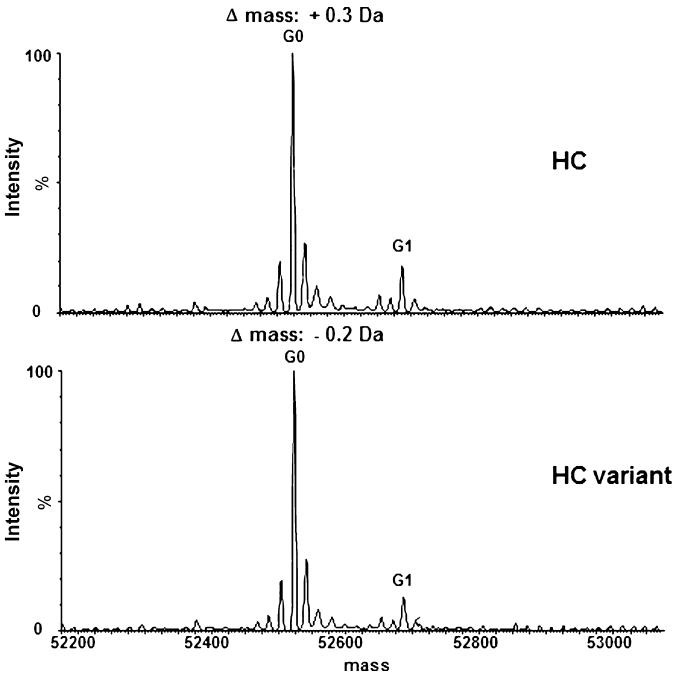

Fig. 5. Deconvoluted mass spectra of the antibody heavy chain variant peak eluting at 37 min (bottom) and the heavy chain peak eluting at 39 min (bottom). The difference (Δ mass) from the theoretical mass of the heavy chain is shown.

24 min species based on a mass increase of 16 Da (data not shown). The oxidation of the heavy chain, which was not observed at such high levels in reference material of the antibody (data not shown), was probably induced by the fraction collection and up concentration of the fractions. Collecting the fraction into vials containing antioxidant methionine reduced the level of oxidation, but could not prevent it completely. Since similar levels of oxidized variants were detected in both fractions, oxidation was ruled out as the source of the modification of the 24 min species fraction.

3.4. Analyses of the fraction after enzymatic digestion

3.4.1. Lys-C digestion

Lys-C digests from the 24 min species and main peak fractions were performed and analyzed by HPLC coupled to MS. The resulting UV chromatograms are shown in Fig. 6. The major difference was observed at the very end of the HPLC gradient. The large hydrophobic peptides eluting at 108.0 and 108.9 min had identical mass and were both identified as a peptide of the heavy chain spanning the

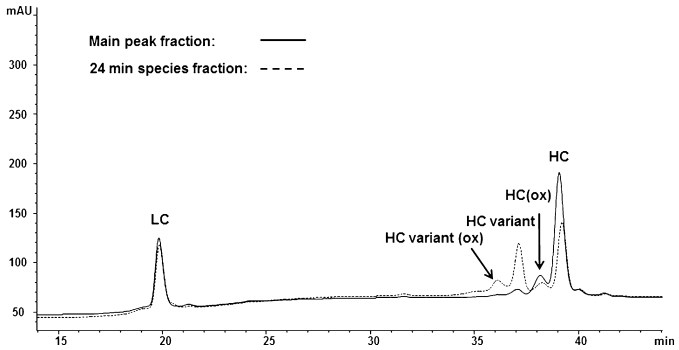

Fig. 4. UV chromatograms overlay recorded at 214 nm of the IP-RP-HPLC analyses after reduction of the main peak fraction (solid line —) and the 24 min species peak fraction (dotted line ·····).

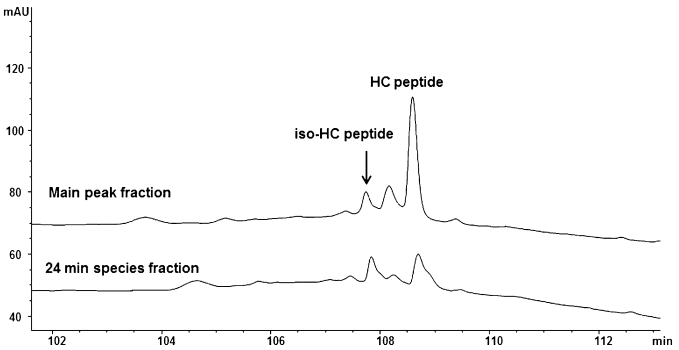

Fig. 6. UV chromatograms recorded at 214 nm of the IP-RP-HPLC analyses after Lys-C digestion of the main peak fraction (top) and the 24 min species peak fraction (bottom).

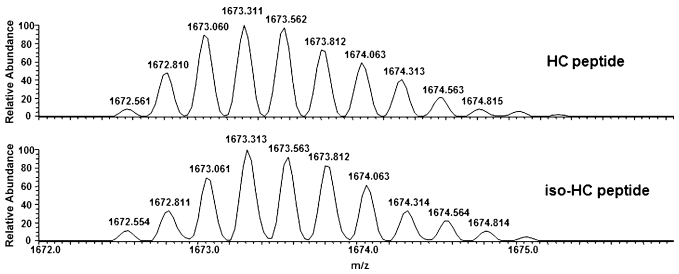

Fig. 7. Mass spectra of the antibody heavy chain isomer peptide eluting at 107.8 min (bottom) and the heavy chain peptide eluting at 109.3 min (top). The peptide was observed in multiple charge states (4, 5, and 6). The most intense ion ($[M+4H]^{4+}$) is shown in a zoom.

CDR-H3 region of the antibody. The mass spectra of both peaks are shown in Fig. 7 (zoom at the $[M+4]^{4+}$ ions), demonstrating that both peaks had identical mass. The theoretical monoisotopic mass of the heavy chain peptide is 6686.188 Da. The observed monoisotopic mass of the heavy chain peptide eluting at 108.9 min was 6686.244 calculated from the monoisotopic peak of the $[M+4H]^{4+}$ ion of 1672.561. The observed monoisotopic mass of the isoform of the heavy chain peptide eluting at 108.0 min was 6686.216 calculated from the monoisotopic peak of the $[M+4H]^{4+}$ ion of 1672.554. The observed masses of both peptides are within 10 ppm (8 ppm and 4 ppm, respectively) of the theoretical mass. Based on the mass accuracy of the Orbitrap mass spectrometer, we concluded that the modification had the identical mass compared to the native molecule; excluding deamidation as the source of the modification (1 Da mass difference in a peptide of 6686 Da is approximately 150 ppm). Therefore, isomerization of aspartic acid residue was the most likely cause of the modification. Still, identification of the modification site was not achieved. The MS/MS spectra of both peptides were identical, but of low quality (data not shown). The peptide contains five aspartic acid residues and efforts to identify the isoaspartic acid residue based on ETD spectra failed due to the poor quality of the MS/MS spectra. The amount of the isomerized peptide was estimated to be approximately 50% using the UV chromatogram, confirming the results of the reduced antibody analyses showing that the modification was on one of the two heavy chain molecules.

3.4.2. Lys-C plus Asp-N digestion

The Lys-C digests were further digested using Asp-N enzyme and analyzed by LC–MS. Asp-N does not cleave at the N-terminus of isoaspartic acid residues, so the isoaspartic acid enriched fraction should contain increased levels of missed cleavages (due to the presence of isoaspartic acid) compared to the native fraction. The resulting UV chromatograms are shown in Fig. 8. The

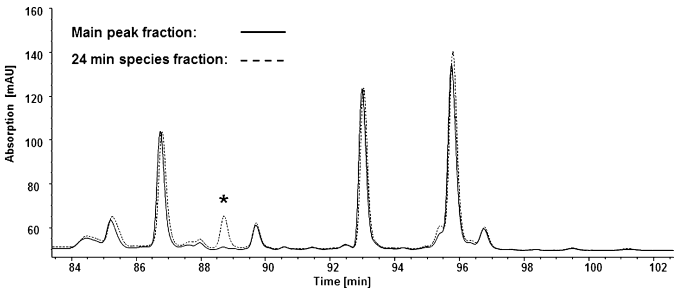

Fig. 8. UV chromatograms overlay recorded at 214 nm of the IP-RP-HPLC analyses after Lys-C and Asp-N digestion of the main peak fraction (solid line —) and the isomer peak fraction (dotted line ·····).

only major difference was a peptide eluting at 88.7 min. The peptide was identified as DXXXisoDYXXXXXXX based on the accurate mass information and collision induced MS/MS spectra (data not shown). As expected the peptide contained a missed cleavage site due to the presence of an isoaspartic acid residue. The presence of isoaspartic acid was confirmed using the ETD spectra performed in the same experiment shown in Fig. 9. The ion at m/z 1296.6 indicates an isoaspartic acid residue as reported by others [16]. We concluded that the modification site was located at DY motif in the CDR-H3 domain of the antibody.

3.5. Isomerization kinetics

The IP-RP-HPLC method of the intact antibody was used to determine the isomerization rate of the DY motif in the CDR of the heavy chain at different temperatures and different pH values. The effect of different pH values on the isomerization reaction is shown in Fig. 10. As expected, isomerization of the aspartic acid residue is favored at lower pH. At 25 °C the amount of isoaspartic acid increased to 6.6% at pH 5.4, 6.0% at pH 5.8, and 5.6% at pH 6.2 after storage for 6 months. At 5 °C storage condition the isomerization rate was significantly lower compared to 25 °C. The results for the two different temperatures are shown in Fig. 10 for samples stored at pH 5.8, which reflected the optimal formulation buffer considering other molecular attributes (physical and chemical degradation) as well. The amount of isoaspartic acid increased to 5.5% from the initial 1.1% over the proposed shelf life of this molecule (24 months).

4. Discussion

Isomerization of aspartic acid residues in monoclonal antibodies has been observed before. Separation of the isomerized antibody from its native counterpart was achieved using ion exchange chromatography or hydrophobic interaction chromatography method [19,30–32]. Cacia et al. [30] reported the identification of an aspartic acid isomerization in the CDR-L1 region of a monoclonal antibody and showed the effect on the binding affinity to the IgE target. The isomerization motif was DG. Dick et al. [31] reported the identification of an isomerization site in the CDR-H2 of an antibody. The researchers used HIC for the separation of the antibody isomer. But neither information about the identification techniques used, nor the isomerization motif was presented. Sreedhara et al. [32] used hydrophobic interaction as well as ion exchange chromatography for the separation and identification of isoaspartic acid in a monoclonal antibody. The isomerization motifs were DG in the CDR region of the antibody with a faster isomerization rate compared to the DS motif in the non-CDR region of that antibody. Wakankar et al. [19] used HIC for the separation of isoaspartic acid containing mAbs. Isomerization of aspartic acid residue was identified in the CDR-1 region of the antibodies at DG motifs. The researchers demonstrated that the differences in the Asp isomerization rates between two mAb molecules were attributed to structural factors including the conformational flexibility and the extent of solvent exposure of the labile aspartic acid residue.

IP-RP-HPLC methods were also employed for the separation of isoaspartic acid containing molecules from the native counterpart, but not of the intact molecule. Rehder et al. [29] reported the identification of aspartic acid isomerization in a monoclonal antibody affecting the biological activities of that molecule and demonstrating the role avidity plays in the biological activity of that particular molecule. The separation was achieved after reduction of the antibody on the approximately 25 kDa light chain of the molecule. The

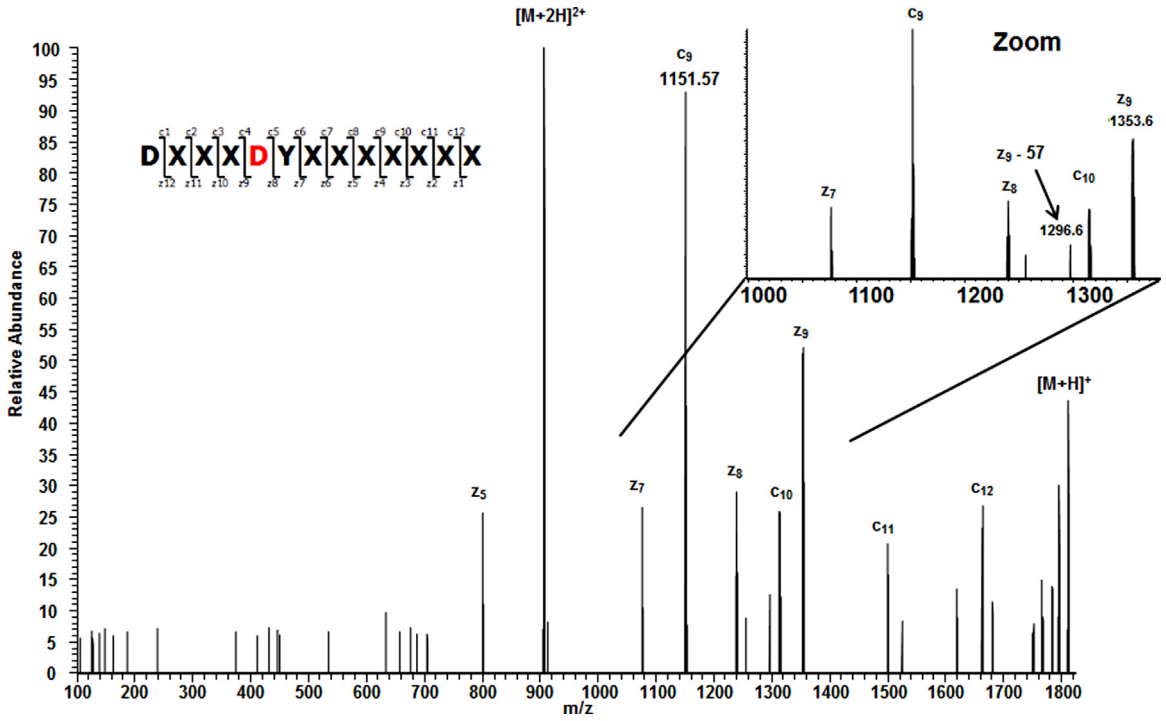

Fig. 9. ETD spectrum of the peptide eluting at 88.7 min. in the IP-RP-HPLC analyses after Lys-C and Asp-N digestion of the isomer peak fraction. The peak was identified as DXXXisoDYXXXXXXX based on a number of consecutive z and c ions. Identification of the isoaspartic acid reside was based on the $[M+1H]^{1+}$ ion at 1296.60, corresponding to the unique isoaspartic acid marker ion z9-57.

isomerization motif was DH located in the CDR-L3 affecting the binding to EGFR.

To our knowledge, here we report for the first time, the separation of a monoclonal antibody variant containing a single isoaspartic acid residue from its native counterpart. Separation of the isoaspartic acid containing molecule from its native counterpart was achieved on the intact molecule (approximately 150 kDa) as well as on the heavy chain of the molecule (approximately 50 kDa). Considering that under the denaturing HPLC separation conditions (high temperature, low pH) it is surprising to achieve such a separation of the isoform; the monoclonal antibody containing over 1300 amino acid residues, which differ only in the backbone and side chain of a single amino acid. Whether this separation can be generalized or whether this was just good luck in this special case remains to be seen. We did not obtain the separation of an intact antibody variant containing a single isoaspartic acid residue from its native counterpart by means of IP-RP-HPLC for any other molecule so far, but have also not encountered other molecules with significant levels of aspartic acid isomerization.

The effect of the column temperature and the low pH on the levels of isoaspartic acid detected by the HPLC method was evaluated in great detail. Increasing the column temperature from 64 °C to 80 °C did not increase the levels of isoaspartic acid detected. Additionally the monoclonal antibody was exposed to the low pH and high temperature for an extended period of time (up to 60 min at 80 °C) without increasing the isoaspartic acid levels. We concluded from these studies, that the levels measured are not method induced artifacts.

For identification of isoaspartic acid, typically the molecules are first enzymatically digested and the resulting isoaspartic acid residue containing peptides are separated from the native peptides by IP-RP-HPLC. Identification is achieved based on the different elution time (the isoaspartic acid residue elutes earlier compared to the native peptide) and the identical MS/MS spectra of both peptides. Classical methods like Edman sequencing [8], the usage of carboxylmethyltransferase [9], or by using Asp-N protease [10], can be used for identification of isoaspartic acid containing peptides. Traditional mass spectrometry technology like collision induced fragmentation, has also been reported to differentiate between

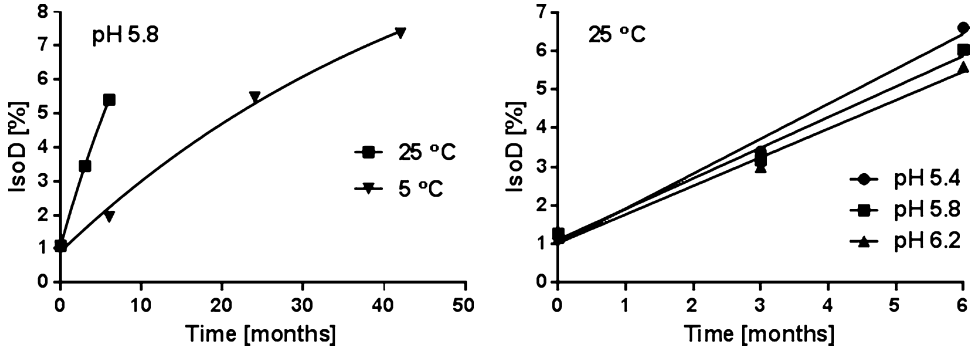

Fig. 10. Percentage of the antibody isomer based on the IP-RP-HPLC analyses of samples stressed at different temperatures (left) and pH (right).

isoaspartic and aspartic acid residues, based on specific marker ions [11–14], or based on the intensity of the b- and y-ion next to the isomerization site [15]. However, in our hands, the reported differences have not been observed for the isomerization site due to the poor quality of the collision induced dissociation MS/MS spectra generated by the LTQ Orbitrap. More recently, ETD and ECD mass spectrometry was used for the identification of isoaspartic acid residues in peptides [16,17]. We successfully identified the isoaspartic acid containing peptide by ETD technology. In our hands, this technology was useful for the identification of isoaspartic acid; however, it is limited by the size of the peptides and the intensity of the mass spectra. For larger peptides (>6000 Da) and for low abundant ions, the quality of the MS/MS ETD spectra was poor and the marker ions could not always be detected using the LTQ Orbitrap mass spectrometer. The marker ion of z-57 next to the isomerization site was only observed in isoaspartic acid containing peptides; hence this allows the unambiguous identification of isoaspartic acid modification. However, the absence of such an ion does not exclude the presence of isoaspartic acid, since the ion was not always present in isoaspartic acid peptides due to the poor quality of the ETD spectra. The identification was complicated by the low abundance of the z-57 ion next to the isomerization site, the fact that the corresponding marker ion of c+57 at the isomerization site was not observed, and the presence of noise signals with similar intensities compared to the marker ion. None of the signals from 100 to 700 m/z units (Fig. 9) could be assigned to fragment ions of the peptide demonstrating the poor quality of the ETD spectrum.

The isomerization site was identified in the CDR region of the antibody at a DY motive. This was surprising, since it is known that large amino acids at the C-terminus of the aspartic acid residue should not favor the isomerization reaction. Additionally, there are three other aspartic acid residues in close vicinity of the isomerization site, one of which has also a tyrosine residue at the C-terminus, the other with even smaller amino acids (isoleucine and leucine), which did not show any detectable isomerization. In total, the antibody contains over 50 aspartic acid residues, some of which are followed by a C-terminal glycine residue. However, none of the other aspartic acid residues isomerized to the extent of the aspartic acid residue in the CDR-H3 domain. The reason for this fast isomerization reaction at this particular site is not clear, but we speculate that the local flexibility, solvent exposure, or other structural features in close vicinity of the binding domain resulted in the increased isomerization rate. This finding is interesting for the development of biopharmaceuticals since so far, only DG, DS, DH, and DD motifs have been considered as isomerization hot spots [33,34]. Clearly, this assumption needs to be revised. At one position in this antibody, structural features other than the nature of N + 1 residue following aspartate play a key role in isomerization rate. We conclude that for monoclonal antibodies, it is not possible to predict the isomerization sites at a particular amino acid, hence, all aspartic acid residues located in the CDR region need to be regarded as possible isomerization hot spots and closely monitored during development. Ideally, developability studies including pH stress conditions are performed for the identification of degradation sites prior to initiation of process development [35]. On the other hand, the aspartic acid residues located in the constant region of the antibody showed little or no isomerization, indicating that it is not necessary to consider these amino acids as hotspots for isomerization even if followed by a C-terminal glycine residue.

The rate of isomerization was determined using the IP-RP-HPLC method of the intact antibody showing an increase to 5.5% from the initial 1.1% over the proposed shelf life of this molecule (24 months). This increase is analytically significant. However, we do not know if the increased level of isoaspartic acid in the molecule is clinically relevant. The effect of the isomerization on the biological activity of the monoclonal antibody could not be determined. The collected HPLC fractions did not show any activity in a cell based bio-assay, probably due to the fact that the antibody was denatured during HPLC analyses (high temperature and low pH). Native analytical methods like ion exchange chromatography or hydrophobic interaction chromatography did not result in separation of the isoaspartic acid containing variant of the antibody. The lack of information about the biological effect of the isomerization reaction required the use of the IP-RP-HPLC method for release and stability testing of the monoclonal antibody. The lack of other suitable methods for the quantification of the antibody isomer highlights the importance of IP-RP-HPLC methods in the analyses of monoclonal antibody biopharmaceuticals.

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