

In Vivo Deamidation Characterization of Monoclonal Antibody by LC/MS/MS

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The spontaneous nonenzymatic deamidation of glutaminy and asparaginy residues of peptides and proteins has been observed both in vitro and in vivo. Deamidation may change the structure and function of a peptide or protein, potentially resulting in decreased bioactivity, as well as alterations in pharmacokinetics and antigenicity of the protein pharmaceutical. Therefore, it is necessary to monitor the effect of storage and formulation conditions on deamidation of a protein drug candidate. Of particular interest is the investigation of in vivo deamidation mechanisms of protein drug candidates. Several methods are available to characterize the deamidation of peptides and proteins. We present here a LC/MS/MS method used to evaluate the deamidation of an antibody after in vivo administration. A humanized monoclonal IgG1 antibody (MAb) has several “hot spots” for spontaneous deamidation. One site, amino acid residue Asn55 located in the CDR2 region of the heavy chain, is of particular interest since deamidation at this site greatly decreases the binding activity. MAb was administered to cynomolgus monkeys by intravenous and subcutaneous routes. At various times after dosing, monkey serum was prepared and MAb captured by the immobilized antigen or a goat anti-human IgG Fc γ antibody. The captured MAb was treated with trypsin followed by endoproteinase Glu-C. The digests were separated on RP-HPLC and analyzed by MS/MS on Q-ToF Global mass spectrometer. Using this method, we were able to determine the deamidation half-life of amino acid residue Asn55 in vivo and the ratio of the deamidated derivatives, i.e., isoAsp55 and Asp55. The method is rapid and sensitive with low-nanogram quantities of protein detected in the biological matrix.

Nonenzymatic deamidation of asparagine (Asn) or glutamine (Gln) is a major degradation pathway of peptides or proteins and can occur spontaneously during manipulation, purification, and long-term storage. Deamidation can cause structurally and biologically important alterations in peptides and proteins through the introduction of unfavorable negative charge. For example, de-

amidation of an Asn residue has been shown to cause a 25–500-fold reduction in the potency of human growth hormone releasing factor.¹ Studies^{2–5} have shown that there may be a close relationship between several disease states and in vivo deamidation of proteins, such as prion disease, Alzheimer’s disease, and cataract formation. Robinson and Robinson⁶ suggested in vivo deamidation of Asn residues of proteins as a molecular timer of biological events and as a mechanism for postsynthetic production of unique proteins of biological significance.

Nonenzymatic deamidation of proteins and peptides involves the formation of a succinimidyl intermediate.^{7,8} The deamidation rate is dependent on the solution pH, temperature, solvent dielectric constant, ionic strength, primary sequence, local polypeptide conformation, and tertiary structure.^{9–11} With regard to the primary sequence, deamidation rates depend on the amino acid residues adjacent to Asn and Gln in the peptide chain. Glycine (Gly) and serine (Ser) are found to be the most destabilizing C-terminal amino acids for deamidation of Asn. Serine, and to a lesser extent threonine (Thr) and lysine (Lys), also appeared to favor deamidation of Asn when they are located at its N-terminal side.^{7,12} Although Gln residues can also be deamidated, the rate of deamidation is much slower than the Asn residue under the same circumstances. Deamidation half-times of Asn and Gln in synthetic pentapeptide chains at neutral pHs and 37 °C are in the range of 1–500 days for Asn and 100–5000 days for Gln.⁹ Deamidation of Asn residues of proteins and peptides results in the formation of both isomeric α - and β -aspartyl residues, with the β -derivatives typically predominate in a ~3:1 ratio.^{2,7}

- (1) Friedman, A. R.; Ichhpurani, A. K.; Brown, D. M.; Hillman, R. M.; Krabill, L. F.; Martin, R. A.; Zurcher-Neely, H. A.; Guido, D. M. *Int. J. Pept. Protein Res.* **1991**, *37*, 14–20.
- (2) Lindner, H.; Helliger, W. *Exp. Gerontol.* **2001**, *36*, 1551–1563.
- (3) Sandmeier, E.; Hunziker, P.; Kunz, B.; Sack, R.; Christen, P. *Biochem. Biophys. Res. Commun.* **1999**, *261*, 578–583.
- (4) Watanabe, A.; Takio, K.; Ihara, Y. *J. Biol. Chem.* **1999**, *274*, 7368–7378.
- (5) Schey, K. L.; Little, M.; Fowler, J. G.; Grouch, R. K. *Invest. Ophthalmol. Vision Sci.* **2000**, *41*, 175–182.
- (6) Robinson, A. B.; Robinson, L. R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8880–8884.
- (7) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785–794.
- (8) Meinwald, Y. C.; Stimson, E. R.; Scheraga, H. A. *Int. J. Pept. Protein Res.* **1986**, *28*, 79–84.
- (9) Robinson, N. E.; Robinson, A. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12409–12413.
- (10) Kosky, A. A.; Razaq, U. O.; Treuheit, M. J.; Brems, D. N. *Protein Sci.* **1999**, *8*, 2519–2523.
- (11) Brennan, T. V.; Clarke, S. *Protein Sci.* **1993**, *2*, 331–338.
- (12) Bischoff, R.; Kolbe, H. V. *J. Chromatogr., B* **1994**, *662*, 261–278.

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Table 1. Concentration of MAb in Cynomolgus Monkey Serum Samples Introduced by Intravenous (IV) or Subcutaneous (SC) Injection

time (h)	concentration ($\mu\text{g/mL}$) of MAb					
	IV injection			SC injection		
	monkey 1	monkey 2	monkey 3	monkey 1	monkey 2	monkey 3
0.25	28.3	28.7	31.4			
4				8.2	11.9	9.3
24	10.7	11.7	12.3			
48				14.2	14.3	18.6
120	3.4	2.9	3.9	7.1	7.5	6.2
312	0.43	0.2	1.1	0.76	1.06	0.39

Antibodies are the first line of defense of the adaptive immune response and are found in the blood plasma and extracellular fluids. Many monoclonal antibodies are marketed and being tested in clinical trials as therapeutics in autoimmune and inflammatory diseases, oncology, and other therapeutic areas.^{13,14} Typically, therapeutic monoclonal antibodies have in vivo half-lives of days to weeks, staying in the circulation much longer than therapeutic growth factors, cytokines, and peptides. Hence, in vivo deamidation of a therapeutic monoclonal antibody may potentially have an impact on its efficacy, especially, when deamidation occurs in the binding regions. Characterization of in vivo deamidation of therapeutic monoclonal antibody could aid in development of more robust protein and antibody therapeutics.

Many methods are available to detect and quantify deamidation of Asn residues. Deamidation introduces negative charge and changes the protein mass (NH_2 vs OH , $\Delta = 1$ Da) and hydrophobicity. Separation techniques including electrophoretic (IEF, cIEF,¹⁵ urea gel electrophoresis¹⁶), and chromatographic (reversed-phase,¹⁷ ion exchange,^{18,19} and hydrophilic interaction²⁰) methods can be used to separate or isolate deamidated forms. Identification of deamidated forms can readily be achieved using mass spectrometry,^{21,22} and N-terminal sequencing, as well as enzymatically using protein L-isoaspartyl/D-aspartyl carboxyl methyltransferase.²³ In this paper, we present a sensitive and rapid LC/MS/MS method for the evaluation of in vivo deamidation in therapeutic monoclonal antibody (MAb).

EXPERIMENTAL SECTION

Materials. MAb, mutants, and human antigen were recombinant products of Eli Lilly and Co. Trypsin, TPCK treated, was

bought from Worthington Biochemical Corp. (Freehold, NJ), and endoproteinase Glu-C was a product of Roche Diagnostics Corp. (Indianapolis, IN). CNBr-activated Sepharose 4 Fast Flow was bought from Amersham Pharmacia Biotech AB (Uppsala, Sweden). AffiniPure goat anti-human IgG, $\text{Fc}\gamma$ is a product of Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The immobilized antigen (2 mg/mL resin) or goat anti-human IgG, $\text{Fc}\gamma$ (10 mg/mL resin) resin was prepared according to the standard procedure provided by the vendor. All other chemicals were analytical grade and commercially available.

Experiments. Cation Exchange Chromatogram of MAb. Sterile-filtered MAb samples, at 1 mg/mL in PBS (10 mM sodium phosphate, 150 mM sodium chloride buffer, pH 7.4), were kept at 4 °C and 37 °C for one week and analyzed by cation exchange chromatography (CEX). Prior to loading, MAb samples were exchanged into 20 mM sodium phosphate buffer at pH 6.5 (pump A buffer) using a Millipore centrifugal filter with 10 000 MWCO membrane. Samples were run on a Dionex Propac WCX-10 column with a flow rate of 1 mL/min using a linear gradient from 0 to 45% of 20 mM sodium phosphate, 250 mM NaCl, pH 6.5 (pump B buffer).

Monkey pK Study of MAb. MAb was administered to male cynomolgus monkeys ($n = 2/\text{group}$) as a single intravenous bolus or subcutaneous administration of 1 mg/kg. Blood samples were collected over a 4-week period and serum prepared. Concentrations (see Table 1 for sample information) of MAb in serum samples were determined by an antigen-capture ELISA method.

MAb in Vitro Experiment. One-milliliter aliquots of monkey serum were spiked with 1 or 10 μg of MAb, respectively, and the samples were then incubated at 37 °C for 96 h. The serum was either directly subjected to capture and LC/MS/MS analysis or stored at -20 °C for later analysis.

MAb Capture from Monkey Serum. Approximately 200 μL of serum from each monkey, collected during the monkey pK study, was mixed with ~ 2 μL of immobilized antigen or anti-human IgG $\text{Fc}\gamma$ antibody resin and the solution was then incubated at ambient temperature, gently shaking for 2 h. The resin was separated by centrifugation, and the supernatant was removed. The resin was washed with PBS buffer containing 0.2% Tween 20 followed with three PBS buffer washes. The antibody was released from the resin by treatment with 15 μL of 0.15% formic acid aqueous solution at ambient temperature for 15 min. The supernatant containing the captured antibody was lyophilized to dryness using a centrifugation vacuum system.

- (13) Andreaskos, E.; Taylor, P. C.; Feldmann, M. *Curr. Opin. Biotechnol.* **2002**, *13*, 615–620.
- (14) Trikha, M.; Yan, L.; Nakada, M. T. *Curr. Opin. Biotechnol.* **2002**, *13*, 609–614.
- (15) Bischoff, R.; Roecklin, D.; Roitsch, C. *Electrophoresis* **1992**, *13*, 214–219.
- (16) Groenen, P. J. T. A.; van Dongen, M. J. P.; Voorter, C. E. M.; Bloemendal, H.; de Jong, W. W. *FEBS Lett.* **1993**, *322*, 69–72.
- (17) Tsuji, K.; Baczynskyj, L.; Bronson, G. E. *Anal. Chem.* **1992**, *64*, 1864–1870.
- (18) Teshima, G.; Stults, J. T.; Ling, V.; Canova-Davis, E. J. *Biol. Chem.* **1991**, *266*, 13544–13547.
- (19) Aswad, D. W. *J. Biol. Chem.* **1984**, *259*, 10714–10721.
- (20) Lindner, H.; Sarg, B.; Grunicke, H.; Helliger, W. *J. Cancer Res. Clin. Oncol.* **1999**, *125*, 182–186.
- (21) Jedrzejewski, P. T.; Girod, A.; Tholey, A.; König, N.; Thullner, S.; Kinzel, V.; Bossemeyer, D. *Protein Sci.* **1998**, *7*, 457–469.
- (22) Carvalho, R. N.; Solstad, T.; Bjørge, E.; Barroso, J. F.; Flatmark, T. *J. Biol. Chem.* **2003**, *278*, 15142–15152.
- (23) Johnson, B. A.; Aswad, D. W. *Anal. Biochem.* **1991**, *192*, 384–391.

Enzymatic Digestions of Captured Monoclonal Antibody. The lyophilized material of each sample was reconstituted in 2 μL of 8 M urea and 0.5 M Tris buffer, pH 8.0, with 2.5 mg/mL dithiothreitol (DTT), and the solution was then incubated at 37 $^{\circ}\text{C}$ for 30 min. To the solution, 2 μL of a solution containing 10 mM ammonium bicarbonate buffer and 10 mg/mL iodoacetamide was added and the sample was incubated at ambient temperature in the dark for 10 min. The solution was diluted with 35 μL of 10 mM ammonium bicarbonate buffer and treated with 1 μL of 0.1 mg/mL trypsin solution at 37 $^{\circ}\text{C}$ for 90 min, followed by treatment with 1 μL of 0.1 mg/mL Glu-C solution at 37 $^{\circ}\text{C}$ for an additional 60 min. The purpose of subjecting the sample to a subsequent Glu-C digestion was to further reduce the peptide size thereby facilitating the separation of native and deamidated peptides and enhancing the sensitivity of LC/MS/MS method. The enzymatic activity was quenched by adding 1 μL of 10% acetic acid solution, and the samples were either immediately analyzed by LC/MS/MS or stored at -20°C until LC/MS/MS analysis.

Enzymatic Digestion of Stressed MAb. Deamidated MAb was generated *in vitro* by thermally stressing the protein at pH 7.4. The protocol subjected a 50- μL antibody sample at 1 mg/mL to 37 $^{\circ}\text{C}$ for one week in PBS (10 mM sodium phosphate, 150 mM sodium chloride buffer, pH 7.4). The sample was subsequently denatured and reduced by the addition of 50 mg of urea, 1 μL of 50 mg/mL DTT, and 20 μL of 3 M Tris buffer, pH 8.0. The resulting solution was incubated at 37 $^{\circ}\text{C}$ for 30 min. The protein was alkylated by adding 6 μL of 50 mg/mL iodoacetamide solution and incubating at room temperature in the dark for 15 min. The solution was desalted on a 1-mL spin column packed with P-6 resin (Bio-Rad; Hercules, CA). The desalting columns were washed and eluted with 0.025 M NH_4HCO_3 buffer. About 250 μL of protein fraction was collected for each sample from the column. Each protein fraction was mixed with 1 μL of 1 mg/mL trypsin solution, and then the mixture was incubated at 37 $^{\circ}\text{C}$ for ~ 3 h. A 5- μL aliquot of 0.1 mg/mL endoproteinase Glu-C solution was added to a ~ 100 - μL aliquot of the tryptic digests, and the mixtures were incubated at 37 $^{\circ}\text{C}$ for 2 h. The reaction was quenched by adjusting the solution pH with acetic acid.

Reversed-Phase HPLC Chromatography. A Waters Alliance HPLC system, model 2960, was used to separate the enzymatic digests of antibody starting material or captured material from monkey serum. The HPLC column was a Zorbax 300SB C18, 2.1 \times 150 mm, 5-mm particle size column. The flow rate was 0.2 mL/min and the column was at ambient temperature. The mobile phase was as follows: solution A, 0.15% (v/v) formic acid aqueous solution; solution B, 0.12% formic acid in acetonitrile. Prior to sample injection, the column was equilibrated at 12% solution B for 15 min. After the injection of sample, a gradient elution was performed from 12 to 25% solution B over 12 min, then to 90% solution B over 1 min, held at 90% solution B for 1 min, and then rapidly returned to initial conditions. An Agilent 1100 HPLC system was used to analyze the enzymatic digests of the stressed samples with the same conditions except a different gradient was used. The column was equilibrated at 5% solution B, and elution achieved by increasing solution B to 30% over 50 min, then 90% over 3 min, and subsequently held at 90% solution B for 1 min before being rapidly returned to initial conditions.

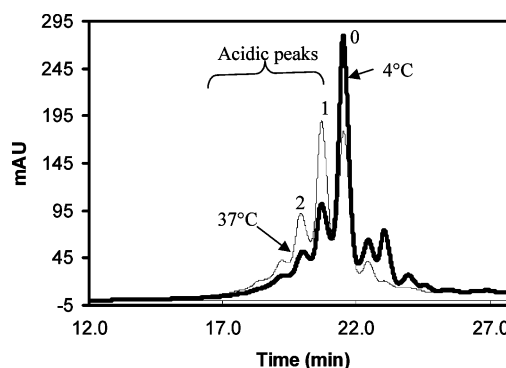


Figure 1. Cation exchange chromatography of MAb. Control sample at 4 $^{\circ}\text{C}$ vs a stressed sample kept at 37 $^{\circ}\text{C}$ for 7 days in PBS buffer at pH 7.4.

Mass Spectrometry. An Applied Biosystem Sciex API 150EX (Thornhill, ON, Canada) single-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source was used for LC/MS peptide map analysis of thermally stressed MAb samples. The single-quadrupole mass spectrometer was operated in the positive-ion mode with a mass scan range of 300–1500 Da, using a declustering potential of 30 V, focusing potential of 250 V, and scan rate of 2 s/scan. LC/MS/MS was performed on a Micromass Q-TOF Ultima Global with the following settings: a capillary voltage of 3.46 kV, a cone voltage of 100 V, a collision energy of 22 eV, a mass range of 100–2000, a desolvation temperature of 400 $^{\circ}\text{C}$, and a desolvation gas flow of 600 L/h. For single-quadrupole mass spectrometry, the HPLC stream was split $\sim 1:4$ with the smaller fraction of the stream diverted into the mass spectrometer. For the Micromass Q-TOF mass spectrometer, the HPLC effluent was introduced directly into the mass spectrometer without splitting the stream.

RESULTS AND DISCUSSION

Nonenzymatic deamidation of Asn or Gln residues of a peptide or protein is greatly affected by the amino acid residues near the Asn and Gln in the peptide chain. Gly and Ser located C-terminally to the deamidation site were found to be the most destabilizing.^{7,12} The MAb investigated in this study contains three Asn-Gly sequences. One of these sequences, Asn55 of the heavy chain, is in the binding region (CDR2), while the other two are in the Fc region. Figure 1 shows the CEX chromatograms of the thermally stressed MAb, in PBS buffer at 37 $^{\circ}\text{C}$ for one week, relative to a control sample at 4 $^{\circ}\text{C}$. The main peak (peak 0) corresponds to antibody lacking Lys residues at the C-terminus of both heavy chains. The acidic peaks (peaks 1 and 2) were greatly increased after a one-week incubation at 37 $^{\circ}\text{C}$ and thus were attributed to deamidation in the antibody.

Identification of Deamidation at Asn55 of MAb. To confirm that the increases in acidic peaks were due to Asn deamidation, LC/MS was used to analyze the enzymatic digest of the thermally stressed samples. The total ion chromatograms (TICs) of the control and thermally stressed MAb samples are shown in Figure 2. Based on mass analysis, the three peaks identified correspond to peptide masses (1444.8 for peak 1, 1445.7 for peak 2, and 1445.8 for peak 3), which are consistent with the native form and deamidated derivatives of heavy-chain peptide 51–63 ($_{51}\text{ILPGNG-NINYNEK}_{63}$). As shown in Figure 2, the deamidated

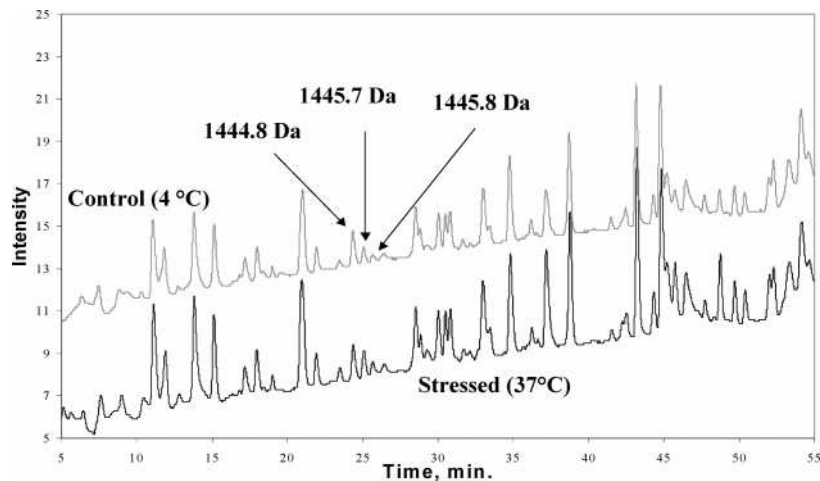


Figure 2. TICs of the digests of the control and stressed MAb.

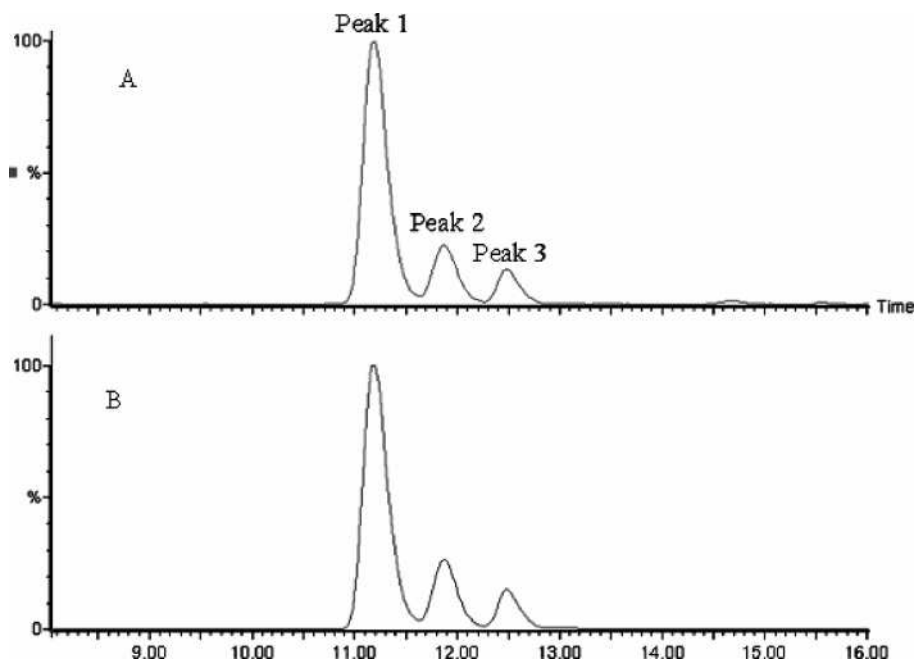


Figure 3. LC/MS/MS TIC (A) with the set mass (m/z) at 723 (equal to the doubly charged ion of peptide 51–63) and (B) the XIC from the ion at m/z 610–613, which covers the doubly charged ion of the fragment (y11) of peptide 51–63.

derivatives were more abundant in the thermally stressed sample. The half-life of deamidation in PBS at 37 °C was estimated to be ~6 days by CEX analysis. BIAcore analysis for the deamidated form of the MAb or the MAb mu₁ chain (N55D), in which Asn55 was recombinantly replaced with Asp55, indicated that deamidation at Asn55 greatly reduced the binding affinity to antigen by 14× (data not shown). Asn residues in the other two Asn-Gly sites in the Fc region were also deamidated, but at a much slower rate compared with that of the Asn55 residue; however, the deamidation in the Fc region is not expected to influence the binding affinity to antigen. In this paper, the focus is solely on Asn deamidation in the Fab region. Nonetheless, the method discussed is generally applicable to the characterization of Asn deamidation in the Fc region or in other proteins or peptides.

Heavy-chain peptide 51–63, which contains the Asn55 residue, was obtained by tryptic digestion followed by treatment with endoproteinase, Glu-C. The native peptide 51–63 and its deamidated derivatives were separated into three peaks by reversed-

phase HPLC. Figure 3 is the LC/MS/MS TIC for the doubly charged ion of peptide 51–63 and its derivatives. MS/MS spectra for each peak are shown in Figure 4. The y-series of ions are the predominant fragments. From these fragment (y-series) ions, peaks 1–3 contain the same y-series (≤ 8) of ion for low-mass fragments, indicating that the C-terminal amino acid residues between Gly56 and Lys 61 are the same for all three peaks; but the y9–y11 ions from peaks 2 and 3 are 1 Da higher than the corresponding ions from peak 1. The mass difference between the y9 and y8 is 114 Da for peak 1, matching an Asn residue, and 115 Da for peaks 2 and 3, 1 Da higher than Asn residue mass, thus corresponding to either deamidated derivative, Asp or IsoAsp of the Asn residue. Asp and IsoAsp possess isobaric mass and are generally indistinguishable by mass spectrometry. However, the area of peak 2 is twice that of peak 3. Under neutral or alkaline pH, a deamidation derivative ratio of IsoAsp/Asp of ~3/1 has been observed.^{2,7} Assuming this ratio of IsoAsp/Asp holds for this study and that the ionization efficiency of the peptides are not altered

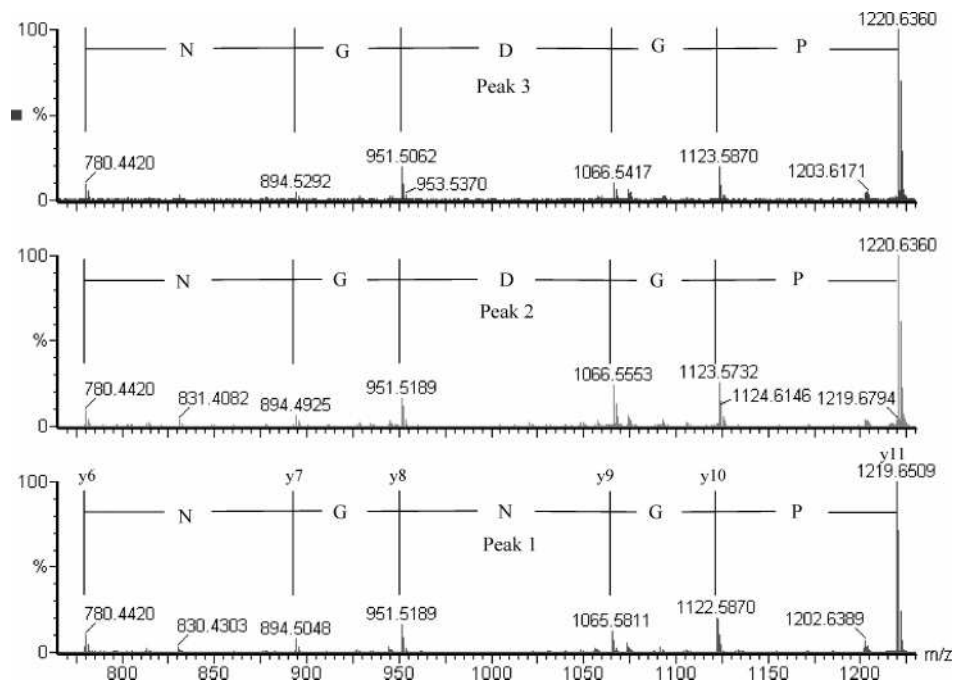


Figure 4. MS/MS spectra of peptide 51–63 peaks 1–3.

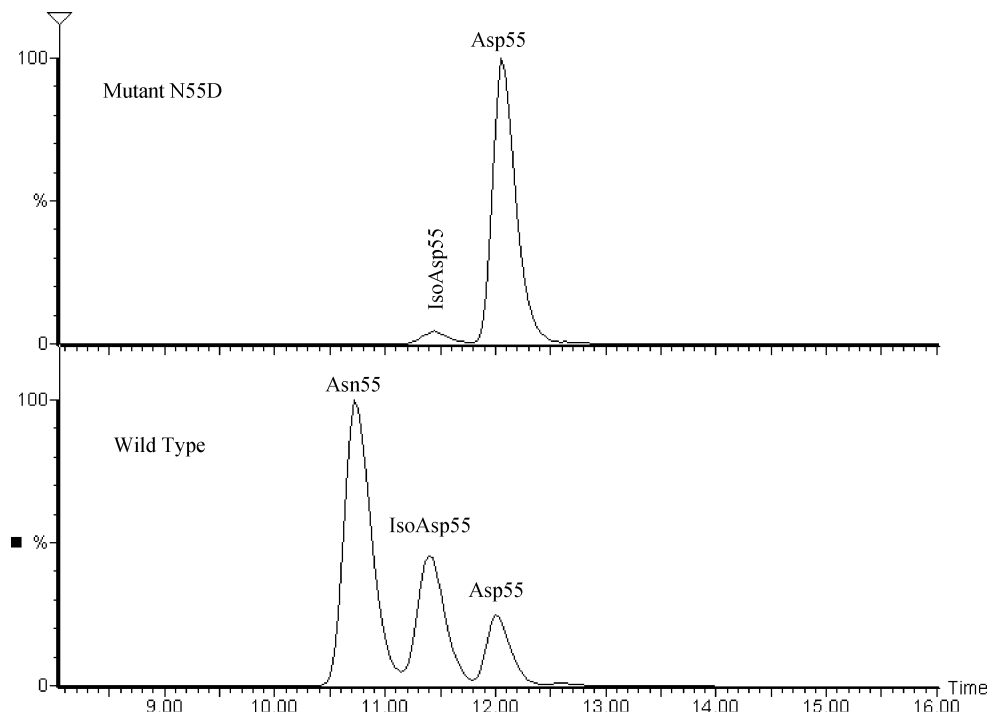


Figure 5. XICs of the double-charged ion of the fragment (y11) (the ion from m/z 610 to 613), of peptide 51–63 of wild-type MAb and its Asn55Asp mutagen.

by the deamidation, peak 2 is assigned as the IsoAsp derivative and peak 3 the Asp derivative. To unambiguously assign the peaks and assess the assumptions made regarding the deamidation derivative ratio and ionization efficiencies, LC/MS/MS analysis of N55D mutagen was used to confirm peak 3 as the Asp derivative (Figure 5) and thus by deduction, peak 2 is the IsoAsp derivative of the peptide 51–63.

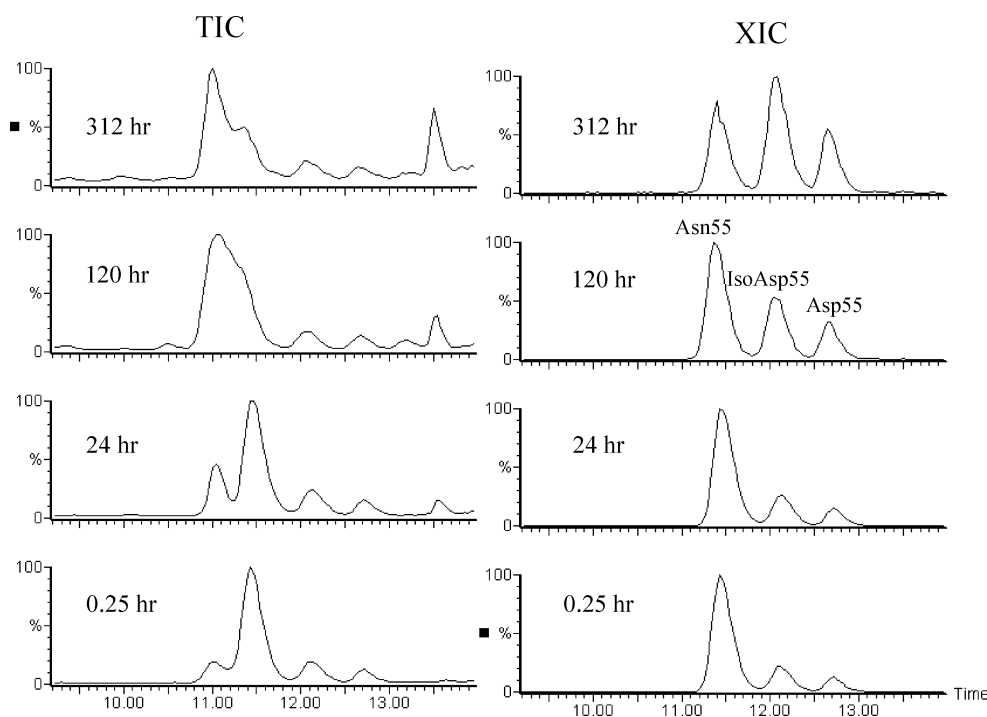
Method Qualification for Use in *In Vivo* Sample Analysis.

The MAb immunoextracted from monkey serum was not very pure, particularly in samples with very low MAb concentration

(for example, $<1 \mu\text{g/mL}$). Consequently, peptides from the contaminating proteins could interfere with the quantitative determination of the TIC from LC/MS/MS. Since the doubly charged ion of the y11 (data not shown) is the dominant fragment in the MS/MS spectrum of the peptide 51–63, the quantitative results of deamidation in the peptide 51–63 can be determined from the extracted ion chromatogram (XIC) of the y11 ion (see Figure 3b). Based on LC/MS/MS analysis for a standard sample, i.e., the MAb spiked into monkey serum, the quantitative results obtained from TIC and XIC for the deamidation of the Asn55 are

Table 2. LC/MS/MS Data Obtained for the Peptide 51–63 at Different Concentrations of MAb

amount (ng) injected	peak area from XIC			relative %			IsoAsp/Asp
	Asn55	IsoAsp55	Asp55	Asn55	IsoAsp55	Asp55	
10	211.3	65.9	29.2	69.0	21.5	9.5	2.3
50	911.7	278.1	117.1	69.8	21.3	9.0	2.4
100	1505.6	457.1	217.2	69.1	21.0	10.0	2.1
500	7089.2	2292.1	1088.3	67.7	21.9	10.4	2.1
av				68.9	21.4	9.7	2.2
SD				0.9	0.4	0.6	0.1
RSD, %				1	2	6	6

**Figure 6.** TICs of the double charged ion of peptide 51–63 and XICs of the double-charged ion of γ 11 fragment of peptide 51–63 from monkey serum samples at different time points.

very similar (data not shown). Furthermore, the quantitative results obtained by LC/MS/MS are consistent with those obtained by the other methods, such as CEX and capillary isoelectric focusing for a standard sample. Again, the results suggested that the ionization efficiency of both native and deamidated peptide 51–63 was very similar.

To evaluate the limit of quantitation and linearity of the LC/MS/MS method for deamidation characterization of peptide 51–63, a series of digests with different concentrations of MAb were analyzed. The area for each of three peaks was integrated from XIC to calculate the relative percentage of each peak. The results are shown in Table 2. Linear regression analysis for the data indicated that the response is linear ($R^2 > 0.99$) from 10 to 500 ng/injection of MAb for the native peptide 51–63 and its deamidated derivatives. In fact, quantitative determination of deamidation of peptide 51–63 only requires relative percent and not absolute value. Data shown in Table 2 demonstrate that the percent of deamidation of peptide 51–63 is independent of the amount injected over the range studied. These results show that LC/MS/MS can accurately determine the relative percent of deamidation of peptide 51–63 when > 10 -ng amounts of MAb are available.

Analysis of in Vivo Monkey Serum Samples. Serum samples were obtained from cynomolgus monkeys at various time points after subcutaneous or intravenous administration of the MAb and were analyzed by LC/MS/MS to determine the extent of deamidation of amino acid residue Asn 55 of the heavy chain. Most of the time points had more than enough antibody ($> 1 \mu\text{g/mL}$) for LC/MS/MS analysis. However, several of the later time point samples had MAb concentrations as low as 200 ng/mL, and only 200 μL of serum was available for analysis. However even under these circumstances, if the capture efficiency is sufficient, enough antibody is present to determine deamidation by LC/MS/MS. Although we did not determine the capture efficiency, the quantitative results (see below) indicated that enough MAb could be captured from the serum for quantitative analysis. Figure 6 is the TIC with the set mass (m/z) equal to 723 (which equals the doubly charged ion mass of the peptide 51–63) and XIC from the ion (m/z) 610–613 (which covers the doubly charged fragment 53–63 (i.e., γ 11)) at time points 0.25, 24, 120, and 312 h. For the 312- and 120-h time points, an impurity peak dominated the TIC tracing; thus, it was difficult to quantify the extent of deamidation at Asn55 from the TICs. However, only three peaks, all related to peptide 51–63, were present in the XICs. The XICs

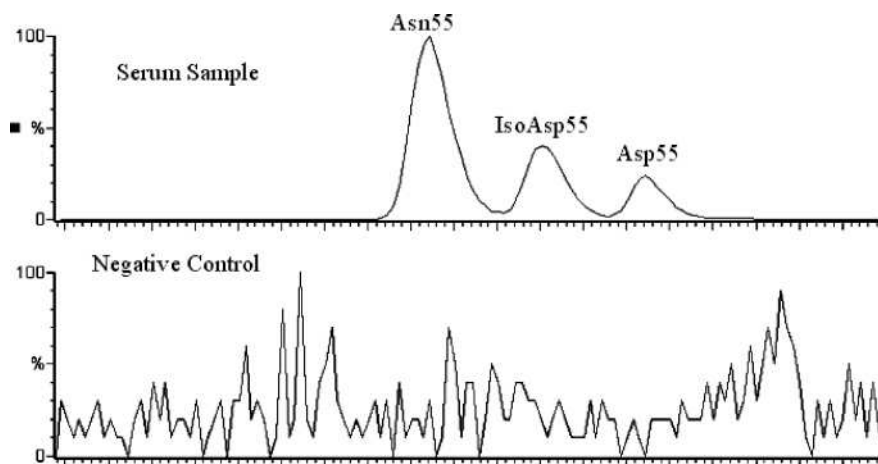


Figure 7. XICs of monkey serum sample of MAb and negative controls (vehicle serum).

for monkey serum MAb sample and the negative control (vehicle serum) are shown in Figure 7. No significant peaks for vehicle serum were detected, indicating that the method is specific for peptide 51–63. For the monkey serum MAb sample, the area of each peak was determined from the XIC and the calculated relative percent of native peptide 51–63 and its deamidated derivative ascertained. The results are summarized in Table 3. As shown in the table, the extent of deamidation was ~25% in the time 0.25- and 4-h samples. This is the amount of deamidation occurred during the protein production and storage.

With increasing circulation time of the antibody in monkey blood, the percent of deamidation at Asn55 increased, but at all time points the ratio of IsoAsp/Asp derivative is consistent, 2:1. Several conclusions can be drawn from these data. The MAb continuously deamidates in monkey blood, as expected, through a process consistent with a nonenzymatic mechanism. Moreover, the ratio of IsoAsp:Asp remains constant during the time course studied (IsoAsp:Asp = 2:1), suggesting that the clearance rates for IsoAsp derivative and Asp derivative are similar. If this were not the case, the ratio of IsoAsp/Asp would change with increasing circulation time.

Rate of Asn55 Deamidation in Vivo. Assuming that the in vivo clearance of native Mab (Asn55) and its deamidated derivatives (IsoAsp55 and Asp55) are similar, the deamidation rate of MAb in vivo could be calculated from the relative percent of deamidation. Nonenzymatic deamidation of Asn and Gln residue is a first-order reaction, i.e., the rate $[R]_d$ of deamidation derivative formed is proportional to the concentration of the native form;

$$[R]_d = d[D]/dt = -d[N]/dt = k[N]$$

$$d[N]/([N] + [D])/dt = -k[N]/([N] + [D])$$

$$d[N]_r/dt = -k[N]_r$$

$$[N]_r = A \exp(-kt)$$

$$\ln[N]_r = -kt + C \quad (1)$$

where $[N]_r$ is relative percent of native form, $[N]$ and $[D]$ are concentrations of native and deamidated derivatives, respectively. Equation 1 was used to fit the data to obtain parameters of k (rate constant) and C ($\ln(\text{percent of native MAb})$) at time zero.

Table 3. LC/MS/MS Data Obtained from Analysis of Monkey Serum Samples

time (h)	% deamidation for IV				% deamidation for SC			
	M 1	M 2	M 3	IsoAsp/Asp	M 1	M 2	M 3	IsoAsp/Asp
0.25	25.5	24.6	25.8	1.9				
4					25.6	26.1	26.0	1.9
24	28.7	30.8	25.7	2.0				
48					35.7	36.2	35.3	1.9
120	45.5	46.5	40.6	1.9	44.7	44.3	45.0	2.0
312	67.9	55.5	64.0	2.1	68.0	66.7	62.5	2.1

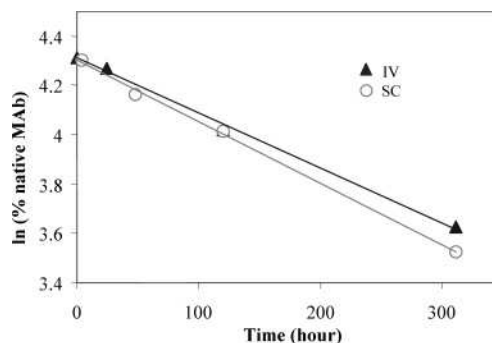


Figure 8. Logarithm of relative native peptide of monkey serum sample vs time for in vivo PK study.

The plot of the logarithm of relative percent of native MAb as a function of time is linear and shown in Figure 8. The linear regression fit predicts that slope ($-k$) equals -0.0022 and -0.0025 for IV and SC administration, respectively, and the intercept (C) equals 4.31 and 4.30 for IV and SC administration, respectively. From these data, the half-life of deamidation of the Asn55 of MAb in monkey is ~277 h for both IV and SC. However, since antibodies are dimers possessing two heavy chains, i.e., two Asn55 residues, and assuming the deamidations at these two Asn55 are independent of each other, the half-life of the deamidation of intact MAb is half of that ascribed to the Asn55 peptide, or 140 h or 6 days in monkey. The rate of deamidation does not appear to be dependent on the route of administration (SC vs IV).

As discussed above, this LC/MS/MS methodology enabled the quantitative determination of Asn and Gln deamidation in a peptide or protein from a complex biological matrix. In this methodology, it is beneficial that the chromatographic separation

be of high enough resolution to resolve amidated and deamidated forms of the same peptide. If the amidated and deamidated forms coelute, the single mass unit increase due to deamidation will only be observable, in most instruments, by an alteration in the isotopic distribution pattern. The methodology also requires that a sufficient number of MS/MS spectra for a specific parent ion be acquired for quantitation. This may preclude simultaneous quantitation of the relative extent of deamidation at multiple sites within a mixture of proteins in a single LC/MS/MS run. In some cases it may also be advantageous to add an alternate enzymatic digestion protocol to generate suitable sized peptides before LC/MS/MS analysis.

CONCLUSION

Nonenzymatic deamidation of Asn or Gln of a peptide or protein is one of the major degradation pathways observed in vivo as well in the manufacturing and storage of biopharmaceuticals. Deamidation of a protein or peptide introduces additional negative charge, which may cause structural changes in the molecule. These structural changes may further introduce changes in biological function, leading to a decrease in activity or function.

Deamidation of a protein or peptide introduces an unnatural amino acid residue, IsoAsp, and may change its antigenicity. Hence, deamidation of a protein or peptide biopharmaceutical must be carefully monitored. There are many available methods to characterize and quantify deamidation of protein or peptide. However, these methods require pure materials or large quantities of samples and cannot handle complex mixtures.

As we discussed in this paper, by combining HPLC/MS/MS and microscale immunopurification, it is possible to characterize deamidation in vivo with low-picomole or high-femtomole amounts of material. Specifically, we have described a LC/MS/MS method to characterize deamidation of Asn55 of the heavy chain of a monoclonal antibody, MAb, in vivo. The method is sensitive and rapid with analysis accomplished in less than one day. To our knowledge, this is the first paper describing a rapid and sensitive method to characterize in vivo deamidation of a protein or peptide pharmaceutical.

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