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# Development of an analytical reversed-phase high-performance liquid chromatography–electrospray ionization mass spectrometry method for characterization of recombinant antibodies

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**Abstract**

Analytical characterization of monoclonal antibodies has been hindered by the lack of appropriate chromatographic methods to be used in conjunction with high-resolution MS. Current methodologies for standard RP-HPLC are incompatible with antibodies due to irreproducibility, low recovery, short column lifetimes, and poor resolution of degradation products. An analytical RP-HPLC–MS method was developed for monitoring and characterizing intact IgG1antibodies. Key parameters required for improved chromatographic resolution included long alkyl chains of the stationary phase (Zorbax SB300 $C_8$), column temperatures elevated to 65–70 °C and combination of trifluoroacetic acid and heptafluorobutyric acid ion-pairing agents. RP chromatographic separation of degradation species and C-terminal lysine variants along with the characterization of glycosylation profile by mass spectrometry demonstrates the capability of this method for whole antibody analysis.


*Keywords:* Recombinant antibody; Charge variants; Characterization; Heterogeneity; Degradation

## 1. Introduction

Recombinant monoclonal antibodies (mAbs) are an emerging therapeutic modality and subject of exhaustive characterization in pharmaceutics. MAbs are heterogeneous glycoproteins composed of two identical light and heavy chains connected by multiple interchain disulfide bonds. Structural heterogeneity can arise by the following three common post-translational modifications, creating several structural variants of the molecules (Fig. 1). First, incomplete galactosylation and fucosylation of the two N-linked biantennary oligosaccharides attached to the second constant domain (CH2) of the heavy chain [1]. Second, an N-terminal glutamine residue of the heavy chain is converted post-translationally to pyroglutamate [2]. Third, although coded genetically, C-terminal lysine(s) are often completely or partially absent in proteins because of the activity of carboxypeptidase B [2–5]. In addition to the above, the following modifications can occur during manufacturing and formulation, further increasing heterogeneity of antibody molecules and which may have clinical implications: oxidation [6], deamidation [7], isomerization of aspartic acid [7], disulfide bond scrambling [8,9], cleavages [10–12], dimer formation [13], and others.

Although successfully analyzed by several separation techniques, mAbs have been intractable to standard RP-HPLC analytical methods. Insufficient recovery of protein from the column, poor resolution, irreproducibility, and rapid decrease of column performance have led most analytical chemists to rely on alternative methods of chemical characterization. Cation-exchange chromatography (CEX), and isoelectric focusing (IEF) have typically been used to detect deamidation [3,14] and hydrophobic interaction chromatography (HIC) to detect oxidation and aspartic acid isomerization [7,15,16]. Besides deamidation, charge heterogeneity in antibodies from glycosylation and C-terminal lysine variants are monitored primarily using CEX–HPLC, capillary isoelectric focusing (cIEF), and capillary zone electrophoresis (CZE) [2,3,5,17–21]. Often, several antibody modifications take place simultaneously, which complicates reliable assignment of the multiple chromatographic or electrophoretic

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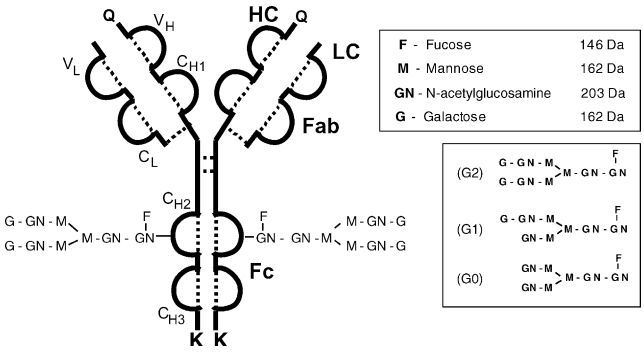


Fig. 1. A structure of a recombinant monoclonal IgG1 antibody. The antibody has one N-linked biantennary carbohydrate in each of the CH2 domains of the heavy chain (HC). The possible number of terminal galactose residues ranges from zero to a maximum of four (G2 + G2). The IgG1 structure contains lysine residues (K) on the C-termini of the heavy chains and glutamine residues (Q) on the N-termini of the heavy chains, which are susceptible to conversion to pyroglutamate.

bands. The use of orthogonal separation methods and more specialized detectors is often required to characterize these variants. Having a high-resolution mass spectrometer in tandem with the separation techniques can provide the required additional information needed for the reliable assignment of antibody variants. Unfortunately, the salts frequently used in solvents of the above chromatographic techniques are not amenable to online mass spectrometry. Attachment of capillary electrophoresis to mass spectrometry for detection of large proteins is still an art and requires careful selection of buffers [22].

Reversed-phase chromatography is known to be amenable to online mass spectrometric detection, but because of the large size (molecular mass ca. 150 000) and hydrophobic nature of antibodies, only limited success has been reported in using RP-HPLC to resolve smaller antibody fragments from the intact species [23,24]. In addition, efforts in using perfusion chromatography or HIC in RP mode have been adequate as a means of removing buffer salts for MS analysis and separating light and heavy chain antibody fragments [21,23], but these methods are not always analytically rugged for formulation applications nor do they have stability-indicating separation capabilities. Orthogonal methodologies, such as gel filtration and anion exchange [25] or nonporous RP-HPLC and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [26], have been coupled to separate large hydrophobic proteins; however, the presence of salt and detergent can interfere with online mass spectral analysis. Although whole antibody analysis by RP-HPLC has been problematic, RP analysis of antibody fragments and peptide maps have been successful [21,23,27–29]. Since proteolytic digest procedures are time-consuming, a viable RP-HPLC method for whole antibodies is needed for an efficient means of separation and identification of chemical degradants and heterogeneity.

In RP-HPLC method development, the important parameters for choosing a column include the type of bonded phase, column dimensions, particle size, pore size, carbon load, and the degree of end-capping. For large proteins of molecular mass ca. 100 000–200 000, the scope of RP-HPLC method development has been limited to wide pore, silica-based columns of shorter alkyl chain length and Poros perfusion resins of highly cross-linked polystyrene-divinylbenzene to minimize recovery losses of hydrophobic species. RP-HPLC applications for large hydrophobic proteins typically employ *n*-butyl ($C_4$) silica-based columns of 5 μm particle size, ≥300 Å pore size to obtain adequate loading capacity, recovery, backpressure, and flow rate [21].

Ion-pairing reagents are often used in RP-HPLC analysis to shield the effective charge of functional groups on a protein. Within the pH range for chromatography on silica-based columns, the ionizable functional groups include carboxylates (p$K_a$ of 2 and 4), sulfhydryls (p$K_a$ of 8), amines (p$K_a$ of 6, 9 and 10, or 11), and guanidines (p$K_a$ of 12 and 13). Two approaches to neutralizing these functional groups are to lower the pH to about 2 to reduce the carboxylate charge and to use anionic ion-pairing reagents to neutralize the positive charged functional groups, thereby increasing the hydrophobic nature of the separation. Although standard RP-HPLC methods for protein characterization almost exclusively employ mobile phases containing a default level of 0.1% trifluoroacetic acid (TFA) with a linear gradient of acetonitrile, the resulting TFA suppression of the mass spectrometric signal [30] makes identification and characterization of low levels of protein modifications unfeasible. Lower amounts of TFA can be used for MS applications at the expense of chromatographic peak resolution. Other ion-pairing reagents used less frequently include heptafluorobutyric acid (HFBA) and pentafluoropropionic acid (PFPA). Until recently, HFBA has been used for peptides of low hydrophobicity [31,32] and small proteins [33] and PFPA for peptides intermediate hydrophobicity. In this report, we optimized reversed-phase chromatography of intact antibodies by making several counterintuitive selections of the key separation parameters: we employed $C_8$ stationary phase and TFA/HFBA combination of ion-pairing agents, which have not been previously used for separation of large proteins.

## 2. Experimental

### 2.1. Materials

Recombinant monoclonal IgG antibodies were produced and purified at Amgen Inc. The purified protein was stored in formulation buffer at 4 °C. Other commercially available mAbs were tested for comparison. Carboxypeptidase B was purchased from Worthington Biochemical and stored at −20 °C. Trypsinogen from bovine pancrease was purchased from Sigma, part T-1143, molecular mass 23 981.0. All chemicals and reagents were of analytical grade and were purchased from Sigma and VWR Scientific.

### 2.2. Reversed-phase HPLC

RP-HPLC was performed on Agilent 1090 and 1100 HPLC systems. Numerous column chemistries, flow rates, mobile phases, and sample conditions were tested during method optimization as discussed subsequently. The optimized methods employed a Zorbax SB300 $C_8$ column (150 mm × 4.6 mm, 3.5 μm particle size, 300 Å pore size) and a Polaris $C_8$-ether column (150 mm × 4.6 mm, 3.0 μm particle size, 200 Å pore size). The column temperature was 70 °C for the Zorbax and 65 °C for the Polaris column, and the solvent flow rate was 1.0 mL/min. Solvent A was water containing ion-pairing agent, and solvent B was 90% acetonitrile with ion-pairing agent. The ion-pairing agents were varied combinations of trifluoroacetic acid (0.01–0.2%, v/v; TFA) and heptafluorobutyric acid (0.001–0.03%, v/v; HFBA), with a preferred level of 0.11% TFA in mobile phase A and 0.09% TFA in mobile phase B. The Polaris $C_8$-ether column was used for LC–MS analysis using the same conditions but with lower levels of TFA (0.01–0.05%) in order to minimize ion suppression during the electrospray process. The initial conditions ranged from 0 to 28% B. The two-stage gradient was Δ2.5% B per min from 28 to 38% B, followed by a second gradient of Δ0.5% B per min from 38 to 44% B. A flush step was performed with 90% B and 10% isopropanol (IPA) for 5 min. The absorbance of the eluent was monitored at 214 and 230 nm.

### 2.3. Electrospray ionization mass spectrometry

LC–MS data were acquired using an Agilent 1100 capillary-LC system with micro-bore columns (1 mm × 50 mm) packed with the same stationary phases as specified above and in tandem with a Micromass Micro quadrupole-time-of-flight (Q-TOF) mass spectrometer through an electrospray ionization (ESI) atmosphere–vacuum interface. The ESI-Q-TOF mass spectrometer was set to run in positive ion mode with a capillary voltage of 3400 V, sample cone voltage of 99 V, *m/z* range of 800–5500, mass resolution of 5000, and was tuned and calibrated using multiply charged ions of the bovine trypsinogen. The deconvolution of electrospray ionization mass spectra was performed using MaxEnt1 algorithm, which is part of the MassLynx software from Micromass. After deconvolution, the mass spectral data were further processed by using the centering and smoothing functions in the same software.

### 2.4. Size-exclusion chromatography (SEC)

SEC analysis was performed on an 1100 HPLC system fitted with two Tosohaas TSK G3000 columns in series. The columns were at room temperature. The buffer was 100 mM sodium phosphate, 500 mM NaCl, and 5% ethanol at pH 7.0. Elution was isocratic at a flow rate of 0.5 mL/min. The absorbance of the eluent was monitored at 214 and 230 nm.

### 2.5. Carboxypeptidase B treatment

Carboxypeptidase B was used to cleave lysine residues from the C-terminus of the Fc moiety of recombinant monoclonal IgG1 antibodies produced in hybridoma cells. The antibody was incubated for 2 h at 37 °C at an enzyme:protein ratio of 1:10 (w/w). The protein was diluted to 1 mg/mL in phosphate buffered saline pH 7.5.

## 3. Results

During the reversed-phase method development, a variety of column chemistries and running conditions were explored. Parameters that were thought to be critical for full recovery of large proteins were low carbon load, high particle size, and large pore size. In addition, preserving the end-capping of the silica-based columns by adjusting the pH or using stable bond chemistry was believed to minimize recovery losses. Column temperature, initial conditions of the column, and choice of ion-pairing agents were also suspected to be critical in minimizing column interactions and in preserving the protein integrity and solubility. These and other parameters were examined for their effect on RP-HPLC of antibodies.

### 3.1. Method development

As a starting point, Fig. 2 depicts an example of typical chromatographic results that standard reversed-phase methodologies yield for whole antibody analysis. The percentage of solvent B is overlaid with a broken line. A Jupiter $C_4$ column 150 mm × 2.0 mm, 5 μm, 300 Å was maintained at 40 °C. The mobile phase solvents contained 0.1% aqueous TFA in A and acetonitrile–water–TFA (90:9.9:0.1) in B. The flow rate was 0.35 mL/min. Low recovery, severe peak tailing, and large amounts of material that elute in the flush are evident.

To address these issues of poor chromatography, analytical method development was conducted to optimize an RP-

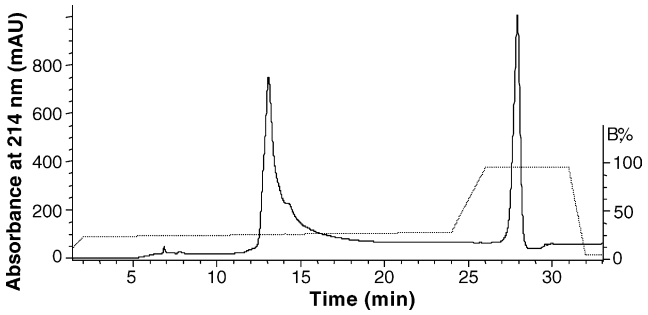


Fig. 2. A reversed-phase chromatogram of an IgG1 monoclonal antibody (solid line). The percentage of solvent B is overlaid with a broken line. A Jupiter $C_4$ column 150 mm × 2.0 mm, 5 μm, 300 Å was maintained at 40 °C. The mobile phase solvents contained 0.1% aqueous TFA in A and acetonitrile–water–TFA (90:9.9:0.1) in B. The flow rate was 0.35 mL/min.

HPLC method for antibody characterization. Stable bond column chemistry (Zorbax SB) was utilized to ensure that the bonded phase remained stable at low pH and high temperature. More polar column chemistries (Polaris embedded ether group columns) also achieved favorable recovery with additional selectivity but were only used at higher pH and lower temperature as per manufacturer recommendations. General guidance from column manufacturers suggested columns of lower alkyl chain length ($C_4$), larger particle size (5 μm), and larger pore size (>500 Å) for large proteins such as antibodies. Unexpectedly, columns of higher alkyl chain length ($C_8$), lower particle size (3.0–3.5 μm), and smaller pore size (200–300 Å) were found to provide good recovery with excellent resolution capabilities when run under optimized conditions, as described below.

Method development work demonstrated that column temperature had a major impact on separation and recovery of antibodies. A recombinant monoclonal IgG1 antibody was run on RP-HPLC using column temperatures ranging from 35 to 80 °C (Fig. 3). The recovery and peak shape was optimized at approximately 70 °C, while at lower temperatures excessive peak tailing, poor recovery, and column fouling were observed. The example shown in Fig. 3 was obtained using the Zorbax SB300 $C_8$ column of 3.5 μm particle size and 300 Å pore size described in Section 2. With an optimized column temperature of 70 °C, excellent separation of the main component and the degradation products was achieved using a column of relatively high alkyl chain length, low particle size, and small pore size, contrary to manufacturer's guidelines. These column properties provided improved separation capabilities while maintaining good recovery.

To evaluate the quantitative nature of the method, a series of controls were run to test the linearity, range, and recovery. Using a Zorbax SB300 $C_8$ 150 mm × 4.6 mm column, linearity was assessed by injecting seven IgG1 standards that ranged in concentration from 1 to 100 μg. The integrated peak areas were plotted versus amount injected. The calibration curve was found to be linear with a correlation coefficient of 0.9996. Carry-over following the 100-μg standard injections was determined to be less than 0.2%.

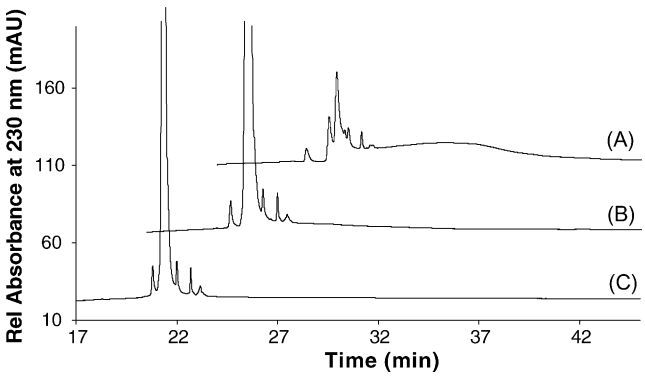


Fig. 3. The effect of column temperature on reversed-phase chromatographic properties of a recombinant monoclonal antibody is shown. Data are shown for a Zorbax SB300 $C_8$ column with temperature values of: (A) 35 °C; (B) 55 °C; (C) 70 °C.

Additional analysis was performed to assess the injection reproducibility and robustness of the chromatography. One of the major hurdles for developing a rugged reversed-phase method for antibodies is column fouling and irreproducibility. Using the conditions as described above, the Zorbax SB300 $C_8$ column lifetime was nearly one thousand injections when properly flushed, using 10% IPA, and stored (following manufacturer's guidelines). The Zorbax SB column was also analyzed for lot-to-lot variability using four different manufacturer lot numbers. The results indicated (data not shown) that two out of four lots had significant differences in resolution but could be compensated by increasing the level of ion-pairing agents in solvent. As mentioned above, another column that worked well with the reversed-phase method was the Polaris $C_8$-ether column. Although this column was not tested as extensively for column lifetime compared to the Zorbax $C_8$ column, initial results indicated no lot-to-lot variability in resolution capability. Therefore, preliminary screening results suggested that although the stable bond chemistry of Zorbax SB columns allowed the implementation of higher temperatures (70 °C) and lower pH (1.7) than the Polaris ether columns, the Polaris columns offered improved lot-to-lot reproducibility, favorable recovery, and comparable selectivity when using lower levels of ion-pairing agents.

To summarize, the optimized method for the separation of IgG1 antibodies required relatively high column temperatures of 65–70 °C. The preferred columns were a Zorbax SB300 $C_8$ column (150 mm × 4.6 mm, 3.5 μm particle size, 300 Å pore size) and a Polaris $C_8$-ether column (150 mm × 4.6 mm, 3.0 μm particle size, 200 Å pore size). The mobile phases were 0.11% TFA in water for mobile phase A and 0.09% TFA and 90% acetonitrile for mobile phase B. A lower level of TFA (0.01–0.05%) is preferred when coupled with online mass spectral analysis in order to minimize ion suppression during the electrospray process with a minimal loss in resolution capabilities. The column should be equilibrated with up to 28% mobile phase B. The preferred two-stage gradient was Δ2.5% B per min from 28 to 38% B, followed by a second gradient of Δ0.5% B per min from 38 to 44% B. A flush step was performed with 90% B and 10% isopropanol for 5 min.

### 3.2. Applications

The optimized reversed-phase chromatography in combination with mass spectrometry was used to characterize and identify heterogeneity and variants of the antibodies, both charged and electrically neutral. For mAbs, charge heterogeneity may arise from differences in the processing of lysines at the carboxy-terminus of each of the heavy chains, creating a distribution of molecules containing 0, 1, or 2 C-terminal lysines (Fig. 1). By using for example CEX–HPLC, the lysine variants may not be distinguished from the variants caused by the formation of N-terminal pyroglutamate

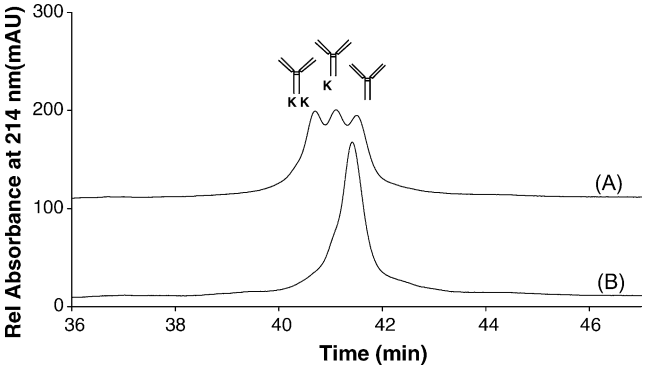


Fig. 4. Separation of lysine variants from an intact IgG1 antibody by RP-HPLC using a Zorbax SB300 $C_8$ column with a mobile phase containing 0.2% TFA/0.03% HFBA; (A) control; (B) after treatment with carboxypepsidase B for 2 h at 37 °C.

or deamidation. Figs. 4 and 5 demonstrate the capability of RP-HPLC–MS to separate and identify the lysine variants. Fig. 4A shows separation of the lysine variants of an intact IgG1 antibody using an RP-HPLC method utilizing Zorbax SB300 $C_8$ 150 mm × 4.6 mm column and relatively high concentrations of TFA (0.2%) and HFBA (0.03%) in the mobile phase. Treatment with carboxypeptidase B cleaved the C-terminal lysines, resulting in a homogeneous population of antibodies containing no C-terminal lysines (Fig. 4B). Similar separation was achieved on a Polaris $C_8$ column using the above mobile phase. We observed MS signal reduction when using the 0.2% TFA and 0.03% HFBA mobile phase. By trading off some chromatographic resolution in order to increase the MS signal intensity, the percentage of the acids was decreased to 0.05% TFA and 0.005% HFBA. The Polaris $C_8$ column performed better using this low-acid mobile phase. Fig. 5 displays the deconvoluted electrospray ionization mass spectra of the three lysine variants (0K, 1K, and 2K) of the native intact IgG1 antibody separated on a Polaris $C_8$ column. The separation on Polaris $C_8$ was slightly less than with the Zorbax SB300, but sufficient for obtaining mass spectra of individual lysine variants. The loss of each C-terminal lysine residue shifted the molecular mass of each variant by 128 u. If these variants were due to the formation of N-terminal pyroglutamate or deamidation, the mass would be decreased by 17 u for each terminal pyrolization or 1 u for deamidation. Also, the lysine variants each contain carbohydrate microheterogeneity caused by the varying number of terminal galactose residues per intact molecule (Fig. 1), with each variant separated by 162 u. An accurate molecular mass measurement of the reduced IgG1 indicated that the N-terminal glutamine residues of heavy chains were completely converted to pyroglutamate.

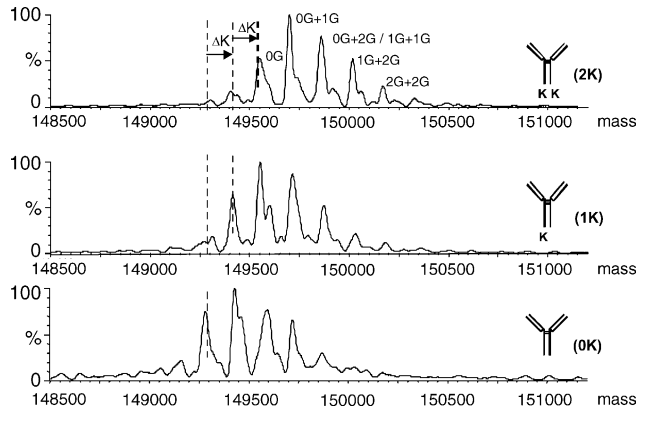


Fig. 5. Deconvoluted electrospray ionization mass spectra of IgG1 lysine variants separated by RP-HPLC in Fig. 4A.The top spectrum represents the earliest eluting LC peak that contains two C-terminal lysines. The middle trace represents the middle LC peak that contains one C-terminal lysine. The bottom trace represents the latest eluting LC peak that contains zero C-terminal lysines. The loss of one lysine residue reduces the mass of antibody by 128 u. Each IgG1 lysine variant also contains microheterogeneity caused by the different number of galactose residues (G) in the carbohydrate moieties, each peak separated by 162 u (Fig. 1).

Note that the combination of HFBA and TFA as ion-pairing agents in the mobile phase was critical for separating lysine variants, as shown in Fig. 4. Another benefit of using TFA in combination with HFBA was the ability to lower the level of TFA without losing chromatographic resolution. This helped to improve ionization in ESI–MS, which provided a greater MS sensitivity for low-level degradation products. Other ion-pairing agents such as PFPA were tested but did not offer any improvements in separation.

In addition to characterizing antibody heterogeneity, the RP-HPLC method in conjunction with mass spectrometry can be used to identify and quantify degradation products that could otherwise remain undetected by conventional methodologies. Fig. 6A shows a typical SEC chromatogram of an entire degraded IgG1 sample stored at 29 °C for 4 months. Two forms of degradation were evident by the appearance of late-eluting species, termed post-peak 1 and post-peak 2,

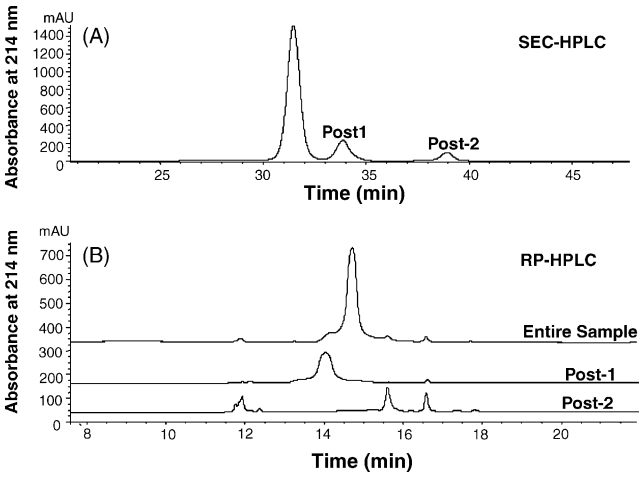


Fig. 6. SEC and RP-HPLC analysis of a degraded antibody (IgG1) sample and RP-HPLC analysis of collected SEC fractions. (A) Size-exclusion chromatogram of a degraded IgG1 antibody sample stored at 29 °C for 4 months. (B) Reversed-phase chromatogram of the same degraded IgG1 antibody (entire sample); RP chromatogram of SEC fraction containing post-peak 1 (post-1) and RP chromatogram of SEC fraction containing post-peak 2 (post-2).

which were presumed to be clipped species. Subsequent characterization of fractions collected from SEC and further analyzed by RP-HPLC (Fig. 6B) demonstrated that post-peak 1 represented an early-eluting species (Fig. 6B, post-1) compared to the main peak (Fig. 6B, entire sample) on reversed-phase. Surprisingly, post-peak 2 contained multiple degradation species (Fig. 6B, post-2).

Further characterization by mass spectrometry was performed to identify the degradation species. The SEC post-peak 2 eluted from the reversed-phase column as three peaks, which we tentatively identified as LC, Fab, and VHCH1 regions of antibody. The remaining antibody fragment, which eluted on SEC as post-peak 1, contained one Fab and one Fc fragment connected by the hinge with the molecular mass value of approximately 100 000. The cleavages at the hinge region of IgG1 are consistent with other recent results in resolving fragments by size-exclusion chromatography [10]; however, the denaturing conditions of the reversed-phase method resolve additional peaks representing the non-covalently bound regions VHCH1 and LC. Therefore, this novel RP-HPLC method provides chromatographic capabilities and direct online mass spectrometric compatibility for the separation and identification of large and small antibody degradants that other single-dimensional separation techniques cannot attain. In this application, we took advantage of the denaturing properties of our RP-HPLC method and its ready online compatibility with mass spectrometry to resolve the multiple non-covalently bound degradation species, which were not resolved by SEC.

Consistent differences in the reversed-phase chromatographic properties of recombinant monoclonal IgG1 and IgG2 antibodies were observed (Fig. 7). Zorbax SB300 $C_8$ column was used for these separations. In general, IgG1 antibodies demonstrated good recovery, excellent peak shape, and minimal column interactions. RP-HPLC of IgG2 antibodies showed reproducible assembly of several peaks and larger percentage of carryover. The underlying mechanism of these differences may be hydrophobic, structural, or charge related.

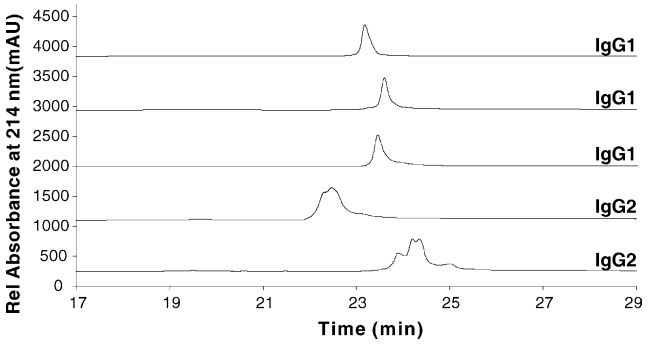


Fig. 7. Comparison of RP chromatograms of different recombinant monoclonal antibodies of IgG1 and IgG2 subclasses. All samples were analyzed using a Zorbax SB300 $C_8$ column 150 mm × 4.6 mm, 5 μm, 300 Å was maintained at 70 °C. The mobile phase solvents contained 0.11% TFA in A and acetonitrile–water–TFA (90:9.9:0.1) in B. The flow rate was 1.0 mL/min.

## 4. Discussion

### 4.1. Critical parameters for RP-HPLC of antibodies

Multiple mechanisms of separation seem to be involved in the RP-HPLC analysis of antibodies. In reversed-phase mode, separation is achieved by a complex interaction of the nonpolar stationary phase and the polar mobile phase. Partitioning can occur between the solvent and the matrix functional groups, and multiple modes of separation can be achieved due to the heterogeneity of silica-based stationary phase and the effect of mobile phase composition.

The improved RP-HPLC peak shape and column recovery with increasing column temperature suggests that structural or hydrophobic forces are involved. The significant improvements in chromatography at elevated temperatures in an acidified organic mobile phase suggest that unfolding the antibody to a more complete denatured state is required. Moreover, decreasing the protein's net affinity with the column stationary phase by increasing its solubility in the mobile phase at higher temperatures also affords improvements in protein recovery.

In RP-HPLC method development for antibodies, the pronounced effect of ion-pairing agents influencing peak capacity and selectivity suggests that an ionic component is present in addition to the hydrophobic mode of separation. In the mode of ion interaction chromatography, hydrophobic anionic ion-pairing reagents neutralize the positive charged groups on the protein, thereby increasing the retention time. The ion-pairing reagent affects to a lesser degree the more neutral species. In separating charge variants of antibodies, dialing in the level of ion-pairing reagents can therefore influence relative peak elution times by differentially modifying the ion interaction component for each species.

Optimization of the mobile phase utilizing a combination of TFA and HFBA ion-pairing agents resulted in improved chromatography and enhanced mass spectrometric signal. Certain levels of TFA and HFBA were effective in ensuring improved chromatographic peak shape and resolution for intact antibodies and antibody fragments.

The combination of ion-pairing agents was able to provide unique resolution of charge variants and antibody fragments that was not possible using TFA or HFBA alone. The improvement in chromatographic resolution also simplified the mass spectra for each variant, which helped to characterize antibody degradation products and heterogeneity. This novel approach allows the identification and characterization of low levels of protein modifications which otherwise could remain undetected by conventional methodologies.

### 4.2. Characterization of antibody heterogeneity and stability

The development of a rugged RP-HPLC method with enhanced mass spectrometric sensitivity represents a major advancement for in-depth analytical characterization of an-

tibody heterogeneity and stability. The method allows for routine RP-HPLC–MS analysis of whole antibodies or antibody fragments with minimal sample preparation compared to peptide mapping. The approach utilizes standard chromatographic equipment with more ruggedness in column lifetimes and separation capabilities compared to typical perfusion chromatography or HIC in reversed-phase mode [21]. The applicability of the method to identify and characterize structural variants and degradation products for antibodies provides a new tool for product comparability analysis and formulation screening.

Significant and reproducible differences between the chromatographic profiles of IgG1 and IgG2 subclasses may indicate differences in the degree of heterogeneity in the charge variants or in the physicochemical properties of the hinge region. As for structural differences between the subclasses, the hinge region of IgG1 is 16 amino acids in length and displays interdomain flexibility to rotate about its two intermolecular disulfide bonds. The hinge region of IgG2 is shorter and more rigid than that of IgG1, consisting of 12 amino acids and four disulfide bonds in a proline-rich double helix. Further characterization efforts using this RP-HPLC method in conjunction with MS analysis may elucidate the nature of the differences in physicochemical properties amongst antibody subclasses.

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