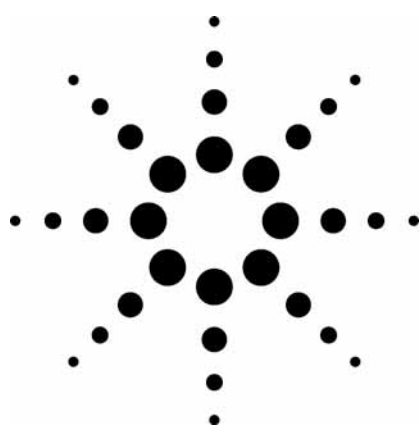




Comparison of ZORBAX StableBond 300 Å LC Columns to Optimize Selectivity for Antibody Separations Using HPLC and LC/MS*

Application

Pharmaceuticals

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Abstract

StableBond 300 Å Rapid Resolution (3.5 µm) LC columns with different bonded phases were used to systematically develop a reversed-phase LC/MS method for reduced antibodies that provides effective separations of light chains and two variants of heavy chains containing N-terminal glutamine and pyroglutamic acid. The best separation was achieved on ZORBAX 300 Å SB-CN LC Columns eluted with increasing percentage of *n*-propanol and acetonitrile in 0.1% trifluoroacetic acid.

Introduction

Characterization of antibodies is an important challenge in pharmaceuticals. Although they can be

characterized by many separation techniques, reversed-phase chromatography LC/MS of these compounds has been difficult. Problems have included poor recovery from the column, poor resolution, and rapid decrease in column performance, leading researchers to choose other separation techniques. In many cases modifications of potential clinical significance can occur simultaneously, making mass spectroscopic analysis highly desirable.

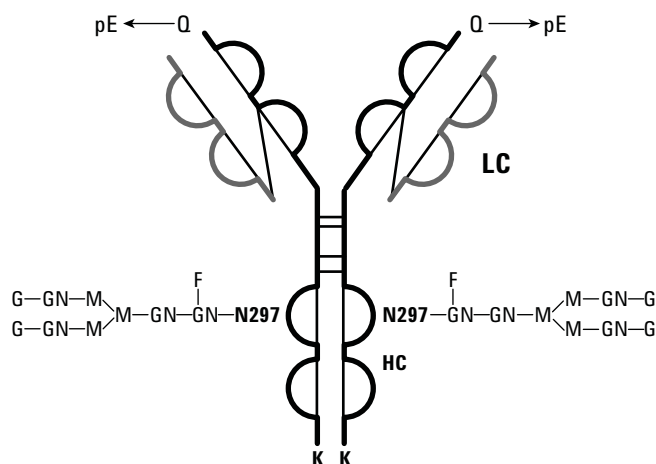
Perfusion chromatography has been frequently used for separation and purification of intact antibodies [1–3] as well as their reduced and alkylated chains [4]. Although an effective preparative technique, analytical perfusion chromatography suffers from two limitations, that is, poor separation of variants and difficulty with interfacing mass spectrometry because of high flow rates required for separation [2]. A method for RPLC/MS analysis of intact antibodies using column temperatures greater than 70 °C and stationary phases with long alkyl chains (C8 and C18) is described in reference [5] and was further optimized by employing propanol and other solvents with high elutropic strength [6]. That RPLC/MS method using rugged StableBond columns for the intact antibodies served as a starting point for method development for reduced antibodies described here.

Human immunoglobulin gamma (IgG) antibodies are glycoproteins containing two identical copies of light chains (LC) and heavy chains (HC) connected by external disulfide bonds (Figure 1). The light chains can be either kappa or lambda type and the heavy chains are gamma. The N-terminal

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Code	Glycan structure	Mono mass	Average mass
G2	$\begin{array}{c} \text{G}-\text{GN}-\text{M} \\ \text{G}-\text{GN}-\text{M} \end{array} \text{M}-\text{GN}-\text{GN}-\text{F}$	1768.6	1769.6
G1	$\begin{array}{c} \text{G}-\text{GN}-\text{M} \\ \text{GN}-\text{M} \end{array} \text{M}-\text{GN}-\text{GN}-\text{F}$	1606.6	1607.5
G0	$\begin{array}{c} \text{GN}-\text{M} \\ \text{GN}-\text{M} \end{array} \text{M}-\text{GN}-\text{GN}-\text{F}$	1444.5	1445.4
MAN5	$\begin{array}{c} \text{M} \\ \text{M} \\ \text{M} \end{array} \text{M}-\text{GN}-\text{GN}-\text{F}$	1216.4	1217.2
F	Fucose	146.0579	146.1430
G	Galactose	162.0528	162.1424
M	Mannose	162.0528	162.1424
GN	N-acetylglucosamine	203.0794	203.1950

Figure 1. Structure of recombinant monoclonal IgG antibody used in this study. Glycosylation profile includes two biantennary glycans with variable number of galactose residues (G). Most common isoforms are G0, G1, and G2. Conversion of N-terminal glutamine (Q) to pyroglutamate (pE) of heavy chain (HC) is also shown. Heavy chain C-terminal lysine (K) is often missing in antibodies due to the enzymatic activity. Disulfide bonds are shown in yellow, heavy chains in green, and light chains (LCs) in pink.

residue of immunoglobulin gamma heavy chain is typically either glutamine, glutamic acid, or aspartic acid. The N-terminal residues for kappa light chain are typically either glutamic acid or aspartic acid, while for lambda light chain they are serine, proline, or glutamine [7] [8]. The most typical post-translational modification of monoclonal antibodies is conversion of the N-terminal glutamine (Q) to pyroglutamate (pE).

The formation of pyroglutamate (pE) from Q eliminates N-terminal amine and generates an acidic variant because the remaining amide of the ring is neutral [2]. This modification is associated with the release of ammonia and mass decrease by 17 u (Figure 2). Ion exchange chromatography or isoelectric focusing have been found to be useful in separating these variants, but CEX and IEF are not easily hyphenated with mass spectrometry. The RPLC/MS technique described here has the poten-

tial to distinguish pyroglutamate (−17 u) from other “charged modifications” such as deamidation (+1 u), C-terminal lysine (+128 u), extra sialic acid (+291 u), and partial unfolding (0 u).

Electrospray ionization mass spectrometry (ESI-MS) has been used previously to establish the glycosylation profile of an antibody after reduction/alkylation or limited proteolysis using direct infusion of desalted sample [9]. In this article, we report on the identification of a glycosylation profile by ESI-MS on-line with reversed-phase HPLC separation.

This RPLC/MS method for reduced antibodies clearly separates light and heavy chains, Q and pE variants of heavy chains, and other possible modifications of antibody chains.

Experimental

Materials

The recombinant human monoclonal IgG antibody analyzed in this study was expressed at Amgen (Seattle, WA, USA) and purified using standard manufacturing procedures. A sample of the antibody has been stored for 3 months in a pH 5.8 buffer at 29 °C ($T = 3 \text{ m } 29 \text{ °C}$) and compared to control sample, which was stored frozen at −80 °C (T_0).

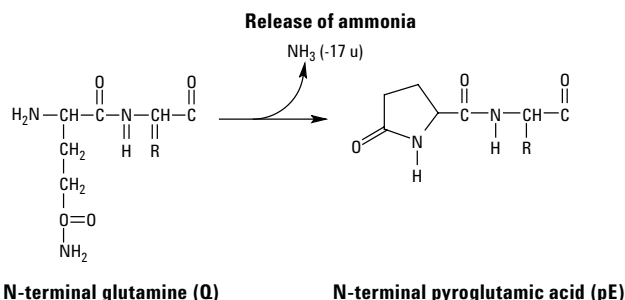


Figure 2. Mechanism of formation of pyroglutamate (pE) from N-terminal glutamine (Q).

Sample Preparation

Reduction and Alkylation

Reduction and alkylation was performed using IgG under denaturing conditions to produce the free heavy and light chains for further analytical characterization. Antibody was diluted to 2 mg/mL with a buffer including 7.5 M guanidine hydrochloride, (Mallinckrodt, Phillipsburg, NJ, USA), 0.1 M Tris-HCl (Sigma, St. Louis, MO, USA), and 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma) pH 7.5 to a volume of 0.5 mL. A 5- μ L aliquot of a 0.5 M dithiothreitol (DTT, Sigma) stock solution was added to obtain 5 mM DTT concentration and the reaction mixture was placed at 37 °C for 30 min. Protein solution was then cooled to room temperature and a 13- μ L aliquot of a 0.5 M iodoacetamide (IAM, Sigma) stock solution was added to reach 13 mM IAM. The alkylation was performed at room temperature for 40 min while being protected from light. The 0.5 mL of buffer of the reduced and alkylated protein was exchanged with 1 mL of a 10 mM sodium acetate (J. T. Baker, Phillipsburg, NJ, USA) solution at pH 5.0 to a final concentration of 1 mg/mL of protein. Buffer exchange was performed using a NAP-5 gel filtration column packed with Sephadex G-26 medium (Amersham Pharmacia Biotech, Orsay, France) following the manufacturer's recommendations.

RPLC of Reduced and Alkylated IgG

Reversed-phase LC/MS of the reduced and alkylated IgG was performed on an Agilent 1100 Capillary HPLC system equipped with a UV detector, autosampler, nano flow cell, and temperature-controlled column compartment. The mobile phase included water solvent A and 80% *n*-propanol, 10% acetonitrile, 9.9% water in solvent B, with 0.1% trifluoroacetic acid added to both solvent bottles. Agilent ZORBAX StableBond SB-300 Å columns with 3.5 μ m particle size, 300 Å pore size, 1 mm $\times$ 50 mm, were used for the LC/MS analysis, including C3, C8, C18, and CN stationary phases. The columns were operated at 75 °C and a flow rate of 50 μ L/min. The column eluate was analyzed by UV detector and then directed to an on-line mass spectrometer. The same types of ZORBAX StableBond 300 Å columns in 4.6 mm $\times$ 150 mm format at 1 mL/min were used for UV detection only.

Comparison of Different StableBond Widepore Reversed-Phase Columns for Reduced and Alkylated Antibody Analysis

Figure 3 shows RP chromatograms of the reduced and alkylated IgG obtained on ZORBAX SB-300 Å columns. The separation was achieved using the same linear gradient from 23% to 35% of solvent B. We determined that the Agilent ZORBAX StableBond 300 Å columns packed with C3, C8, C18, and CN materials provided the high resolution and

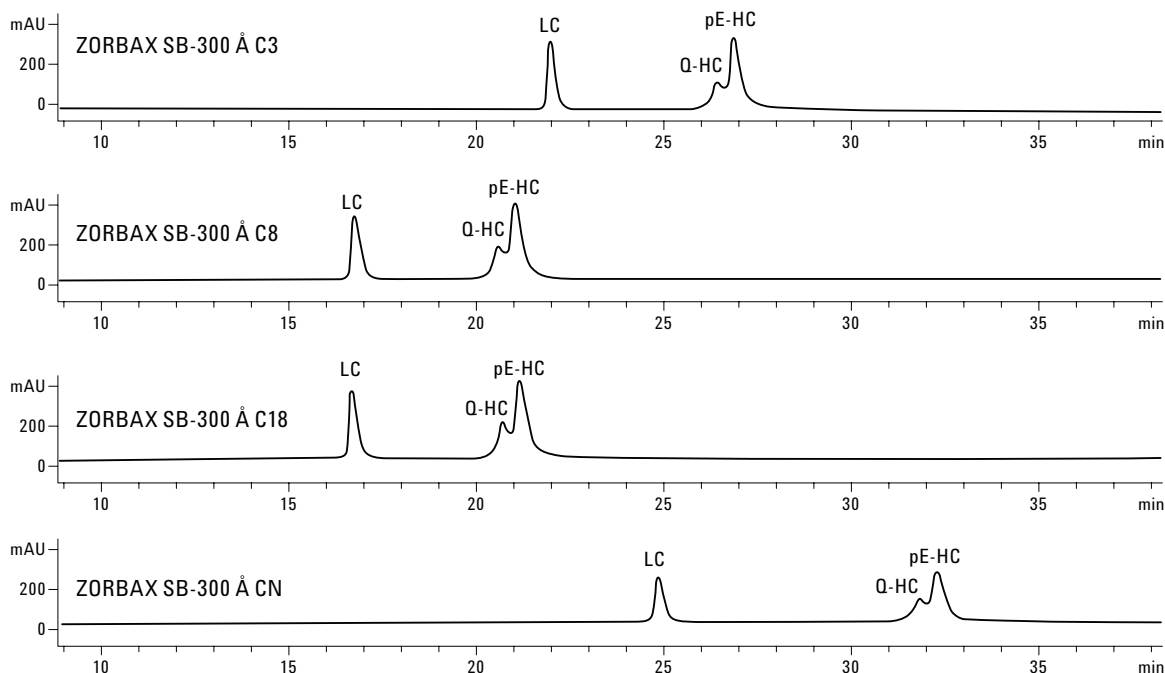


Figure 3. Reversed-phase chromatograms of reduced and alkylated IgG on ZORBAX StableBond SB-300 Å, 4.6 mm $\times$ 150 mm columns packed with 3.5 μ m particles, 300 Å pore size, C3, C8, C18, CN and Varian DiPhenyl 150 mm $\times$ 4.6 mm column. The separation was achieved using the same linear gradient from 23 to 35% B.

Table 1. Resolution, Selectivity Factor and Plate Numbers Calculated for Each Separation in Figure 2 Using Retention Times and Peak Widths of Heavy and Light chains.

Column	Selectivity	Resolution	Plate number for HC
ZORBAX 300 Å SB-C3	1.24	18	130000
ZORBAX 300 Å SB-C8	1.28	16	80000
ZORBAX 300 Å SB-C18	1.29	17	81000
ZORBAX 300 Å SB-CN	1.31	25	155000

selectivity for the light and heavy chains, as well as for the Q and pE variants of heavy chain. Resolution, selectivity factor, and plate number were calculated and summarized for each separation in Figure 3 of heavy and light chains. The calculated quantities are shown in Table 1. ZORBAX StableBond 300 Å CN showed the highest selectivity and resolution. In addition, it provided large space between the light and heavy chains on the chromatogram for the separation of cleavage products and variants, should they appear in degraded samples.

Monitoring Variations of Heavy and Light Chains by LC/MS

The chromatogram (Figure 4) of reduced and alkylated IgG demonstrates the ability to separate Q variant of heavy chain (Q-HC) from pE variant of heavy chain (pE-HC) using a ZORBAX 300 Å SB-CN column at 75 °C with the gradient elution. The mobile phase contained *n*-propanol, acetonitrile, and water with 0.1% TFA as specified in Section 2. The conventional mobile phase containing just acetonitrile and water with 0.1% TFA showed poor separation, poor sample recovery, and significant carryover (Figure 3b). Two samples are compared in Figure 4: a sample frozen for 3 months at -80 °C (T0) and a sample stored for 3 months in a pH 5.8 buffer at 29 °C ($T = 3 \text{ m } 29 \text{ °C}$). The peak area of the pE-HC increased after storage at 29 °C in a pH 5.8 buffer to almost complete conversion after several weeks (Figure 4B). This method enabled monitoring conversion of Q-HC to pE-HC during stability studies and long-term storage.

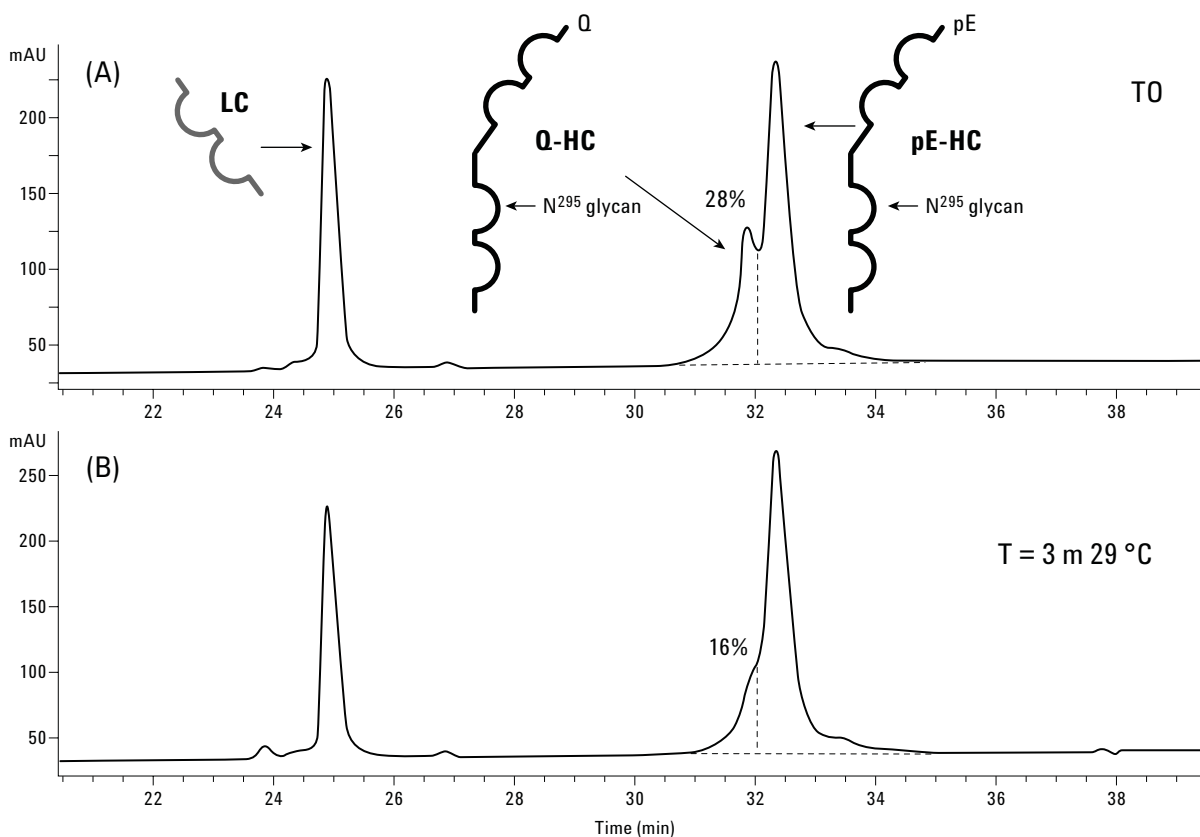


Figure 4. Reversed-phase chromatograms of reduced and alkylated IgG antibody analyzed on an Agilent ZORBAX StableBond SB-300 Å, 3.5-μm particle size, 300 Å pore size, 150 mm × 4.6 mm CN column. A control sample had been frozen for 3 months at -80 °C, T0 (A) and compared with a degraded sample stored for 3 months in pH 5.8 buffer at 29 °C, $T = 3 \text{ m } 29 \text{ °C}$ (B). Labeled on the chromatograms are light chain (LC), heavy chain with N-terminal Q (Q-HC), and heavy chain with N-terminal pE (pE-HC).

Figure 6 shows the chromatogram of a reduced and alkylated IgG sample and the deconvoluted ESI mass spectra of two minor fragments that elute at 13 and 33 min (labeled with asterisks). The analyzed control IgG sample (T0) contains significant abundance of Q-HC (the same as in Figure 5A). The same two minor peaks were present after the material was stored for 3 months at pH 5.8, 29 °C ($T = 3 \text{ m } 29 \text{ }^{\circ}\text{C}$), suggesting that they may be artifacts of the method. The intensity of the peaks increased after holding the sample on column for 30 min before starting the gradient elution (data not shown), identifying these peaks as products of acid hydrolysis at low pH and high temperature environment of the column. Masses of the two

minor fragment peaks (minus water 18 u) add up to the mass of intact heavy chain, confirming that these species are cleavage products of the heavy chain. Deconvoluted ESI mass spectra of these peaks (Figure 6B and C) unambiguously identified them as products of acid hydrolysis between aspartic acid and proline residues according to Edelman et al. [10] of heavy chain. The mass spectrum in Figure 6B shows a C-terminal fragment of HC with glycosylation variants: G0, G1, G2, and MAN5. Figure 5C is the complementary N-terminal fragment of HC containing a smaller peak with mass 29,208 u for Q-HC variant and a larger peak with mass.

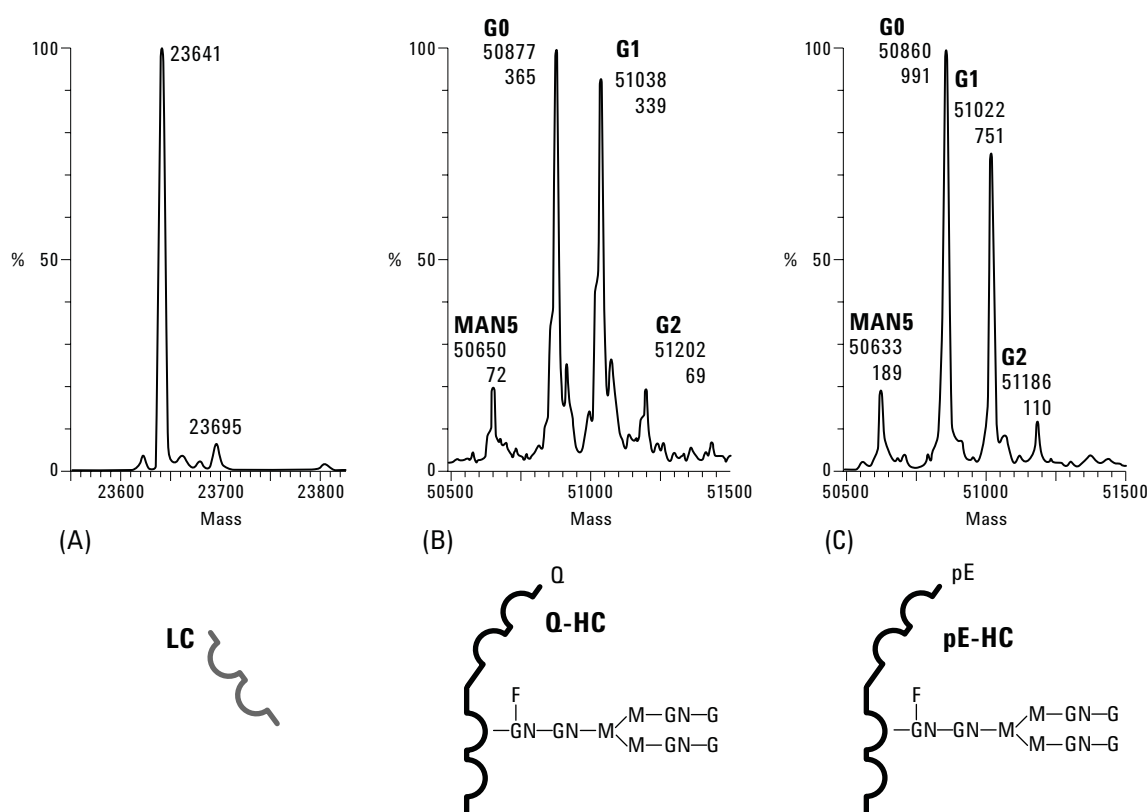


Figure 5. Deconvoluted ESI mass spectra of reduced and alkylated IgG antibody chains separated on the chromatogram in Fig. 4A: (A) light chain, LC; (B) 50,877 u heavy chain variant with N-terminal Q, Q-HC, and (C) 50,860 u heavy chain variant with N-terminal pE, pE-HC. Deconvoluted mass spectra show glycosylation profiles of Q-HC and pE-HC including G0, G1, G2, and MAN5 sugar moieties.

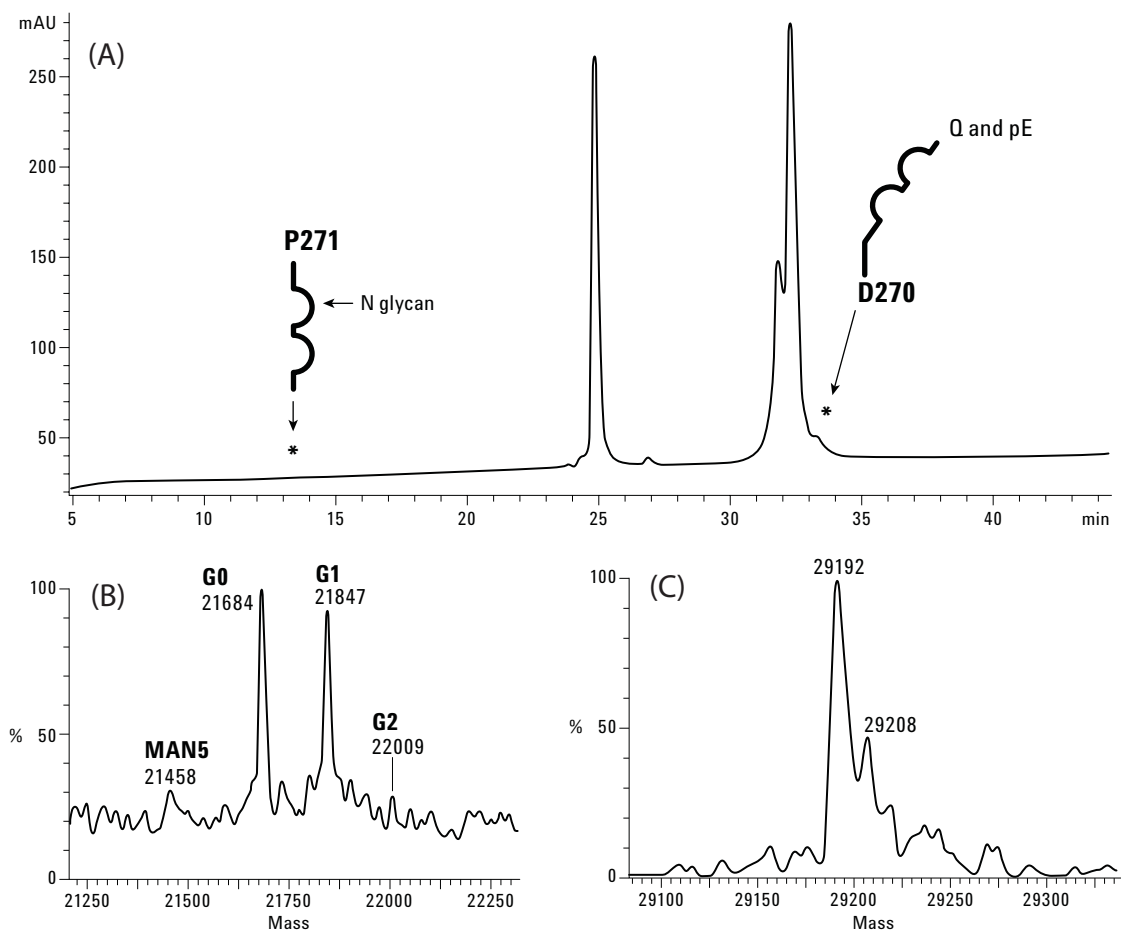


Figure 6. (A) RP chromatogram of a reduced and alkylated IgG control sample frozen for 3 months at -80°C . (B and C) Deconvoluted ESI mass spectra of two heavy chain fragments (labeled with asterisks) generated by the on-column acid hydrolysis between aspartic acid 270 and proline 271(D270/P271). (B) Deconvoluted ESI mass spectrum of C-terminal fragment of HC containing glycosylation profile due to the *N*-glycosylation. (C) Deconvoluted ESI mass spectrum of a complementary *N*-terminal fragment of HC created after the D/P cleavage.

Conclusions

A reversed-phase LC/UV/MS method was developed for characterization and quantitation of two common modifications of reduced monoclonal antibodies: formation of N-terminal pyroglutamate (pE) and heterogeneous glycosylation profile. The light and heavy chains were clearly separated on the chromatograms. A heavy chain variant with N-terminal glutamine (Q-HC) was quantitatively separated from the pE-HC variant. The optimal chromatographic separation was achieved with increased column temperature (75 °C), strong elutropic solvent in the mobile phase (*n*-propanol), ZORBAX LC columns with long alkyl chains (C8, C18), and CN functionality. TFA (0.1%) was used in the mobile phase for optimal chromatographic separation. Although TFA reportedly causes suppression of the electrospray ionization, in our method using propanol, it allowed obtaining high ion intensity and accurate mass measurements. Although peptide mapping can be potentially used to assess the status of N-terminal glutamine, it is a time-consuming procedure and also contains a risk of cyclization during the digestion. The described RPLC/MS analysis of the light and heavy chains of IgG antibodies is less prone to modifications because of the shortened sample preparation procedure.

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