PLRP-S Polymeric Reversed-Phase Column for LC/MS Separation of mAbs and ADC

Analysis of Intact and Fragmented mAbs and ADC

Application Note

Biotherapeutics and Biologics

Author

Suresh Babu C.V.

Agilent Technologies, Inc.

Abstract

This application note describes the application of polymeric-based reversed-phase columns for the characterization of large biomolecules such as monoclonal antibodies (mAbs) and antibody drug conjugate (ADC). Studies of both intact and fragment levels of mAbs were carried out to understand the performance of the polymeric column. The results demonstrate the superior separation performance of the column, and its suitability for routine LC/MS analysis of mAbs and ADC.

Introduction

Monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs) are therapeutic molecules that are heterogeneous in nature [1]. These molecules can undergo various modifications during the drug development process requiring thorough characterization to ensure safety and efficacy. Reversed-phase liquid chromatography with mass spectrometry detection is most commonly used for primary characterization of mAbs and ADCs. The correct choice of LC column and method is critical to achieve reproducible high-resolution separations and high-quality MS data. Typically, using formic acid (FA), an MS-friendly ion-pairing agent, in the mobile phase leads to poor total ion chromatogram (TIC) peak shape with traditional silica-based reversed-phase columns. This affects LC/MS results (resolution, sensitivity, MS signal, accurate molecular weight information, and so forth). Hence, there is a critical requirement for an LC column compatible with formic acid conditions for enhanced LC/MS analysis of biomolecules.

Agilent Technologies

This study used a polymeric reversed-phase column (Agilent PLRP-S) to demonstrate the LC/MS analysis of mAbs and ADC. PLRP-S columns contain rigid, macroporous, spherical particles of polystyrene and divinylbenzene. They are physically and chemically stable across the complete pH range. The particles are inherently hydrophobic, therefore, neither bonded phases nor alkyl ligands are required for reversed-phase separations. This gives a highly reproducible material that is free from silanols and heavy metal ions. In this work, multiple therapeutic mAbs, including ADC, were analyzed using the PLRP-S column in an LC/MS method. This approach delivered better LC/MS results, and accurate mass determination of intact and fragmented mAbs and ADC.

Materials and methods

Samples

- Therapeutic mAb1, mAb2, and ADC (lysine-conjugated) were purchased from a local pharmacy, and stored according to the manufacturer's instructions.
- **Intact**: mAb1, mAb2, and ADC were diluted to a concentration of 2 µg/µL using 0.1% formic acid in 3% acetonitrile (ACN), and 1 µL was injected.
- **Reduction**: 20 µL of mAb (2 µg/µL) sample was mixed with 5 µL dithiothreitol (DTT) (1 M) followed by incubation at 37 °C for 1 hour.
- **Papain digestion**: 10 µL of mAb (2 µg/µL) sample was mixed with 5 µL digestion buffer (with cysteine), 5 µL of activated papain (Sigma). The mixture was incubated at 37 °C for 3 hours.
- **IdeS proteolytic digestion**: 20 µL of mAb (2 µg/µL) sample was mixed with 0.5 µL FabRICATOR (30 Units) (Sigma) and the mixture was incubated at 37 °C for 1 hour.

Instrumentation

- **LC**: Agilent 1290 Infinity LC system
- **MS**: Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) with an Agilent Jet Stream ion source

Conditions

Agilent 1290 Infinity LC system		
Column:	Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å (p/n PL 1912-1502)	
Injection volume:	1 µL	
Sample thermostat:	5 °C	
Mobile phase A:	0.1% FA in water	
Mobile phase B:	0.1% FA in ACN	
Gradient:	Intact	Fragments
	at 0 min → 20% B	at 0 min → 20% B
	at 4 min → 20% B	at 10 min → 50% B
	at 5 min → 40% B	at 10.1 min → 85% B
	at 10 min → 70% B	at 11 min → 85% B
	at 11 min → 90% B	at 11.1 min → 20% B
	at 11.1 min → 20% B	
Stop time:	11.1 min	
Post time:	4 min	
Column temperature:	80 °C	
Flow rate:	0.6 mL/min	

Agilent 6530 Accurate-Mass Q-TOF LC/MS	
Ion mode:	Positive ion mode, dual AJS ESI (profile)
Drying gas temperature:	350 °C
Drying gas flow:	8 L/min
Sheath gas temperature:	400 °C
Sheath gas flow:	11 L/min
Nebulizer:	35 psi
Capillary voltage:	5,500 V
Fragmentor voltage:	380 V
Skimmer voltage:	65 V
Oct RF Vpp:	750 V
Acquisition parameters MS mode:	Data acquired at 1 GHz, MS only mode, mass range 600-4,000 *m/z* (fragments), 2,000-6,000 *m/z* (intact)

Results and Discussion

To achieve better chromatographic performance, trifluoroacetic acid (TFA) is typically added to the mobile phase as an ion-pairing agent to provide sharper chromatographic peaks. However, TFA is not suitable for MS analysis due to its signal suppression effect [2]. Formic acid is the preferred MS-friendly ion-pairing agent for biomolecule applications, but can cause poor peak shape on traditional silica-based columns. Achieving good chromatographic peak shape and a high signal-to-noise ratio (S/N) *m/z* mass spectrum is a tradeoff when working with high molecular weight proteins such as mAbs [3]. It is important to make the right LC column choice for MS compatibility and improved separation of mAbs and ADC. For guidance on using silica-based columns with LC/MS for mAb separations, refer to Agilent application notes: 5991-6296EN, 5991-4266EN, 5991-2116EN, and 5990-9631EN [3-6].

In this study, the polymeric-based PLRP-S column was used to analyze mAbs and ADC at both the intact and fragment level. PLRP-S is robust and mechanically stable, and available in a range of pore and particle sizes suited for large biomolecule applications.

Intact analysis

Figure 1 shows the LC/MS analysis of the intact mAbs and ADC using a PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. The column provided excellent TIC peak shapes with ≤0.1 minutes full width at half maximum (FWHM) mAbs and 0.25 minutes FWHM ADC. The narrow TIC peak width was obtained using a standard RP mobile phase system (ACN + FA). Similar peak widths were observed for two mAbs, demonstrating the suitability of the method for various mAb samples. Narrow peak width was also demonstrated with the ADC sample, which is highly heterogeneous. The same method conditions and column were also tested with UV detection, and the results were satisfactory with identical retention time profiles (data not shown).

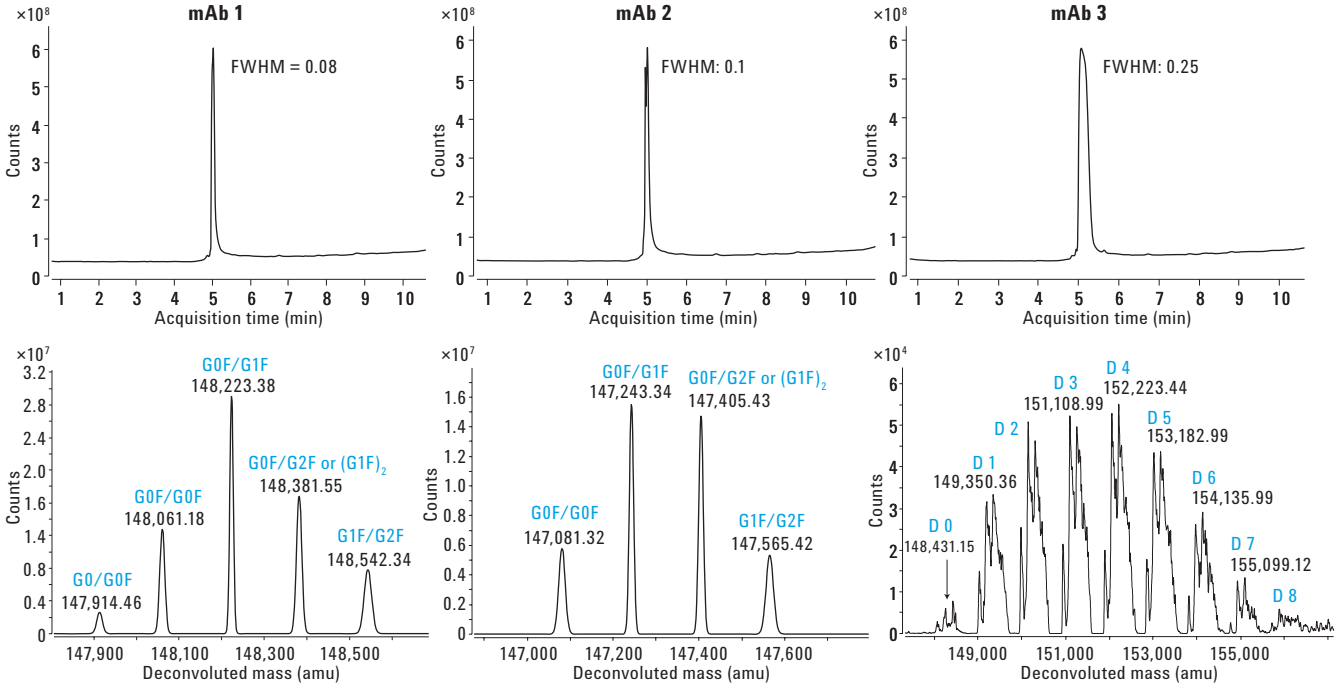

Figure 1. Intact mAb/ADC LC/MS analysis on an Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. Top: Total ion chromatogram; bottom: Deconvoluted spectrum. FWHM: full width at half maximum.

The raw mass spectra were converted to zero-charge mass spectra using Agilent MassHunter BioConfirm software. Figure 1 shows the deconvoluted spectra. Five major glycoforms are seen in the mAb1 deconvoluted spectrum, while four major glycoforms are evident in the mAb2 spectrum. The deconvoluted mass spectra for ADC showed increasing payload trend in steps of one drug load with eight major drug conjugations (D0 to D8).

Fragment analysis

To analyze the generated fragments, the samples were subjected to chemical and enzymatic reactions. Figure 2 shows the LC/MS separation of reduced, IdeS and papain-digested mAbs and ADC using a PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. The fragment peaks (LC, HC, ScFc, F(ab')2, Fc, 2 * Fab2, and ADC fragments with different drug conjugates species) are well separated on the PLRP-S column using the standard mobile phase system (ACN + FA). As expected, the two mAb samples resulted in two major fragments post digestion.

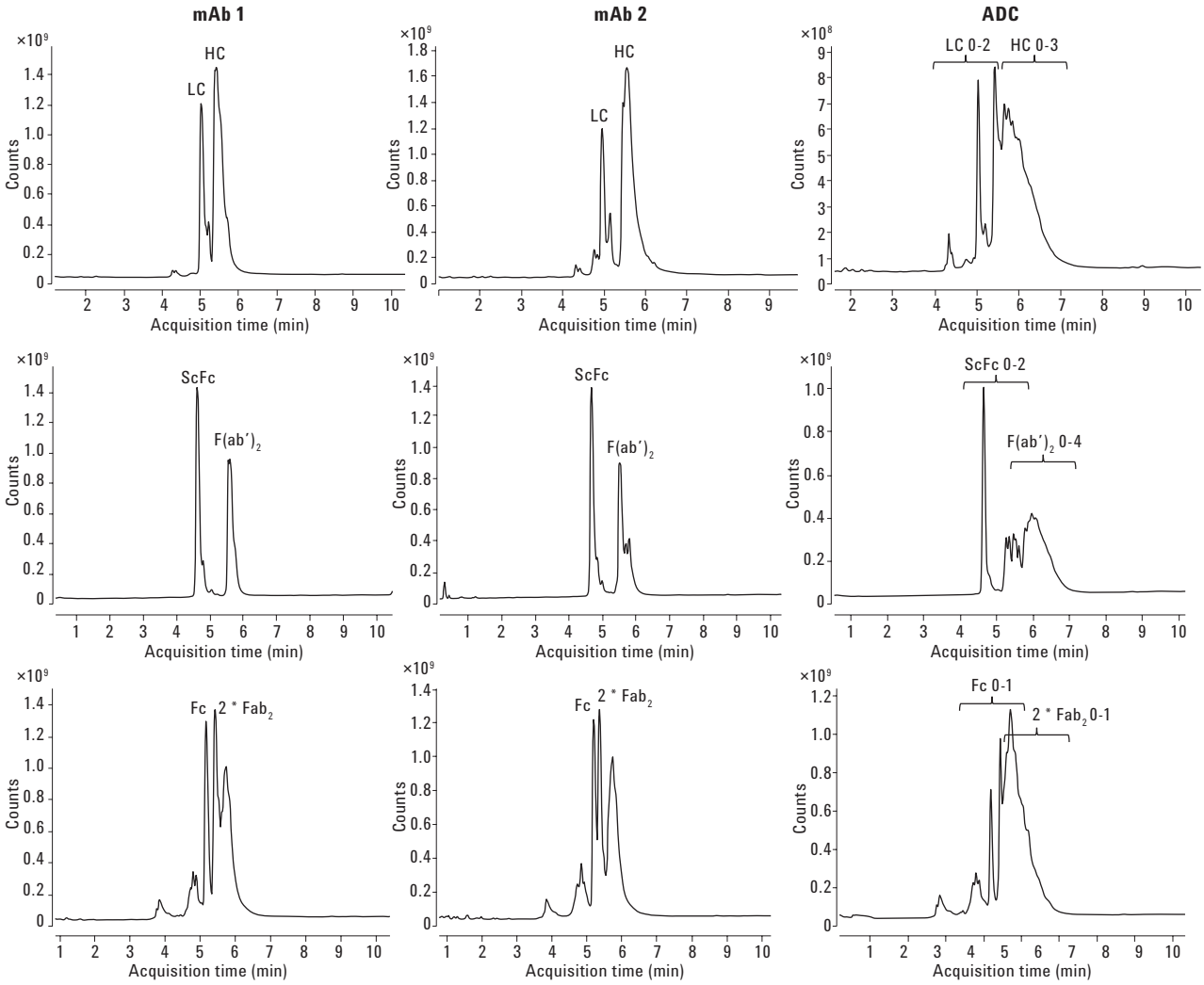

Figure 2. mAb fragment LC/MS analysis on an Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. Top: reduction; middle: IdeS digestion; bottom: papain digestion.

The separations between these major fragment peaks are sufficient to enable better MS sensitivity and accurate molecular weight determination (Figure 3). Separation of the fragment species is very challenging with ADC samples, as conjugation at the various available lysine residues results in a high degree of heterogeneity. Figure 2 shows the separation of glycosylated ADC fragments on the PLRP-S column. It is evident that different drug conjugated species are reasonably separated under the discussed LC/MS conditions, which demonstrates the separation performance of the PLRP-S column. Figure 3 shows the representative deconvoluted spectra for mAb1 and ADC fragments.

Conclusion

- The Agilent PLRP-S column demonstrates superior separation performance for the analysis of mAbs and ADC at both intact and fragment levels.
- The Agilent PLRP-S provided better chromatographic performance and high-quality MS response with formic acid-containing mobile phases.
- Analyses of mAbs and ADC using Agilent PLRP-S columns coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS has been demonstrated.

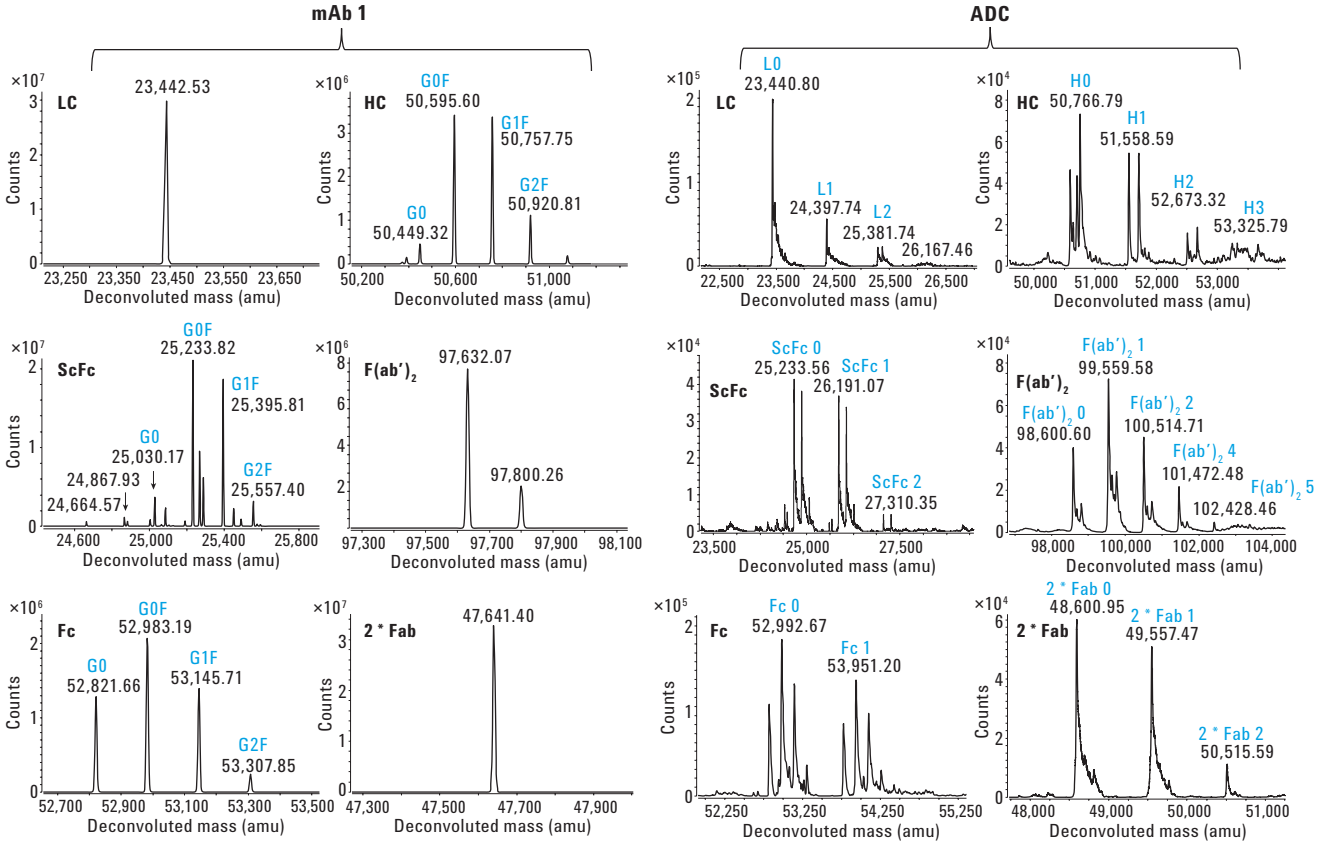

Figure 3. Representative deconvoluted mass spectrum. Top: reduction; middle: IdeS digestion; bottom: papain digestion.

References

1. Beck, A.; Reichert, J. M. Antibody-drug conjugates. *mAbs* **2014**, *6:1*, 15-17.
2. McCalley, D. V. Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography. *Journal of Chromatography A* **2004**, *1038*, 77–84.
3. Gudihal, R.; Suresh Babu C. V.; Tang, N. *Analysis of Monoclonal Antibody(mAb) Using Agilent 1290 InfinityLC System Coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF)*; Application note, Agilent Technologies, Inc. Publication number 5991-4266EN, **2014**.
4. Suresh Babu, C. V. *LC/MS of Intact TherapeuticMonoclonal Antibodies Using Agilent AdvanceBio RP-mAb*. Application note, Agilent Technologies, Inc. Publication number 5991 6296EN, **2015**.
5. Gudihal, R.; Suresh Babu, C. V.; Tang, N.; Madhavi H. N.; Uma, M. *Intact Protein Analysis using an Agilent 6550 Q-TOF Mass Spectrometer*. Application note, Agilent Technologies, Inc. Publication number 5991-2116EN, **2013.**
6. Martosella, J.; Duong, P.; Moyer, S. Rapid UHPLC *Analysis of Reduced Monoclonal Antibodies using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 Column*. Application note, Agilent Technologies, Inc. Publication number 5990-9631EN, **2012.**

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem/advancebio

Printed in the USA
August 1, 2016
5991-7163EN

Agilent Technologies