PROTEOMICS METABOLOMICS GENOMICS INFORMATICS
GLYILEVALCYSGLUGLNALASERLEUASPARG
CYSVALLYSPROLYSPHETYRTHRLEUHISLYS

Antibody Analysis by ESI-TOF LC/MS

Authors

Lorenzo Chen, Merck & Company, West Point, Pennsylvania USA

Andy Gieschen, Greg W. Kilby, and Jim Lau, Agilent Technologies, Wilmington, Delaware USA

Abstract

One of the challenges in the development of therapeutic antibodies is the development of a fast and precise method to determine the molecular weight (MW) of antibodies. Historically, gel analysis, size exclusion, analytical ultracentrifugation, and MALDI-TOF MS have been used to determine the MW of either the entire molecule, or of its separate components. However, each of these methods suffers from either imprecise MW determination or from the lengthy time required to perform the analysis. In this study, we determined the capability of the Agilent 1200 series HPLC coupled with the 6210 TOF MS to analyze IgG4 antibody samples. For analysis of intact IgG4, results indicated a short retention time of 2.78 min, with very good chromatographic peak shape. Furthermore, the mass spectral analyses demonstrated the ability of the system to resolve the various antibody subpopulations present in a sample. The routine mass accuracy demonstrated by the TOF MS was better than 25 ppm. The results of this study demonstrate that the combination of Agilent Technologies Poroshell column, 1200 HPLC, and 6210 TOF MS provides a total system solution for efficient, accurate antibody analysis.

Introduction

Significance of monoclonal antibody therapy

Monoclonal antibodies (mAbs) are one of the most promising classes of therapeutics being developed using biotechnology and is a research area of significant investment. One reason for the interest is the exquisite specificity of mAbs; mAbs specific for a particular antigen have structurally identical antigen-binding regions and thus will bind to the same site of the antigen. This specificity can lead to highly selective interactions with antigens or targets such as receptors within cells, tissues, and organs involved in the pathology of disease, while minimizing potential side effects. In addition, the success rate for mAb therapy is estimated at a favorable 18–29%, whereas small molecule drugs have a success rate of 11%.[1,2] Current therapeutic areas for mAbs are oncological, immunological, and anti-infective, but potential clinical uses are rapidly developing in disease fields traditionally targeted by smaller chemical entities. Pharmaceutical sales for mAbs in 2005 were $14 billion, a 36% increase from 2004.[3]

The number of mAbs in development for therapy is expected to increase. Since the approval of Johnson & Johnson's Orthoclone OKT3 in 1986 for organ transplant rejection, 17 antibodies have been approved for commercial sale in the U.S. and other countries. In addition, there are more than 150 antibodies currently in clinical trials.[1] Over the next several years, this list of mAbs is expected to expand significantly, with mAb therapies for respiratory, cardiovascular, and ophthalmology disorders expected to be approved by the Food and Drug Administration (FDA).[1,4]

mAbs are complex structures

An antibody is a large Y-shaped protein used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. Each antibody recognizes a unique antigen target. A generic representation of an antibody is shown in Figure 1. It consists of two identical light chains and two identical heavy chains held together by inter-chain disulfides and non-covalent interactions. Furthermore, post-translational modifications occur during cellular development and production, and these can affect the properties of the antibody. For example, if the amino terminus of the heavy chain starts with a glutamine residue, it can be converted to pyroglutamate. Also on the heavy chains, the C-terminal residue is often a lysine residue that may be post-translationally excised. Of additional importance is the addition of various glycans to an asparagine residue in the heavy chain, generating an N-glycan linkage. mAbs and their modifications need to be characterized quickly and accurately as part of bioprocess development for the evaluation of product consistency. A critical component of characterization is an accurate determination of the antibody's molecular weight, which is important because the mass provides a glimpse of the integrity of the structure.

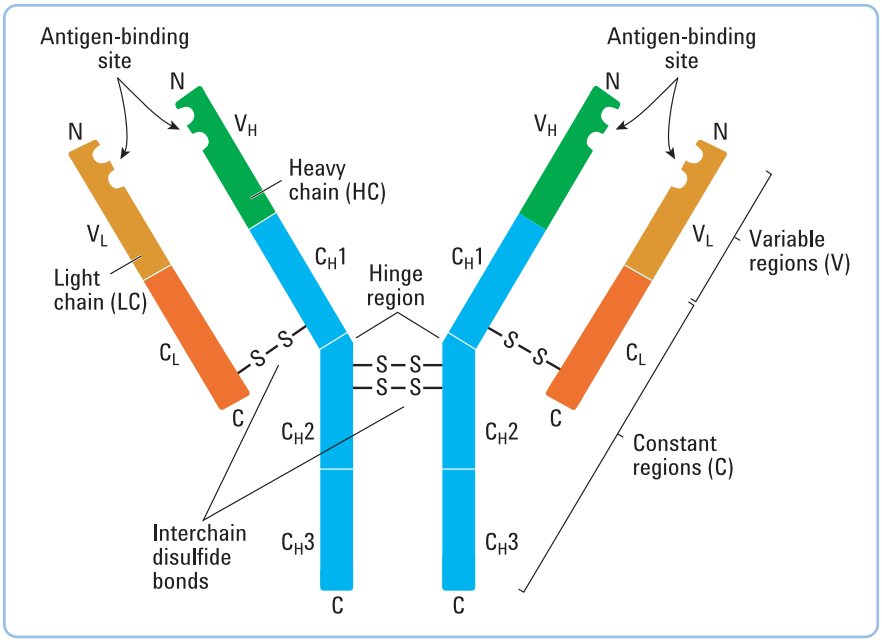

Figure 1. Generic representation of an antibody. The structure, along with any post-translational modifications, presents a challenge in experimentally determining accurate molecular weight as a part of mAb characterization.

Characterization of mAbs by determining molecular weight

One of the challenges in the development of therapeutic antibodies is the development of a fast and precise method to determine the molecular weight (MW) of antibodies. Historically, gel analysis, size exclusion, analytical ultracentrifugation, and MALDI-TOF MS have been used to determine the MW of either the entire molecule, or of its separate components. However, each of these methods suffers from either imprecise MW determination or from the lengthy time required to perform the analysis. This application note demonstrates the capability of the Agilent 1200 series HPLC (incorporating a Poroshell SB 300, 5 µm C8 column) and an Agilent 6210 Time-of-Flight mass spectrometer to determine accurate MWs of IgG4 antibodies in a 9-minute analysis.

Experimental

Sample preparation

Human IgG4 was provided as 1 mg/mL buffered solutions by Merck & Company, West Point, PA. For intact antibody method development, the antibodies were diluted 1:10 with 20% acetonitrile (ACN), 0.1% aqueous trifluoroacetic acid (TFA). For antibody heavy and light chain studies, the antibody samples were reduced under a variety of conditions ranging from 20 mM to 50 mM dithiothreitol (DTT) at pH 8.6 for 15 min to 1 hr at 60–80°C. Samples were then diluted 1:10 with 20% ACN, 0.1% aqueous TFA.

Analysis

All experiments were performed using an Agilent 1200 series HPLC system, comprising a capillary HPLC pump, micro vacuum degasser, thermostatted high-pressure well-plate autosampler, column oven and diode-array detector (DAD), coupled to an Agilent 6210 Time-of-Flight (TOF) mass spectrometer with a dual-nebulizer electrospray ion source.

HPLC conditions for intact and reduced antibodies

Mobile phase: Solvent A = 0.1% aqueous formic acid (FA) or 0.1% aqueous TFA; Solvent B = ACN/0.1% aqueous FA or ACN/0.1% aqueous TFA.

Initial conditions: 20% B at indicated temperature and flow.

The optimal flow rate and column temperature were determined experimentally to be ~500 µl/min and 90°C.

The following 5-minute gradient (total 9-minute run time) was used.

Gradient: 20% B at 0.5 min
90% B at 5.5 min
90% B at 6.5 min
20% B at 6.6 min

TOF MS conditions

Capillary voltage: 5500 V
Drying gas flow: 13 L/min
Drying gas temperature: 300°C
Nebulizer gas flow: 60 L/min
Fragmentor voltage: 350 V for intact antibodies and 200 V for reduced antibodies

Results and discussion

Typical chromatographic and mass spectral analysis

To determine the capability of the Agilent 1200 series HPLC and 6210 TOF MS to analyze antibody samples, intact IgG4 samples were studied. (The most widely used antibody class for therapeutic development is immunoglobulin G [IgG].)

Results indicated a short retention time of 2.78 min, with very good chromatographic peak shape for IgG4 when using the Poroshell C8 column (Figure 2). Furthermore, the mass spectral analysis demonstrated the ability of the system to resolve the various antibody subpopulations present in the sample (Figure 3, Panels A and B).

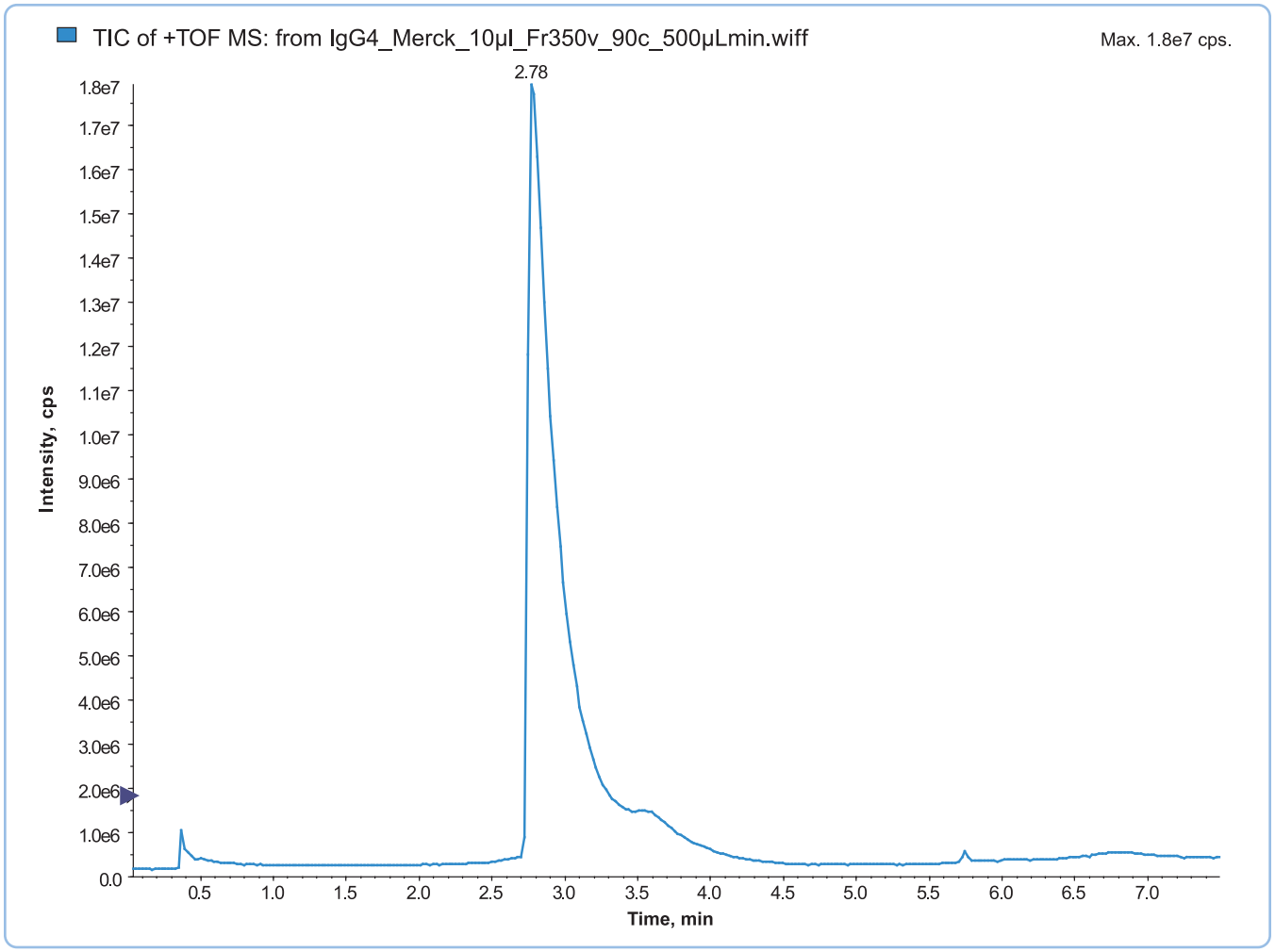

Figure 2. Analysis of intact IgG4 antibody using the Agilent 1200 series HPLC and 6210 TOF MS. Total ion chromatograph of intact IgG4.

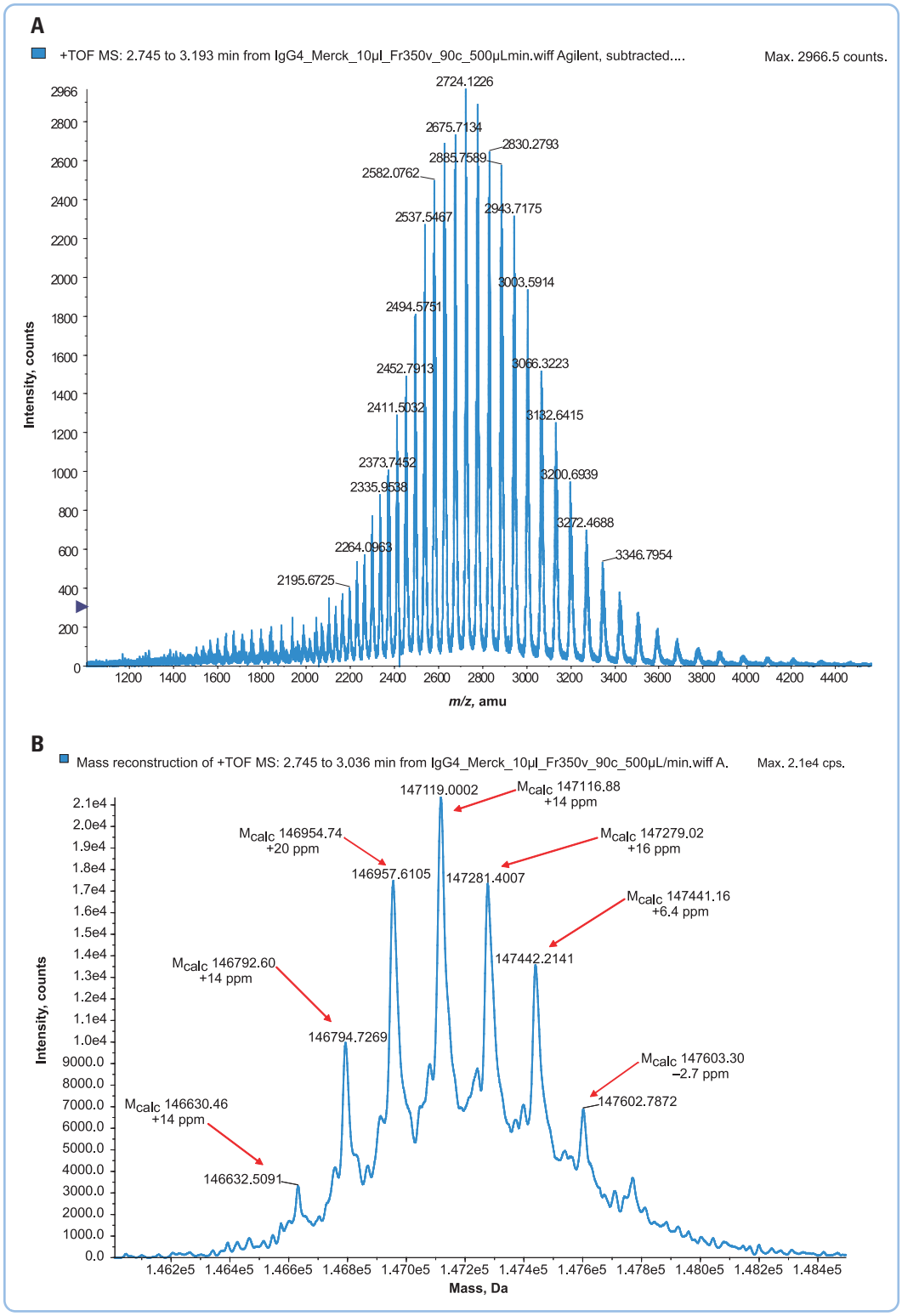

Figure 3. Analysis of intact IgG4 antibody using the Agilent 1200 series HPLC and 6210 TOF MS. Panel A: Mass spectrum of intact IgG4. Panel B: Deconvoluted spectrum of intact IgG4.

Next, we studied the ability of the Agilent system to analyze antibody samples that had been reduced with DTT. Results indicated that although the heavy- and light-chain forms (HC and LC, respectively) were not completely resolved, the individual mass spectrum for each form (and subsequent mass resolution) of the HC and LC was clearly detected and the individual masses were easily determined (Figures 4–7).

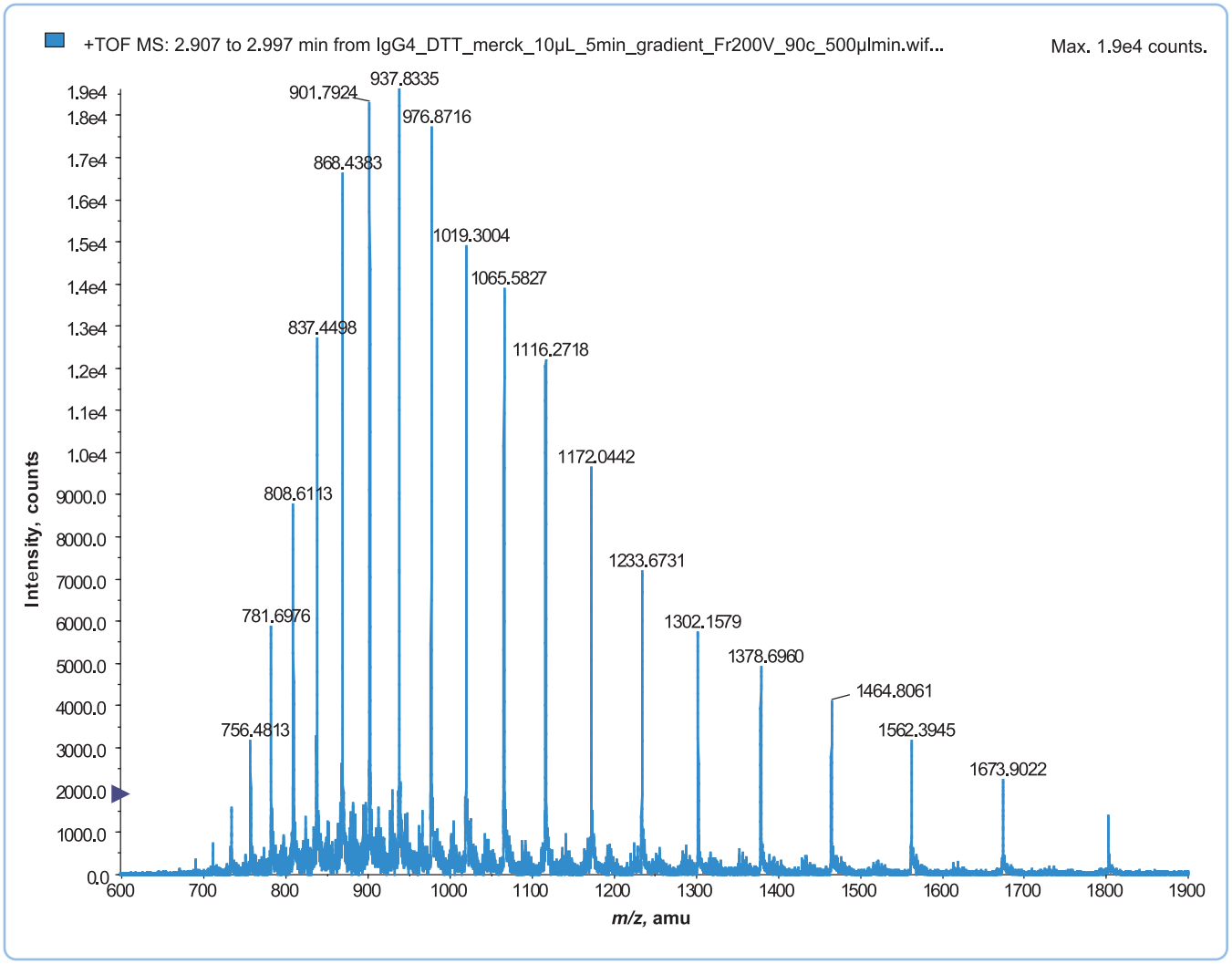

Figure 4. Mass spectrum of IgG4 light chain generated by the Agilent 1200 series HPLC and 6210 TOF MS systems.
IgG4 was reduced with 20 mM DTT at 80°C for 1 hr into heavy and light chains.

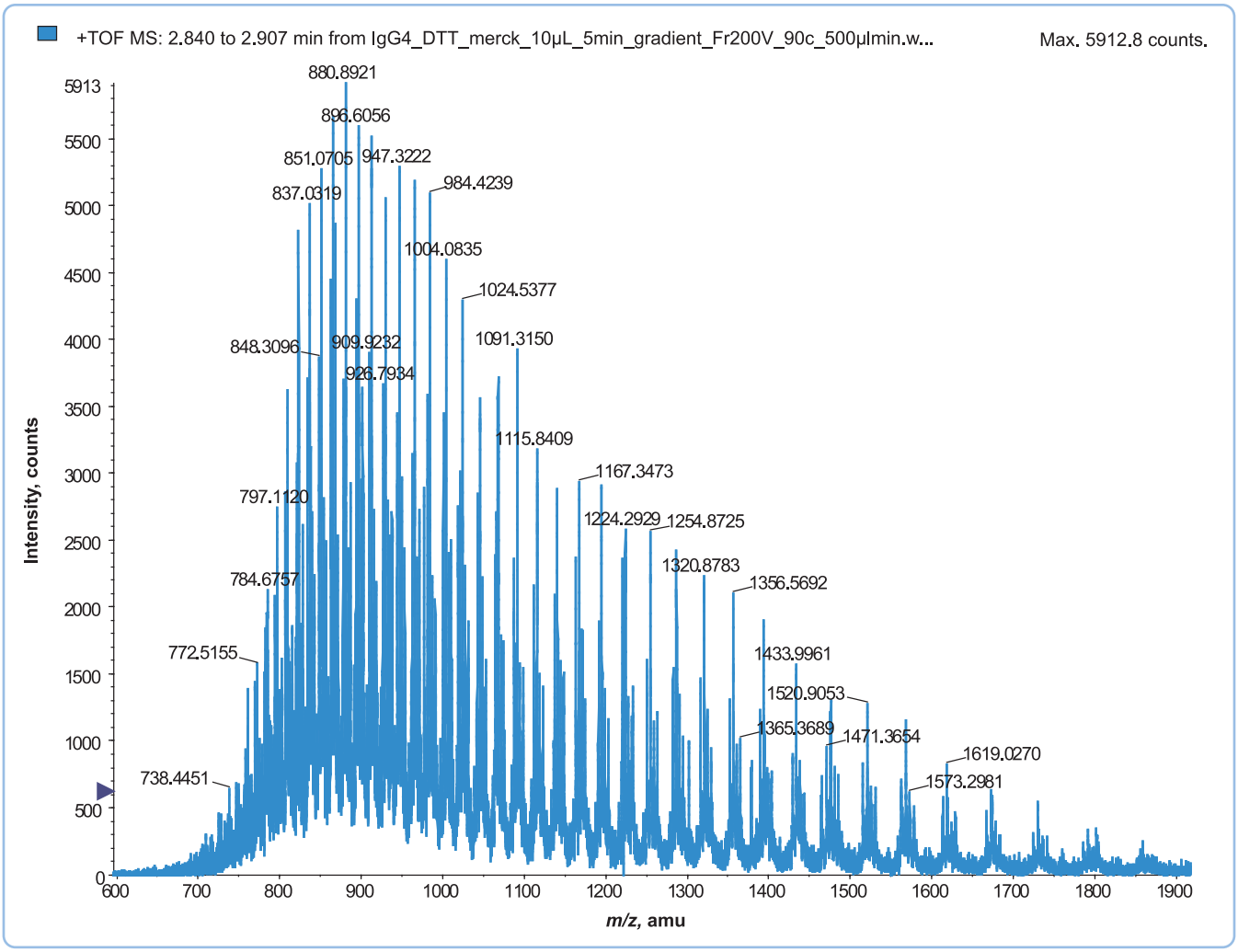

Figure 5. Mass spectrum of IgG4 heavy chain generated by the Agilent 1200 series HPLC and 6210 TOF MS systems.
See Figure 4 for reduction conditions.

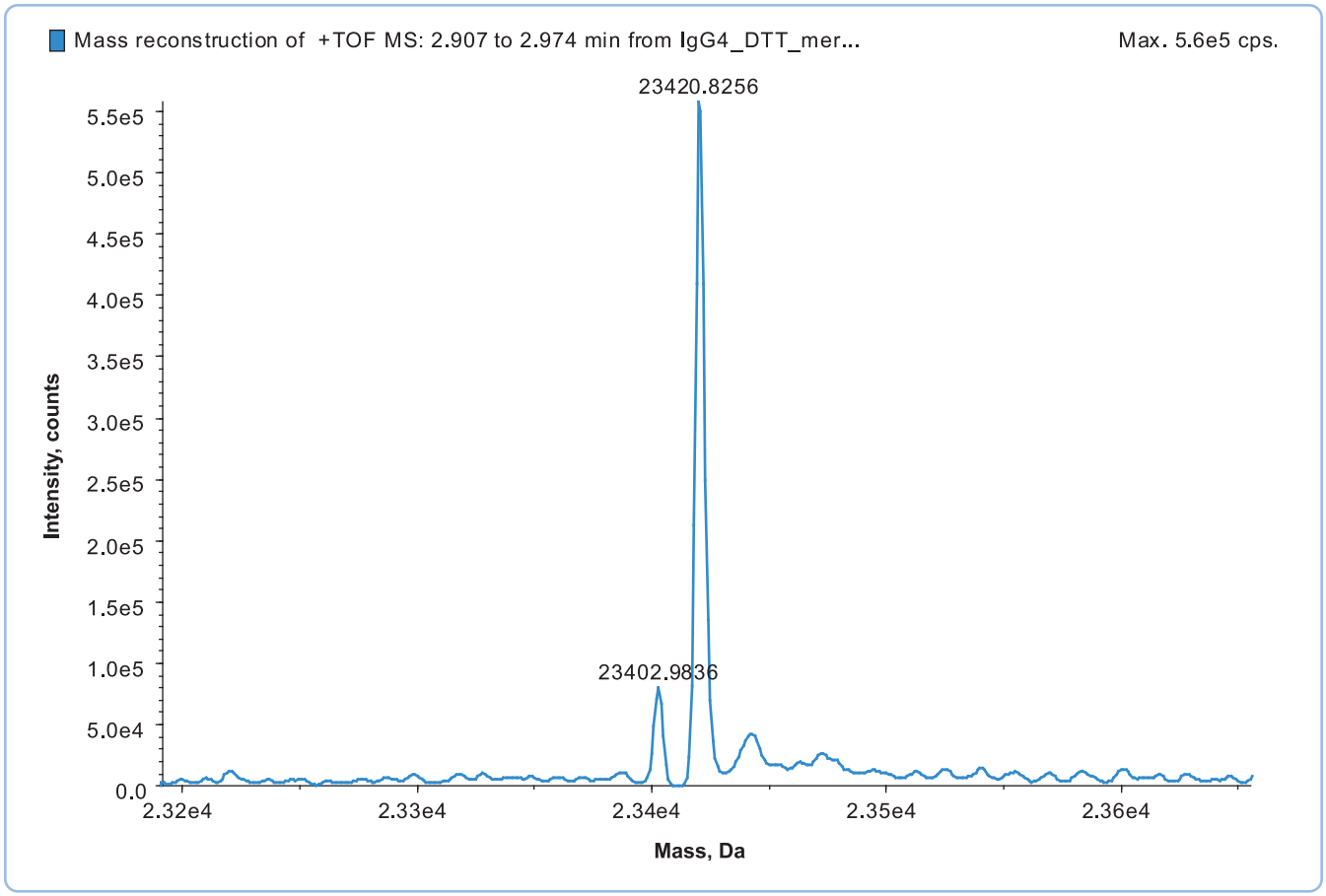

Figure 6. Deconvoluted spectrum of IgG4 light chain generated from the Agilent 6210 TOF MS system.
IgG4 was reduced with 20 mM DTT at 80°C for 1 hr into heavy and light chains.

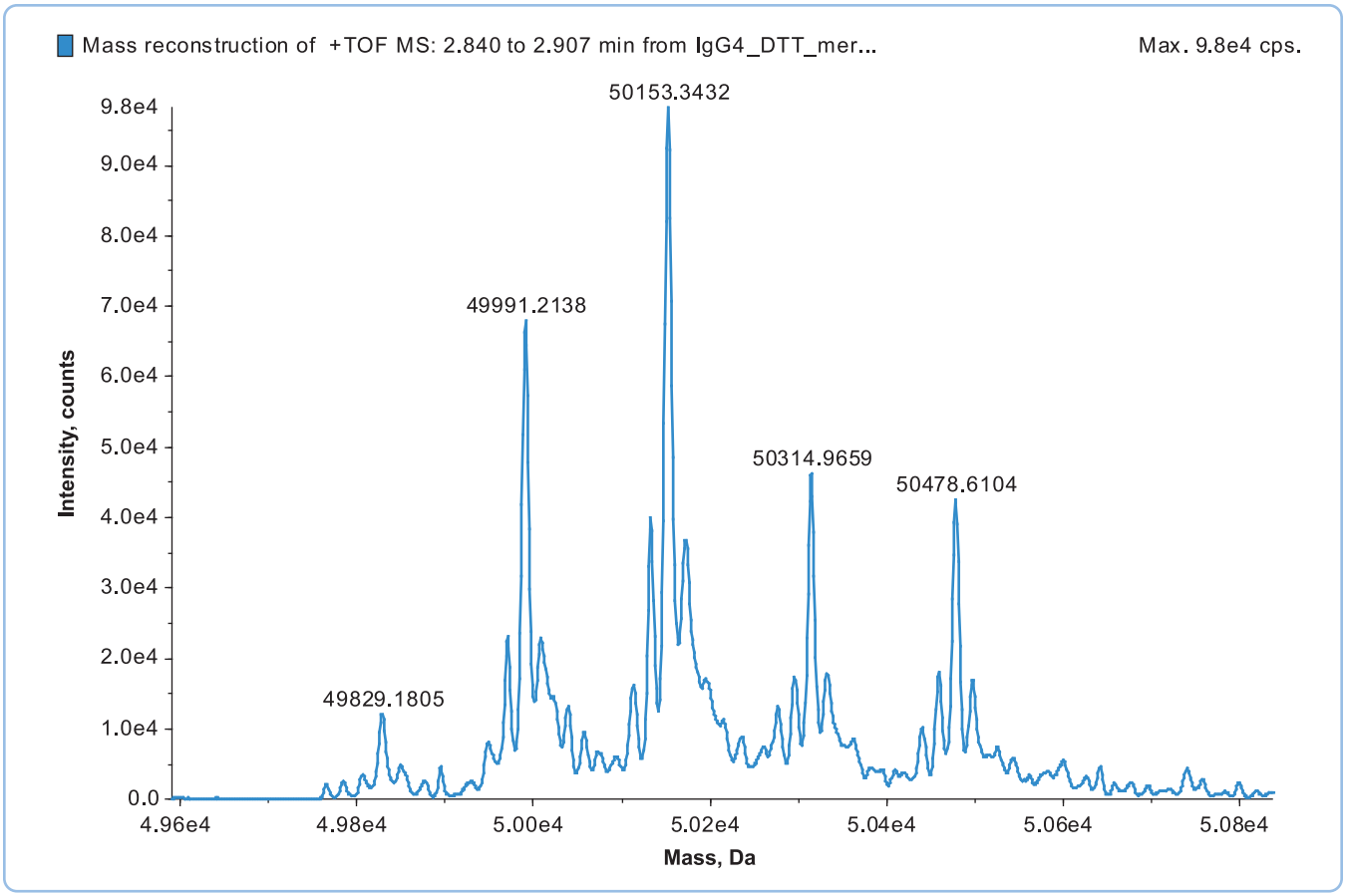

Figure 7. Deconvoluted spectrum of IgG4 heavy chain generated from the Agilent 6210 TOF MS system.
See Figure 6 for reduction conditions.

Mass accuracy calculations for IgG4 reduced with DTT

As shown in Table 1, the routine mass accuracy demonstrated by the TOF MS was better than 25 ppm. Moreover, the data showed the ability of the TOF MS to resolve the various glycanated antibody moieties present in the sample.

Table 1. Reduced IgG4—mass accuracy results

Sample	Calculated mass (Da)	Experimentally determined mass (Da)	Error (ppm)
Light chain	23421.15	23420.82	–15
Heavy chain	M_{calc} naked = 48383.77 (no glycan attached)		
Heavy chain with 1 glyc*	49829.01	49829.18	3.4
Heavy chain with 1 glyc + 1 hex†	49991.15	49991.21	1.2
Heavy chain with 1 glyc + 2 hex	50153.29	50153.34	2.2
Heavy chain with 1 glyc + 3 hex	50315.43	50314.96	–9.3
Heavy chain with 1 glyc + 4 hex	50477.57	50478.61	21

*glyc = Manα6(Manα3)Manβ4GlcNAcβ4(Fucα6)GlcNAc

†hex = hexose

Conclusions

Effective analysis of antibodies requires speed and precision. In this study, the Agilent 1200 series HPLC coupled with the 6210 TOF MS was used to analyze IgG4 antibody samples. IgG4 antibodies were analyzed with mass accuracy better than 25 ppm in a 9-minute total analysis time. Excellent peak shapes and rapid LC separations for antibodies were obtained with the Agilent Poroshell C8 column. The results of this study demonstrate that the combination of Agilent Technologies Poroshell column, 1200 HPLC, and 6210 TOF MS provides a total system solution for efficient, accurate antibody analysis.

References

1. Reichert, J.M., Rosensweig, C.J., Faden, L.B., & Dewitz, M.C. (2005) *Nat Biotechnol.* **23**(9):1073–1078.
2. Kola, I. & Landis, J. (2004) *Nat Rev Drug Discov.* **3**(8):711–715.
3. Riley, S. *The Future of Monoclonal Antibody Therapeutics: Innovation in antibody engineering, key growth strategies and forecasts to 2011.* ©2006 Business Insights Ltd.
4. From *Monoclonal Antibodies in Asthma.* ©2006 LeadDiscovery Ltd.

About Agilent Technologies

Agilent Technologies is a leading supplier of life science research systems that enable scientists to understand complex biological processes, determine disease mechanisms, and speed drug discovery. Engineered for sensitivity, reproducibility, and workflow productivity, Agilent's life science solutions include instrumentation, microfluidics, software, microarrays, consumables, and services for genomics, proteomics, and metabolomics applications.

Buy online:
www.agilent.com/chem/store

Find an Agilent customer center in your country:
www.agilent.com/chem/contactus

U.S. and Canada
1-800-227-9770
agilent_inquiries@agilent.com

Europe
info_agilent@agilent.com

Asia Pacific
adinquiry_aplsca@agilent.com

Printed in the U.S.A. January 31, 2007

5989-5893EN

Agilent Technologies