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Quantification and characterization of antibody deamidation by peptide mapping with mass spectrometry

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ABSTRACT

Capturing a complete assessment of deamidation in monoclonal antibodies is challenging due to the structural complexity of multiple potential deamidation sites and deamidation pathways. In this study, a peptide mapping approach has been developed to quantify the extent of deamidation of a therapeutic recombinant monoclonal antibody. To obtain an accurate measurement, a rapid sample preparation procedure was developed to minimize formation of deamidation during sample preparation and analysis. Ammonium formate mobile phase was used in the reversed phase separation to completely separate deamidated peptides from their native peptides. To improve detection sensitivity and prevent interference from chemical background noise and coeluting peptides, mass spectrometry (MS) was utilized to quantify the low levels of deamidation in the product. The method was subsequently qualified as a characterization test for comparability studies, forced degradation studies and for characterizing reference standards. The method demonstrated suitable linearity, precision and accuracy. The limit of detection (LOD) and the limit of quantification (LOQ) of the method for specific deamidation sites were estimated to be as low as 0.1% and 0.3%, respectively. The deamidation sites and the deamidation products were identified using several orthogonal methods including tandem MS, N-terminal sequencing and protein isoaspartate methyl transferase (PIMT) enzymatic reactions.

1. Introduction

During the past decades, pharmaceutical companies have substantially increased their drug development pipelines of protein-based drugs, especially with monoclonal antibodies, as they generally offer high target specificity with low side effects. Unlike small molecule drugs, protein products are more susceptible to various chemical modifications during manufacturing, shipping, and storage. Despite the maturation and robustness of production platforms, a variety of product-related modifications can occur and may be present in the final drug form. These modifications may be present in varying amounts in the product and can potentially affect safety and efficacy.

Amongst many potential chemical modifications of proteins, deamidation of asparagine (Asn) or glutamine (Gln) and isomerization of aspartic acid (Asp) represent common degradation pathways for monoclonal antibodies. Deamidation/isomerization may induce the alteration of protein properties through the introduction of negative charges, and/or the insertion of a methylene group in the peptide backbone. This may lead to undesirable effects on the protein, including a decrease in biological activity [1–4], a reduction of product stability [5–8], and an increase in concern for immunogenicity [9–11]. Loss of biological function was reported for a recombinant humanized monoclonal antibody, HER2 [1], due to Asp isomerization occurring in complementarity determining regions (CDRs). The isomerized protein was found to be only 9–21% potent compared to the native protein. To avoid the risk of deamidation, mutations of the neighboring amino acids of the deamidation site in the binding region has recently been applied [4]. Understanding deamidation/isomerization adds value to formulation development and the assessment of product stability. It is an intrinsic part of determining the critical quality attributes and appropriate control strategy for therapeutic monoclonal antibodies.

Abbreviations: Asn, asparagine; Asp, aspartic acid; Asu, succinimide intermediate of Asn deamidation; CAD, collisonally activated dissociation; CDRs, complementarity determining regions; CID, collisional induced dissociation; DAD, diode array detector; DTT, 1,4-dithio-DL-threitol; ECD, electron capture dissociation; EDTA, ethylenediaminetetraacetic acid; EIC, extracted ion chromatogram; ESI, electrospray ionization; FA, formic acid; Gln, glutamine; HCl, hydrochloride; HIC, hydrophobic interaction chromatography; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; NaIAA, iodoacetic acid; PIMT, protein isoaspartate methyl transferase; PMSF, phenylmethanesulfonylfluoride; RP-LC, reversed phase liquid chromatography; SAH, S-adenosyl homocystine; SAM, S-adenosyl methionine; TFA, trifluoroacetic acid; Tof, time-of-flight; Tris, (hydroxymethyl) aminomethane; UPLC, ultra performance liquid chromatography.

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Deamidation and isomerization reactions have been extensively studied and well documented in several reviews [12–16]. The mechanism of this non-enzymatic reaction involves the formation of a succinimide intermediate (Asu) at a neutral or alkaline pH [17]. The succinimide intermediate may hydrolyze to form either aspartate (Asp) or isoaspartate (IsoAsp). If Asu remains stable during the manufacturing processes, it can accumulate in the final product along with Asp and IsoAsp degradants. The rate of the deamidation reaction depends on the solution pH, temperature, solvent ionic strength, protein primary sequence and higher order structure [13,17–24].

A variety of analytical methods have been used for monitoring deamidation products. Deamidated proteins tend to be less positively charged and have a lower pI than their native counterparts. Ion-exchange HPLC and isoelectric focusing are routinely used in the pharmaceutical industry to monitor deamidation of monoclonal antibodies. Reversed phase liquid chromatography (RP-LC) [25] and hydrophobic interaction chromatography (HIC) [1,26] may be used to separate deamidation of reduced or papain-digested antibody fragments based on hydrophobicity differences. IsoAsp products can also be quantified by IsoQuant [27], in which protein isoaspartyl methyl transferase (PIMT) converts IsoAsp residues into methyl IsoAsp residues, releasing S-adenosyl homocystine (SAH) as a product of the reaction. The amount of SAH is subsequently quantified by reversed phase HPLC. Because deamidation of monoclonal antibodies can occur at multiple sites, each with potentially multiple products at different rates, none of these methods are able to determine the amount of site-specific deamidation. Peptide mapping involves an enzymatic digestion of the protein followed by reversed phase separation of the resulting peptides. It is a direct and specific approach for assessing deamidation at multiple sites. Coupled with mass spectrometry (MS), peptide mapping is a powerful characterization tool for deamidation.

Although peptide mapping with MS has been successfully used to detect and identify deamidation sites, accurate quantification of low level deamidation remains a challenge for the following reasons: (1) peptide mapping sample preparation procedures are typically performed at pH 8.0 with a long sample preparation time; Under such conditions, the formation of deamidation during sample preparation becomes significant [23,28–30]; (2) hydrophobicities of deamidated peptides and their corresponding native peptides are very similar, leading only to a small difference in retention time and coelution or partial coelution. Unless an ultra high resolution MS technique is used, it is difficult to quantify the deamidated peptide from the native peptide when coelution of both species occurs, due to interference of isotopic peaks.

To overcome the problems outlined above and develop a reliable quantitative assay, we developed a rapid peptide mapping method using a near neutral pH reversed phase separation and MS quantification. The method was applied to quantify low amounts of deamidation present in an IgG1 monoclonal antibody. A qualification study was conducted to evaluate the performance of the method. Qualification parameters assessed included repeatability precision, intermediate precision, accuracy, linearity, limit of quantification (LOQ) and limit of detection (LOD). In addition, deamidation sites and deamidation products were confirmed by orthogonal characterization techniques.

2. Experimental

2.1. Materials

A fully human IgG1 monoclonal antibody was expressed in NS0 cells and purified by Human Genome Sciences (Rockville, MD). Forcibly deamidated IgG1 was generated through incubation with 100 mM ammonium bicarbonate (pH 8.5) at 40 °C for 8 h.

Acetonitrile was purchased from JT Baker (Phillipsburg, NJ). Iodoacetic acid (NaIAA) and Trizma preset crystals were purchased from Sigma–Aldrich Chemical (St. Louis, MO). 1,4-Dithio-DL-threitol (DTT), ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA) and 8 M guanidine hydrochloride (HCl) were obtained from Thermo (Rockford, IL). Ammonium bicarbonate, ammonium formate and formic acid were purchased from Fisher (Pittsburgh, PA). Trypsin and IsoQuant kits were purchased from Promega (Madison, WI).

2.2. Sample preparation

2.2.1. Conventional peptide mapping

IgG1 (2 mg/mL) was denatured and reduced with 6 M Guanidine HCl, 0.2 M (hydroxymethyl) aminomethane (Tris) (pH 8.0) and 30 mM DTT at 37 °C for 1 h. NaIAA was added to the denatured and reduced solution for a final concentration of 60 mM and incubated at 20 °C for 30 min in darkness. After alkylation, desalting and buffer exchange were performed using PD-10 columns from GE (Piscataway, NJ). Trypsin digestion was completed in 3 h at 37 °C with an enzyme to substrate ratio of 1:50 (w/w). After digestion, 3% TFA was added to quench the digestion reaction.

2.2.2. Rapid peptide mapping

IgG1 was diluted with 6 M Guanidine HCl and 0.2 M Tris (pH 7.2) for a final concentration of 10 mg/mL. The sample was then reduced with 20 mM DTT and incubated at 37 °C for 20 min. After incubation, 10 μL reduced solution was diluted with 90 μL of digestion buffer containing 0.2 M Tris (pH 7.2) and 1 mM EDTA. Then trypsin was added to a final enzyme to substrate ratio of 1:10 (w/w). The solution was mixed and incubated at 37 °C for 20 min. Formic acid (1%) was added to quench the digestion reaction.

2.2.3. PIMT enzymatic modification

PIMT reaction reagents from the Promega IsoQuant Kit were used. The tryptic peptides of forcibly deamidated and control IgG1 samples were prepared using the conventional peptide mapping method. After digestion, trypsin was inhibited using 1 mM phenylmethanesulfonylfluoride (PMSF). S-adenosyl methionine stock solution (SAM) was diluted 5-fold. To each 100 μL sample, 20 μL of SAM, 20 μL of PIMT, and 20 μL of reaction buffer were added. All samples were incubated at 30 °C for 45 min. Samples were analyzed by LC–MS.

2.3. LC–MS analysis

An Agilent 1200 HPLC system with diode array detector (DAD) and Agilent 6220 Time-of-flight (Tof) mass spectrometer (New Castle, DE) were used for LC–MS analysis. The digested peptides were separated using a Zorbax 300SB C18 column (Agilent, New Castle, DE). Mobile phase A was 10 mM ammonium formate in H_2O and mobile phase B was 100% acetonitrile. A linear gradient ramped up from 16% to 36% B over 60 min. The column temperature was set at 40 °C and column flow-through (0.3 mL/min) was monitored by UV at a wavelength of 214 nm. A 4% formic acid solution was delivered through a post-column mixer via HPLC. The flow rate of formic acid was controlled at 0.1 mL/min. The HPLC elution containing 1% final concentration of formic acid was analyzed by electrospray ionization (ESI)-Tof mass spectrometry. The mass spectrometer was calibrated with tuning mixture (Agilent, New Castle, DE) prior to each analysis. Both centroid and profile data were acquired in the 2 GHz mode with a mass range of 300–3000 (m/z). Ion source conditions were optimized to sustain a stable ES and achieve maximum

ion transmission. 9 mL/min drying gas flow rate, 350 °C gas temperature, 175 V fragmentor voltage and 2500 V capillary voltage were chosen as the optimal MS conditions for deamidation quantification. Mass spectrometry data was processed using Agilent Mass Hunter Qualitative Analysis Software (Version B.02.00).

2.4. Data analysis

An ion extraction function was applied to generate an extracted ion chromatogram (EIC) of a given mass from MS data. The percent deamidation for the peptide of interest was derived by dividing the total EIC peak area into the sum of EIC peak area of the deamidated and Asu peaks. The total EIC peak area consisted of the peak area under the native peptide, deamidated peptides and Asu. The monoisotopic mass of the most abundant ion for each peptide was chosen for the ion extraction.

2.5. N-terminal sequencing

Samples were subjected to N-terminal amino acid sequence analysis using a PE-Applied Biosystems (PE-ABI) Procise 494 cLC Protein Sequencer operating with the Pulsed-Liquid cycles and equipped with an on-line PTH-amino acid analyzer (PE-ABI 140D solvent delivery system, Perkin Elmer Series 200 UV/vis absorbance detector and Applied Biosystems SequencePro analysis software).

3. Results and discussion

The IgG1 monoclonal antibody used in this study is composed of more than 1300 amino acid residues with 16 disulfide bonds. Three tryptic peptides of this antibody have been identified as the most susceptible to deamidation under stress conditions. Heavy chain peptide T24(H) (VVSVLTVLHQDWL**N**GK) and T36(H) (GFYPSDIAVEWES**N**GQPE**N**NYK) are located in the Fc region of the monoclonal antibody, which share the same sequences with other human IgG1s. T4(H), located in the CDR region, is a peptide unique to the IgG1 monoclonal antibody studied. Multiple Asn residues are present in T4(H) and T36(H) sequences. The deamidation sites of each peptide (in bold and underlined) were identified by several methods (details of the characterization are described in Section 3.5).

The conventional peptide mapping method designed for primary structure characterization is not suitable as a quantitative assay for deamidation. Three aspects needed to be addressed to extend its application to quantify site-specific deamidation: (1) UV detection sensitivity was not sufficient for detecting low amounts of deamidation present in product; (2) deamidated peptides coeluted with their corresponding non-deamidated peptides and thus, affected the accuracy of quantification; and (3) sample handling time (from sample preparation to detection) was too long and lead to method-induced deamidation during the course of analysis.

The following sections describe the optimization of the peptide mapping method for deamidation quantification. In this study, the focus of optimization was on the three peptides most susceptible to deamidation. The methodology discussed here may be generally applicable for the quantification of Asn deamidation in other proteins.

3.1. MS quantification

MS was utilized in this study for deamidation detection and quantification. In addition to the improvement in sensitivity compared with UV detection, MS can further separate the coeluting peptides and reduce chemical background based on their masses, leading to more accurate measurements. To measure the deamidation percentage of a given peptide by MS, an extracted ion chromatogram (EIC) was used to represent the amount of peptide ions detected by MS. In general, ESI-MS detects a tryptic peptide in multiple charge states. It was found that the charge state had no impact on the deamidation calculation, as long as equivalent charge states were selected for deamidated and native peptides. Therefore, with consideration for sensitivity, the monoisotopic mass of the most abundant charge state was chosen for ion extraction. Assuming that ionization efficiency of the deamidated peptide is similar to that of its native peptide, the deamidation percentage can be derived from the EIC peak area of the deamidated peptide and its corresponding native peptide. This assumption was shown to be valid and is described in Section 3.4.

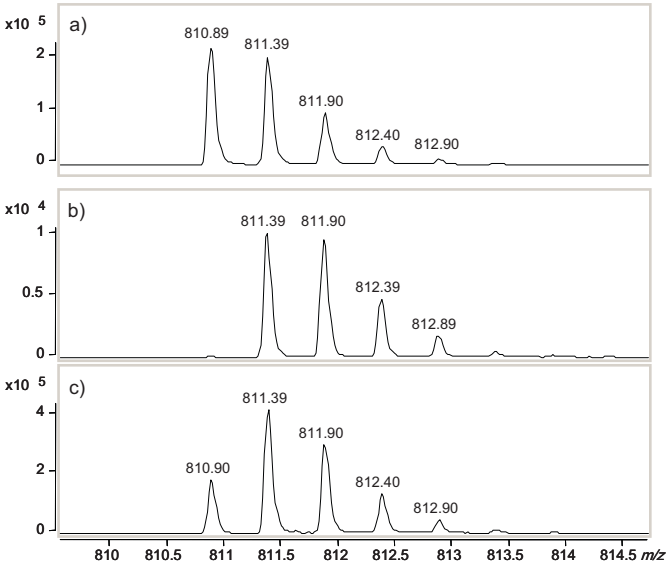

Fig. 1. A comparison of isotopic distributions of the $[M+2H]^{2+}$ ions: (a) native T4(H), (b) deamidated T4(H), and (c) coelution of native T4(H) and deamidated T4(<!– no-mfc –>H)<!– /no-mfc –>.

Use of MS for quantification requires optimization of MS operating parameters. Sustaining a stable electrospray through an entire run is important to obtain an accurate measurement. Because modified and non-modified peptides may elute at different retention times, unstable electrospray can alter the signal intensity recorded at a given time point, which can subsequently influence the relative intensity used for quantification. In addition, minimization of in-source fragmentation was also found to be necessary for accurate quantification. Several MS parameters were tested to assess their impact on the deamidation measurement. MS parameters including drying gas flow rate, drying gas temperature, fragmentor voltage and capillary voltage were tuned in ranges suitable for a flow rate of 0.2–0.5 mL/min. None of the parameters were found to have a significant impact on the deamidation quantification within the tested ranges. Thus, MS can be operated in a wide range without affecting the results.

3.2. RP-HPLC separation

It was found that one of the deamidated isoforms coeluted with its native peptide under the reversed phase conditions using TFA as an ion-pairing reagent. Coelution of deamidated and non-deamidated peptides was revealed through a comparison between the observed isotopic distribution and the theoretical isotopic distribution. As shown in Fig. 1, the isotopic mass distribution of doubly charged non-deamidated T4(H) (Fig. 1a) is different from its deamidated form (Fig. 1b), due to a mass increase of 0.98 Da as

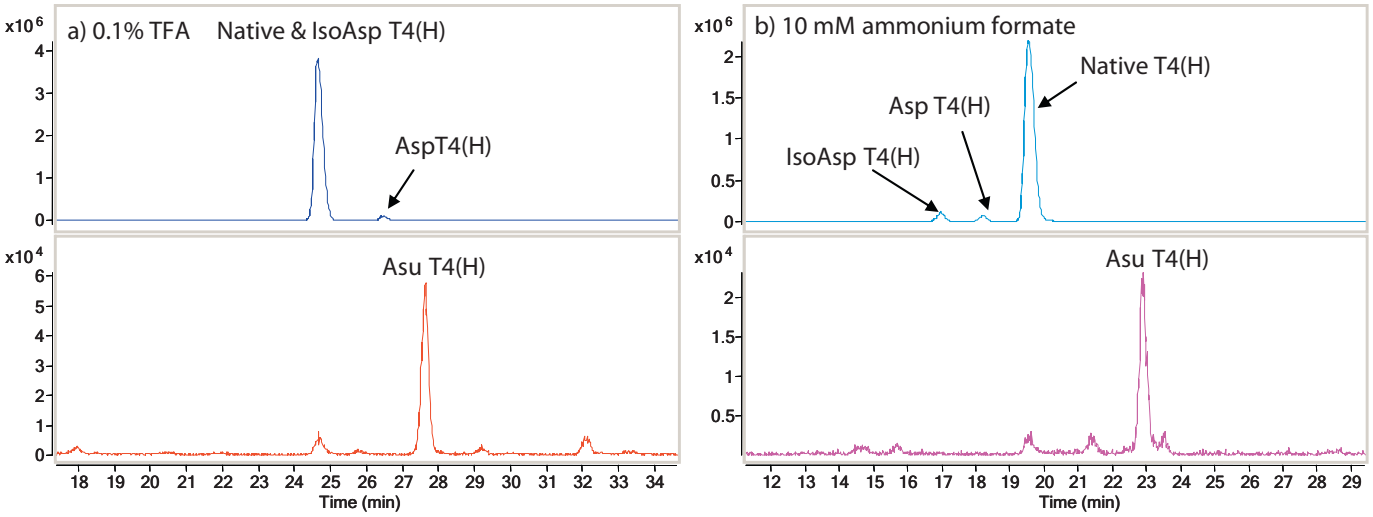

Fig. 2. A comparison of EIC profiles for deamidated T4(H) and Asu intermediate T4(H) in the IgG1 final product obtained from two different RP-HPLC mobile phase conditions: (a) 0.1% TFA and (b) 10 mM ammonium formate.

a result of deamidation. In coelution situations, isotopic mass distributions of two species are merged together as shown in Fig. 1c. Coelution or partial coelution was observed for all three peptides of interest when TFA was used as an ion-paring agent. This phenomenon has also been observed in similar studies [24,31]. As a result, quantification becomes complex and inaccurate.

The reversed phase separation was improved by using an optimized gradient and a small particle size column (1.7 μm vs. 5 μm) with ultra performance liquid chromatography (UPLC). However, the coelution issue still remained. Thus, changing selectivity was considered in order to achieve a base-line separation. When solution pH is greater than the pKa of Asp or IsoAsp, deamidated peptides are expected to be negatively charged and less hydrophobic. Consequently, increasing the mobile phase pH can enhance the selectivity of deamidated peptides. The performance of the peptide mapping carried out at an intermediate or basic pH has been evaluated in several studies [31,32]. Using ammonium formate in the aqueous mobile phase improved the selectivity of peptide separation and the sequence coverage of proteins [32]. The deamidated peptides were found to be well separated from the native peptides using an ammonia containing basic mobile phase (pH = 10) [31].

In this study, a reversed phase separation was performed at a pH of 6.5 by adding 10 mM ammonium formate in the aqueous mobile phase. The peptide mapping profile generated at pH 6.5 is different from that at low pH, due to the change in the overall charge states of the peptides. Fig. 2 shows the separation of peptide T4(H) under both sets of pH conditions. The deamidated isoforms eluted earlier and were well separated from their native peptides. Complete separation was also evident with a close match of isotopic mass distribution to theoretical. As expected, the deamidation of T4(H) measured using the ammonium formate mobile phase is higher by 50% than that measured using the TFA mobile phase. This result suggested more accurate integration of the EIC peak area was achieved due to base-line separation of the deamidated peptides. Low levels of Asu (<0.5%) were also detected under both mobile phase conditions. Asu, which eluted after the native peptide, was well separated under both pH conditions (Fig. 2). Mobile phase pH did not influence the relative elution position of Asu.

Using ammonium formate mobile phase, it was observed that the measured deamidation percentage changed as a function of the sample load amount (Table 1). To understand the cause of this observation, the EIC peak area of a given ion was plotted against the injection volume. Poor linearity was observed for the native peptide, while better linearity was observed for its corresponding deamidated peptide (Supplemental data). This difference was not observed when TFA mobile phase was used, suggesting that electrospray ionization causes poor linearity with the sample loading amounts when using 10 mM ammonium formate mobile phase at pH 6.5. This likely resulted from poor ionization efficiency when a relatively large amount of sample loading is used.

To improve instrument linearity while maintaining the separation, formic acid (FA) was infused into the column eluent through a post-column mixer. Post-column infusion of FA provided additional positive charges and enhanced the ionization efficiency. The pH of the solution delivered into the MS can be adjusted by varying the concentration of FA. Table 1 shows the deamidation of T4(H) measured with and without the post column FA infusion at the increased sample load. Consistent amounts of T4(H) deamidation were obtained from the post-column infusion at both tested FA concentrations. Similar results were observed for other peptides. Consequently, both method robustness and the assay range were improved by post-column infusion of FA.

3.3. Rapid peptide mapping

Numerous studies [7,13] have demonstrated that the rate of a deamidation reaction depends on pH and temperature. In general, high pH and high temperature lead to accelerated rates of deamidation. The conventional peptide mapping method was optimized for complete digestion in a solution of pH 8 at 37 °C for the purpose of global primary structural analysis. Due to the specific method execution parameters necessary for this type of analysis, an increased chance of method-induced deamidation is expected.

Table 1
Percent of T4(H) deamidation (%) measured with different amounts of formic acid (FA) used for post column infusion.

Injection volume (μL)	No FA	0.25% FA[a]	1% FA[b]
10	3.6	4.4	4.6
25	4.0	4.5	4.5
50	4.5	4.6	4.5
60	4.7	4.8	4.6
80	5.1	4.8	4.7
100	5.3	4.9	4.9

[a] 0.25% FA in the final HPLC elution with a pH of 3 was generated by a post-column infusion of 1% FA at a fixed flow rate of 0.1 mL/min.
[b] 1% FA in the final HPLC elution with a pH of 2 was generated by a post-column infusion of 4% FA a fixed flow rate of 0.1 mL/min.

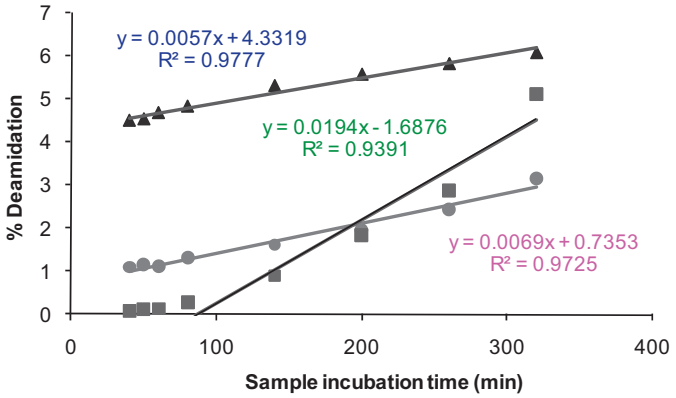

Fig. 3. Deamidation of T4(H) (▲), T25(H) (■) and T36(H) (●) peptides obtained from the IgG1 final product as the function of sample incubation time at 37 °C. Sample incubation time included sample denaturing time (20 min) and digestion time that increased from 20 min to 300 min.

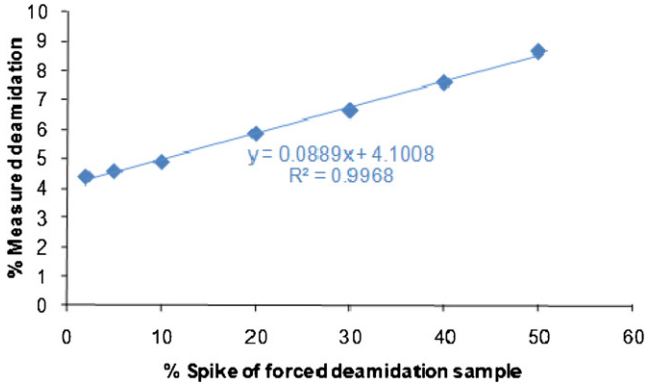

Fig. 4. The linearity plots of T4(H) deamidation of the IgG1 final product measured experimentally as a function of the percentage of spiked forced deamidation sample.

To investigate if the conventional peptide mapping conditions have any impact on the deamidation measurements of the monoclonal antibody, samples were digested at 37 °C (pH = 8) for varying durations ranging from 3 h to 6 h. Indeed, the deamidation levels of the three peptides of interest increased with increasing digestion time. Over the 6 h digestion period, deamidation of T36(H) and T25(H) doubled, and deamidation of T4(H) increased by 1.5 times relative to the 3 h digestion period. These results demonstrated that the conventional peptide mapping experimental procedure induced deamidation and thus, is not suitable for reliable quantification.

To minimize the formation of deamidation during sample preparation, a rapid peptide mapping method was developed. There were two major changes introduced to the sample preparation procedure (sample preparation time and solution pH). The total sample preparation time was reduced from 5 h to 40 min, in which the alkylation and desalting steps were eliminated and the digestion time was reduced to 20 min. To preserve trypsin activity in the presence of guanidine and DTT, the reaction solution was diluted 10 fold after the denaturation and reduction step. Additionally, the pH was adjusted to 7.2 to suppress method-induced deamidation. To compensate for the decrease in enzyme activity, a higher amount of trypsin was used. Although the digestion time was decreased, it was determined that the increased trypsin amount was sufficient to ensure complete digestion of the peptides of interest.

Although the rapid digestion procedure can be completed within 40 min, the possibility of induced deamidation during the sample preparation still remains. Using ^{18}O–water to distinguish between the deamidation that occurred in the sample prior to sample preparation and the deamidation that occurred during sample preparation has been reported [33,34]. In this study, a digestion time course study was performed. The results are shown in Fig. 3. The deamidation of each peptide at time zero (T_0) was determined by extrapolating the linear regression to T_0. The deamidation at T_0 was estimated to be 4.3% for T4(H) peptide, 0.7% for T36(H) peptide and 0.0% for T25(H) peptide. These results suggest that any deamidation of T25(H) detected in the bulk sample is likely method-induced. Using the plots shown in Fig. 3, it is estimated that the rapid peptide mapping procedure (40 min incubated at 37 °C) induces 0.2% deamidation for T4(H) and 0.3% deamidation for T36(H). From the qualification study that followed, both 0.2% method-induced deamidation for T4(H) peptide and 0.3% method-induced deamidation for T36(H) peptide were just slightly above the variability of the method.

3.4. Method qualification

A qualification study was performed to evaluate the reproducibility, precision, accuracy, linearity and sensitivity of the optimized method. Deamidation of T4(H) and T36(H) peptides were measured with six replicates of a representative sample by two separate analysts. An average of 4.6% deamidation was obtained for T4(H) and an average of 1.6% was obtained for T36(H) deamidation. Deamidation measurements for T4(H) and T36(H) were consistent between analysts with intermediate precisions of 3.7% CV and 7.2% CV, respectively. Forcibly deamidated sample was spiked into the control sample at different levels to assess the accuracy of the method. The experimental values were compared to the expected values to determine the recovery. Due to lack of samples with no deamidation, the IgG1 final product was used as the control sample, which contained a low level of endogenous deamidation. Samples were spiked from 1 to 50% with the forced-deamidated sample, and the resulting recoveries were greater than 85%, demonstrating the method has acceptable accuracy.

When the forcibly deamidated sample was spiked into the control sample, the deamidation amount measured in the spiked samples increased correspondingly with the amount of the spike as expected. We found that the total EIC peak area of deamidated peptide and its native peptide remained constant with increasing spiking level. This result indicates the ionization efficiency of deamidated peptide is similar to its native peptide. As a result, the method demonstrated suitable linearity (Fig. 4) with an R^2 value greater than 0.99 for the peptides of interest. The LOD and LOQ were estimated using regression analysis. The noise level was estimated from the ratio of the residual standard deviation of the regression line and the corresponding slope. The noise level was multiplied by factors 3.3 and 10 to calculate the LOD and LOQ, respectively. For T4(H) and T25(H) peptides, LOD and LOQ are 0.1% and 0.3%, respectively. T36(H) peptide has higher LOD and LOQ values, 0.4% and 1.3%, due to coelution with an unrelated peptide. Only LOD and LOQ were assessed for T25(H), since no deamidation above LOQ could be detected in the control sample using the optimized method.

3.5. Characterization of deamidation sites

The deamidation reaction converts Asn residues into two deamidated products, Asp and/or IsoAsp. Due to the identical masses of these two products, MS cannot differentiate an Asp product from an IsoAsp product. In reversed phase separation, an IsoAsp containing peptide usually elutes earlier than the Asp containing peptide. Tandem MS has been applied to differentiate the deamidation products based on the relative peak intensity of fragment ions from collisionally activated dissociation (CAD) [35,36] or based on the signature

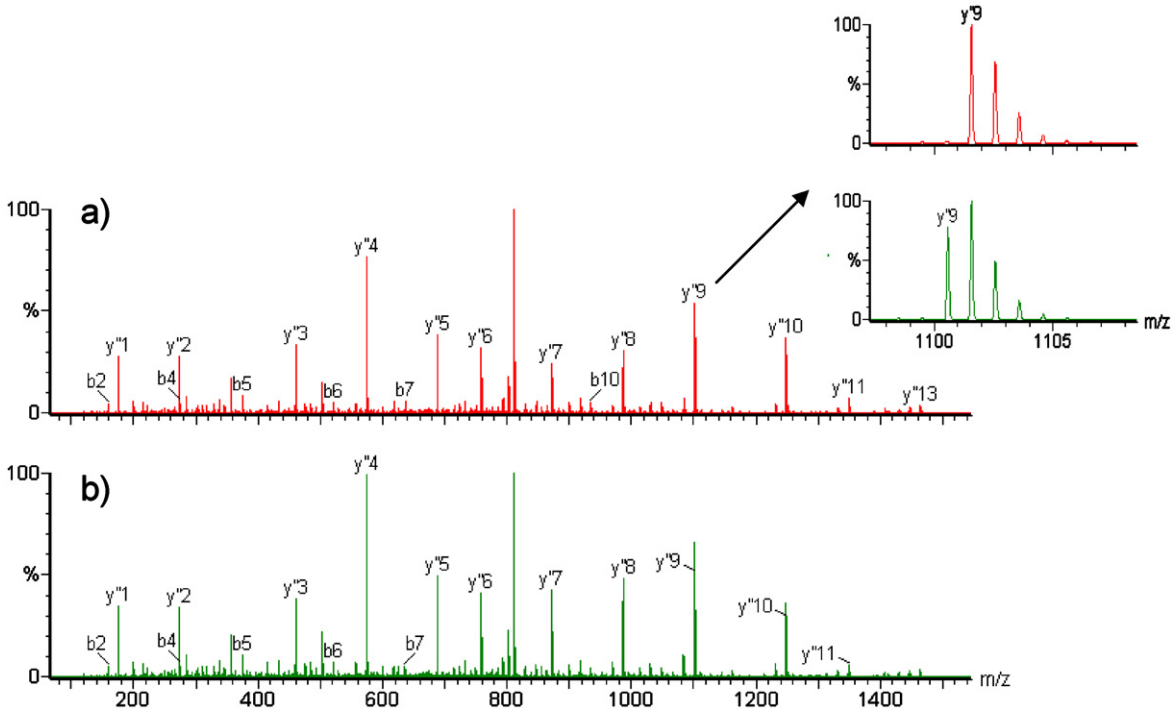

Fig. 5. Tandem mass spectra of the $(M+2H)^{2+}$ precursor ions of T4(H) (a) deamidated T4(H), and (b) non-modified T4(H). The deamidation site of T4(H) was identified from 1 Da mass increase of y''_9 fragment shown in the insert.

isoaspartyl *z* ion by electron capture dissociation (ECD) [37]. In addition to these MS approaches, Edman sequencing has been used to distinguish IsoAsp from Asp, as the presence of an IsoAsp residue in a peptide sequence leads to termination of the Edman degradation reaction. An alternative approach involves modification of deamidated peptides using the enzyme isoaspartyl methyltransferase (PIMT). PIMT selectively modifies IsoAsp residues with a methyl group at the α-carboxyl position. This reaction results in a retention time shift and a +14 Da mass difference between the methylated IsoAsp and Asp. In this study, Edman sequencing and PIMT reactions were applied to distinguish the IsoAsp and Asp deamidation products.

The T4(H) peptide contains four Asn residues. Using the ammonium formate separation conditions, two deamidated peaks were observed in the IgG1 final product (control sample). Edman sequencing was conducted on the deamidated peptides isolated from the LC eluent. Edman sequencing results showed that the first elution peak corresponded to the IsoAsp product and the second peak corresponded to the Asp product (data not shown). This result was confirmed by the PIMT reaction (Supplemental data). Near complete modification with a methyl group was only observed for the first deamidation peak, indicating the first peak contained IsoAsp product, while the second peak remained intact. Sequencing data also showed that the dominant deamidation site was at the first Asn residue. This finding agrees with the result determined by MS/MS (Fig. 5).

The T25(H) peptide has only one Asn residue, located in a favored deamidation sequence, –Asn–Gly– [18]. Thus, it is expected that the Asn residue is the deamidation site. T25(H) deamidation amount is negligible, so the deamidated products of T25(H) peptide have not been further characterized.

The T36(H) peptide contains two well-known deamidation sites in IgGs [23,24]. One is in –Asn–Gly– sequence, and the other is in –Pro–Glu–Asn–Asn–Tyr– (the PENNY sequence). The two deamidated peaks present in the peptide mapping method were characterized by Edman sequencing and the PIMT reaction. Edman sequencing results showed that the deamidation site of the first deamidated peak observed in the LC profile occurred at the first Asn residue (–Asn–Gly–) and the deamidation site of the second deamidated peak occurred at the first Asn residue in the PENNY sequence. Both peaks contained only the Asp product as no IsoAsp was detected by Edman sequencing. This result was verified by the PIMT reaction in which no methylated Asp was detected. The T36(H) peptide is located in the conserved region of the Fc domain. Deamidation products of the conserved T36(H) peptide have been well characterized in several studies [23,24]. Our findings are consistent with the deamidation sites identified in those reports. However, the dominant deamidation product of the first Asn residue (–Asn–Gly–) is IsoAsp in those studies [23,24] as opposed to Asp in our studies. The difference is likely due to the type of samples used for the study. Aged (accelerated stability) samples were used in the referenced studies. However, in this study, it is observed that non-aged samples exhibit the Asp deamidated product while aged samples degrade primarily to the isoAsp deamidation product.

4. Conclusions

A rapid peptide mapping method with MS detection was developed to quantify site-specific deamidation. Three distinct peptides of an IgG1 monoclonal antibody, that were found to be the most susceptible to deamidation under stressed conditions, were used as targeted sites for the development work. The developed rapid sample preparation procedure minimized method-induced deamidation. The reversed phase separation was modified using ammonium formate mobile phases to significantly reduce coelution of deamidated peptides and native peptides. A post-column infusion of formic acid was employed to aid electrospray ionization, and to improve method robustness and to enhance the linear range of the assay. The method has been demonstrated to quantify low levels of deamidation present at multiple sites each with potentially multiple deamidation products. The method was qualified demonstrating suitable linearity, precision and accuracy. LOD

and LOQ for specific peptides were estimated to be 0.1% and 0.3%, respectively. The strategy outlined in this report for quantifying site-specific deamidation can be applied to other monoclonal antibodies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.06.006.

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