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APPLICATION NOTE

Determination of the Glycoforms and Deamidation of a Monoclonal Antibody by Ion Exchange Chromatography and Mass Spectrometry

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ABSTRACT

Purpose: The objective of the current study was to evaluate the glycoform composition and the potential deamidation species of an IgG antibody by cation exchange chromatography and mass spectrometry.

Methods: A concentrated solution of an IgG antibody was chromatographed onto a cation exchange column (Biosuite CM) and eluted with NaCl gradient in phosphate buffer on an HPLC to evaluate the glycoform composition of the sample. For determination of deamidated species, the antibody was incubated with basic solutions of either ammonium carbonate or Tris-HCl at different temperatures and time intervals. The resulting forced-deamidated antibody was chromatographed onto the same cation exchange column. Multiple fractions of the eluted antibody peak were concentrated, desalted, and analyzed by LC-TOF mass spectrometer. The protein concentration was determined by UV mass spectrometry at 280 nm.

Results: Three major glycoforms of the test antibody were sequentially eluted on the cation exchanger; the three glycoforms corresponded to the degree of terminal galactosylation as determined by mass spectrometry. The intact mass of the three major N-glycoforms differed by about 162 Da representing each additional terminal galactosyl moiety in the antibody. The high galactose glycoform showed a longer retention timeas compared with the low galactose species on the cation exchanger. With increasing deamidation temperature or time, the main antibody peak shifted to shorter retention times on the cation exchanger, which corresponded to the presence of acidic or deamidated glycoforms of the antibody. By mass spectrometry, the dominant mass of these acidic glycoforms increased by at least 1 Da as compared with the control. These findings are consistent with the presence of multiple deamidated species of the antibody glycoforms in forced-deamidated samples.

Conclusions: A cation exchange method coupled with mass spectrometry was developed to determine the major glycoforms and the corresponding deamidated species of the test antibody. The deamidated species of the antibody were resolved from their corresponding native forms as determined by mass spectrometry.

BACKGROUND

Glycosylation is an important post-translational modification of proteins. Monoclonal antibodies, which are inherently glycosylated, represent a major component of protein therapeutics and biologics in the biopharmaceutical industry. The glycoform profile and deamidation play a major role in the stability, safety, and efficacy of a monoclonal antibody molecule. The sugar chains on glycoproteins can mediate biological activity and can, therefore, be associated with the safety and efficacy attributes of many biopharmaceuticals. Deamidation, or the conversion of asparagine and glutamine to aspartic/isoaspartic acid and glutamic acid, respectively, has been linked to changes in the physicochemical and functional stabilities of the protein molecule. As such, the heterogeneity of the glycosylation patterns and deamidation are typically characterized and monitored at multiple stages of biopharmaceutical drug development.

OBJECTIVE

The objective of the current study is to evaluate the glycoform composition and the potential deamidation species of the test antibody by cation exchange chromatography and mass spectrometry.

METHODS

Materials: The main sample used in this project consisted of a monoclonal IgG antibody, obtained from Morphotek, Inc.

Equipment: The primary equipment used in this project included an HPLC equipped with fraction collector, a Tangential Flow Filtration (TFF) system, and a UPLC- LCT Premier XE TOF MS system.

Preparative HPLC Conditions: The sample was chromatographed onto a BioSuite CM Cation Exchanger 13 µm, 21.5 × 150 mm at ambient temperature. The monoclonal glycoforms were eluted in a sodium chloride gradient with phosphate buffer, pH 6.0. Eluate fractions of the peaks were collected and stored at 2 – 8 °C until further processing.

LC-TOF MS Analysis: The individual eluate fractions were

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analyzed on ESI-LC-TOF MS for intact mass using a micro desalting column and eluted with an acetonitrile gradient in 0.1% formic acid. The representative parameters for the mass spectrometer were set as follows:

- **Ion Mode:** Positive ion detection
- **Scan Range:** 500 to 4000 m/z
- **Capillary:** 3200 V
- **Sample Cone:** 40 V
- **Desolvation Temp:** 350 °C
- **Source Temp:** 120 °C
- **Cone Gas Flow:** 40 L/h
- **Desolvatio Gas Flow:** 800 L/h
- **Lockmass:** 0.5 ppm leucine enkephalin

Ultrafiltration/Diafiltration (UF/DF): The selected eluate fractions were pooled, concentrated, and buffer-exchanged using a TFF system equipped with a 5 K cut-off filter. The retentate was repeatedly circulated, diafiltered, and concentrated with PBS. The final concentration of the protein sample was determined by extinction coefficient at A280 nm. When applicable, a Vivaspin micro-spin filter with 3K MWCO was used to further concentrate the protein samples.

- **Deamidation Conditions:** The representative deamidation conditions for the antibody were carried out as follows:
- **Buffer:** 0.1 M Tris-HCl, pH 9.0 or 0.1 M ammonium carbonate, pH 8.6
- **Incubation Temp:** 25 °C, 37 °C, 45 °C
- **Duration:** 2 to 6 days

Sample Designation:

- CON Untreated IgG antibody
- De1 treated with 0.1 M Tris-HCl, pH 9 at 25 °C for 6 days
- De2 treated with 0.1 M Tris-HCl, pH 9 at 37 °C for 2 days
- De2B treated with 0.1 M Tris-HCl, pH 9 at 37 °C for 4 days
- De3 treated with 0.1 M Ammonium Carbonate, pH 8.6 at 45 °C for 6 days

RESULTS

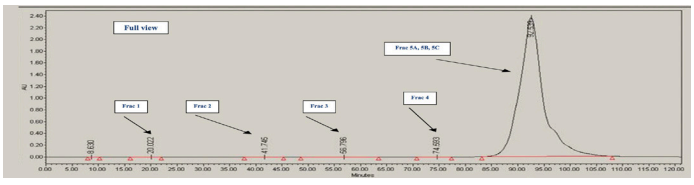

Figure 1. Representative IEX Chromatogram of Sample (Full View)

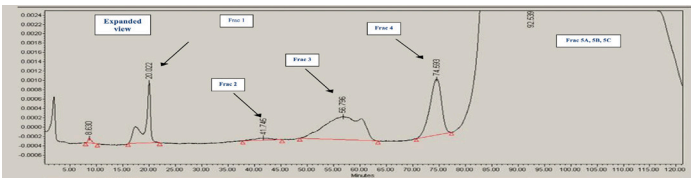

Figure 2. Representative IEX Chromatogram of Sample (Expanded View)

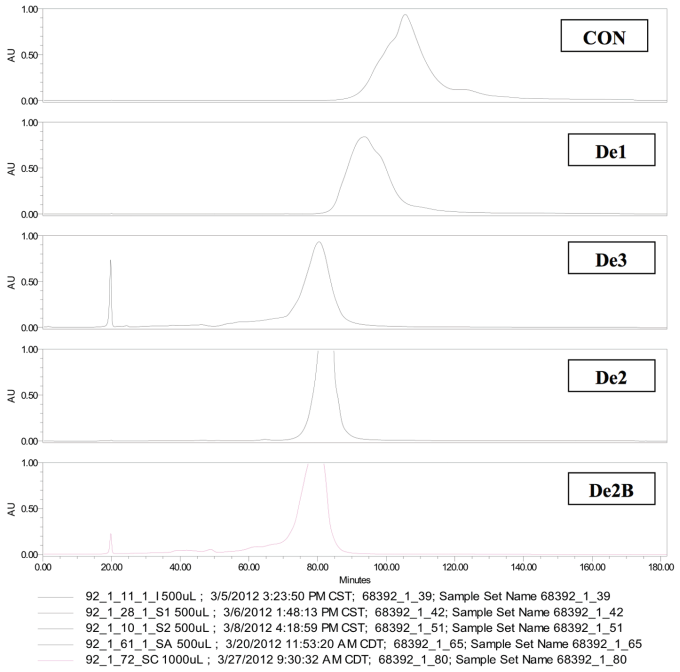

Figure 3. Representative IEX Chromatogram of Forced-Deamidated Sample (Full View)

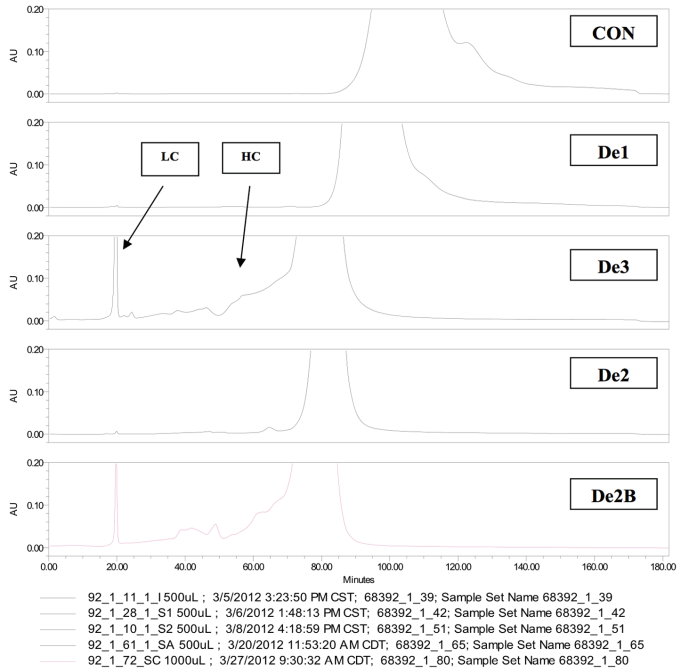

Figure 4. Representative Chromatogram of Forced-Deamidated Sample (Expanded View)

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RESULTS [CONTINUED]

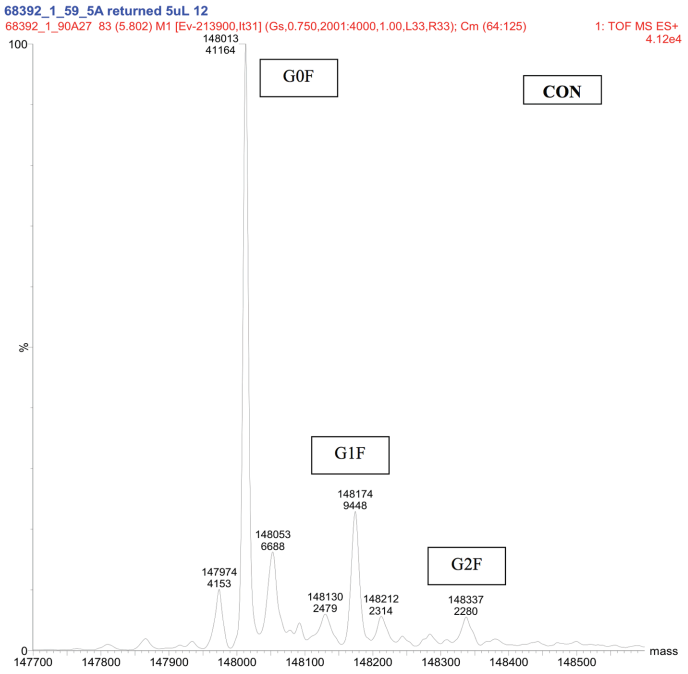

Figure 5. Representative Deconvoluted Mass Spectra of the Control Sample

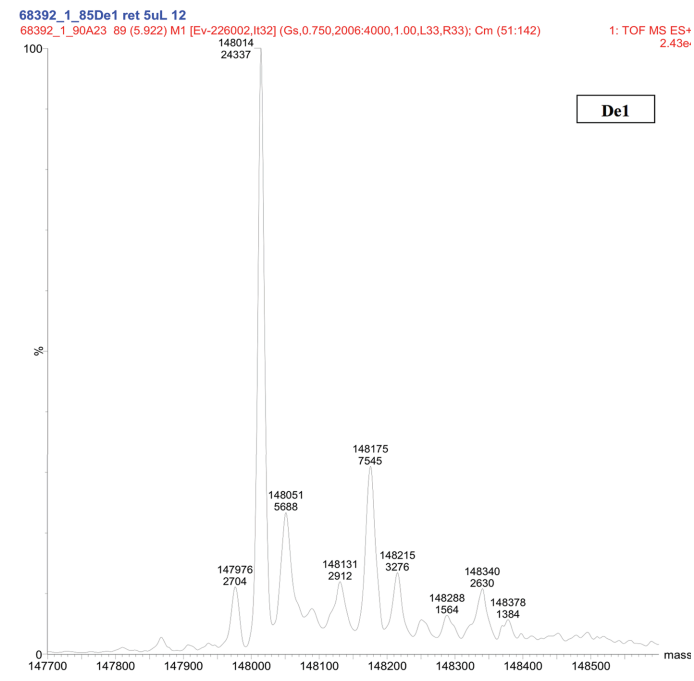

Figure 6. Representative Deconvoluted Mass Spectra of Deamidated Sample (De1)

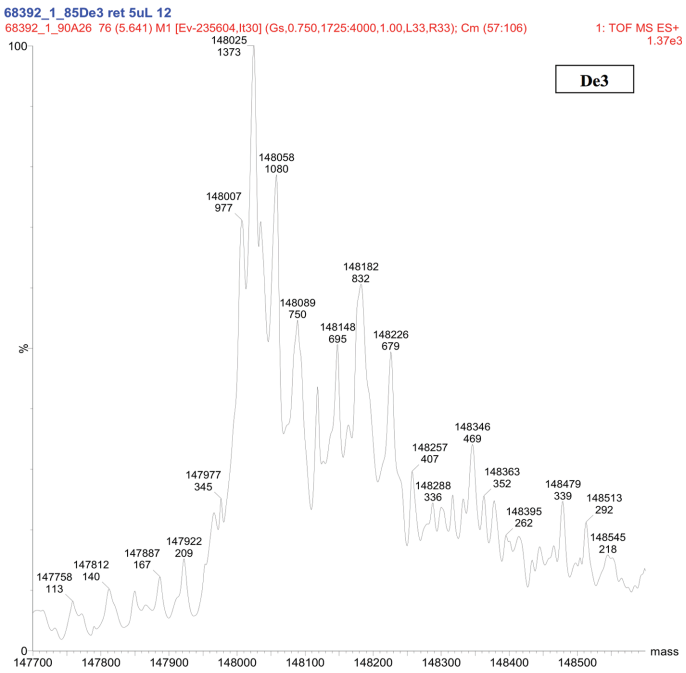

Figure 7. Representative Deconvoluted Mass Spectra of Deamidated Sample (De3)

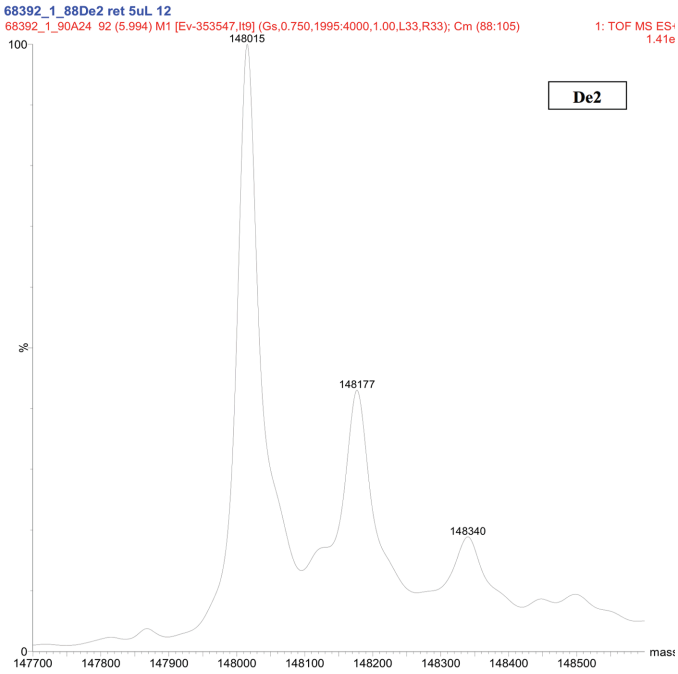

Figure 8. Representative Deconvoluted Mass Spectra of Deamidated Sample (De2)

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RESULTS [CONTINUED]

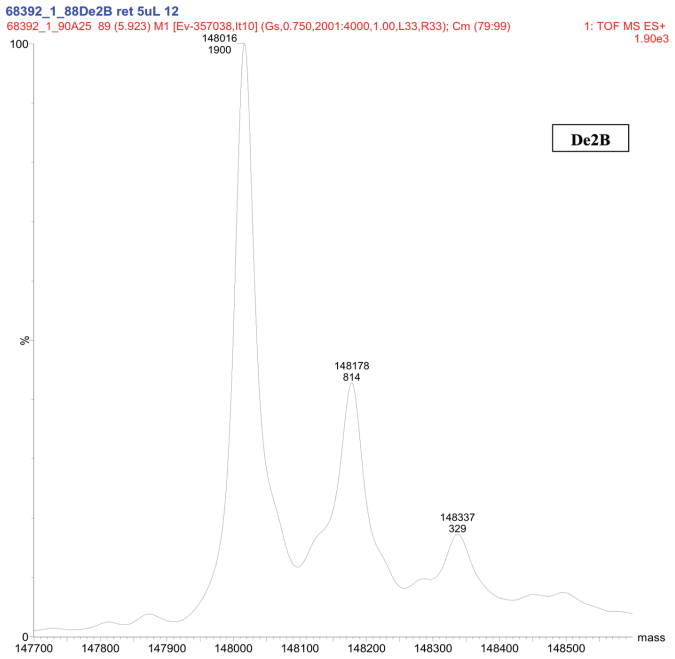

Figure 9. Representative Deconvoluted Mass Spectra of Deamidated Sample (De2B)

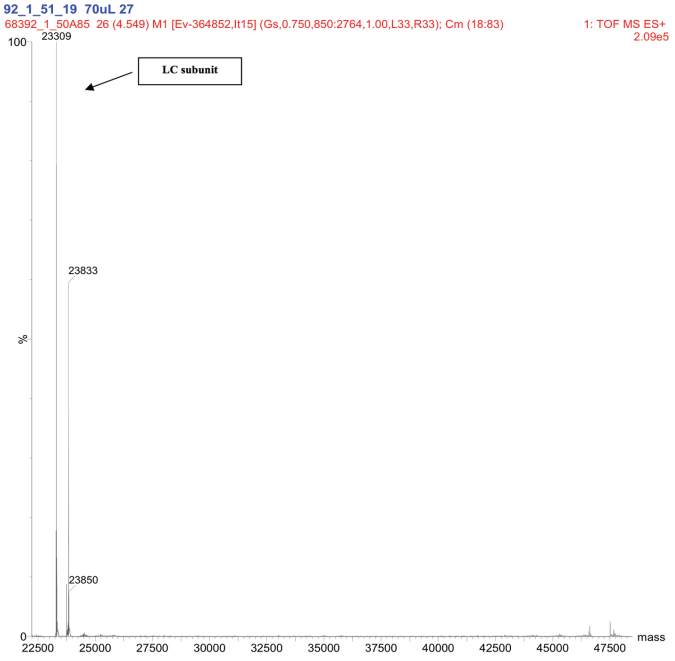

Figure 10. Representative Deconvoluted Mass Spectra of De3 at pk 20 min

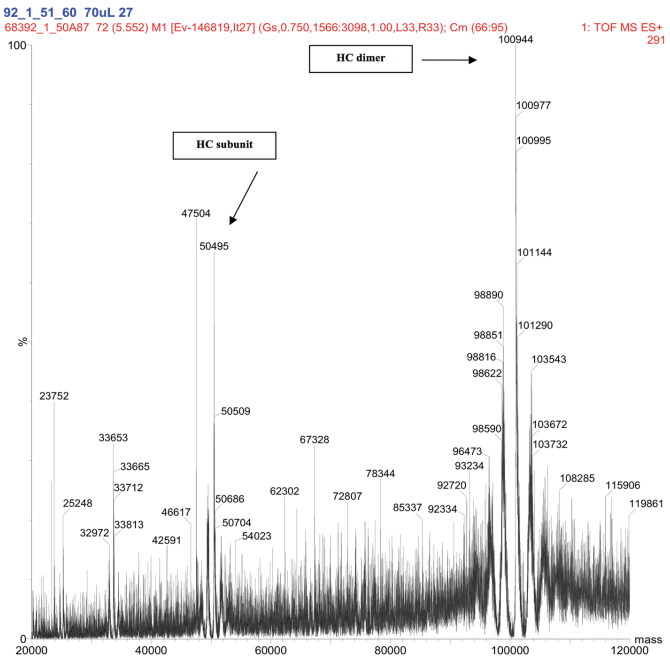

Figure 11. Representative Deconvoluted Mass Spectra of De3 at ~pk 60 min

	Name	Retention Time (min)	Area (µV*sec)	% Area	Height (µV)
1		8.630	4605	0.00	105
2	Frac 1	20.022	99215	0.01	1295
3	Frac 2	41.745	10337	0.00	48
4	Frac 3	56.796	249344	0.03	496
5	Frac 4	74.593	184016	0.02	1200
6	Frac 5	92.539	740825940	99.93	2367561

Table 1. Peak areas of the IEX fractions of the IgG Antibody

DISCUSSIONS

A typical preparative ion exchange chromatogram of the IgG antibody displayed the main peak at about 92 minutes, and four early-eluting minor peaks (Figures 1 and 2). The total peak area of the minorpeaks constituted less than 0.1% of the sample (Table I). The fractions of the four minor peaks were too dilute and were not sufficient to obtain intact mass by mass spectrometry.

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The forced-deamidation profile of the antibody on cation exchange is shown in Figures 3 and 4. In general, the main antibody peak shifted to a shorter retention time on the cation exchanger with increasing deamidation temperature or time. This shift appears to be consistent with the chromatographic behavior of acidic or deamidated glycoform species on a cation exchanger.

In Figure 5, a representative deconvoluted spectra of the control (untreated) exhibited a typical antibody glycoform profile with the main species corresponding presumably to G0F, G1F, and G2F.

The difference in species mass was about 162 Da, which is consistent with terminal galactosylation. With forced-deamidated samples, the intact mass of the glycoforms appeared to increase by at least 1 Da as compared with the control (Figures 6 to 9). This is consistent with the presence of deamidated species. With increasing deamidation time or temperature, the intact mass of the glycoforms increased progressively, suggesting multiple or further deamidation of the sample.

In Figures 10 and 11, the presence of molecular mass corresponding to the light chain (LC) and heavy chain (HC) subunits of the monoclonal antibody molecule were also observed in the forcedeamidated samples. The light chain and heavy chain were eluted before the main peak, at about 20 minutes and 60 minutes, respectively (Figure 4). The light chain occurred as a sharp and distinct peak as compared with the heavy chain subunit, which was observed to have a more complex spectrum.

CONCLUSION

A preparative cation exchange method coupled with mass spectrometry was developed to determine the major glycoforms and the corresponding deamidated species of the antibody. The deamidated species of the antibody were isolated and resolved from their corresponding native forms based on intact mass analysis, as determined by TOF mass spectrometry.

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