Fast and High Resolution Analysis of Intact and Reduced Therapeutic Monoclonal Antibodies (mAbs)

The Agilent Bio-inert LC and AdvanceBio RP-mAb Columns

Application Note

Bio-Pharmaceutical

Author

M. Sundaram Palaniswamy
Agilent Technologies Pvt Ltd
Bangalore, India

Abstract

Therapeutic monoclonal antibodies (mAbs) have become the most rapidly growing class of therapeutics in development for many diseases. Novel mAbs are entering clinical trials at a rate of 40 per year. There is also an urgent need for an analytical method that can be used for high-throughput analysis of large number of samples to support the growing biopharma development. This Application Note describes a fast and high-resolution method for the analysis of intact and reduced therapeutic Innovator and Biosimilar mAbs by reverse phase HPLC. Separation was achieved using an Agilent 1260 Infinity Bio-inert LC system with Agilent AdvanceBio RP-mAb C4 and Diphenyl columns. RP-mAb columns give the advantage of superficially porous 3.5 µm particles with 450Å wide pores for improved accuracy and short analysis time compared to fully porous particles of the same size. The bio-inertness of the system, together with high resolution, and short and reproducible methods makes it highly suitable for biopharma QA/QC analysis.

Agilent Technologies

Introduction

Evaluating the molecular similarity of a biosimilar to the reference or the innovator molecule is crucial during biosimilar development. A number of physicochemical methods are required by regulatory agencies involving a wide range of comparability programs. The authorities want to see comparability data on platforms that the previous company or the innovator submitted, primarily high-performance liquid chromatography (HPLC), TOF, Q-TOF mass spectrometry, and capillary electrophoresis. HPLC is a well-established technique for the determination of intact protein by size exclusion or ion exchange. However, technological developments in the field of reverse phase (RP) chromatographic stationary phases (a large pore size of 300Å or fused core particles with short alkyl chains) have made them promising tools for analyzing intact proteins[1]. Historically, mAbs and their fragments are analyzed with limited success using wide-pore, totally porous particle RP HPLC. Due to their large size and limited diversity, analysis times are often unacceptably long, and mAb peaks can elute as broad bands, compromising resolution. In contrast, high efficiency superficially porous columns easily separate mAbs and their fragments in minutes with high efficiency.

In this work, we have demonstrated the suitability of the Agilent 1260 Bio-inert Quaternary LC system and Agilent AdvanceBio RP-mAb columns to achieve high resolution and rapid separation of intact and fragmented therapeutic innovator and biosimilar rituximab. The unique design of the AdvanceBio RP-mAb column offers unique selectivity due to its superficially porous particles (3.5 µm) with wide pores (450Å). The column delivers a significant speed and resolution advantage while maintaining compatibility with all instruments.

Experimental

Equipment

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60-mm Max-Light high sensitivity flow cell (G4212B option 33)
- Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm (p/n 799775-944)
- Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm (p/n 799775-904)

The complete sample flow path is free of any metal components, therefore, the sample never contacts metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Software

Agilent ChemStation B.04.03 (or higher)

Reverse phase HPLC parameters

Chromatographic parameters for intact and reduced mAb analysis using AdvanceBio RP-mAb columns are shown in Table 1.

Table 1. Chromatographic parameters used for intact and reduced analysis.

Parameter	HPLC (intact and reduced mAbs)
Mobile phase	A) Water + 0.1 % TFA B) IPA:ACN:Water (70:20:10) + 0.09 % TFA
Columns	Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm
Gradient	Time (min) % B 0 15 0.5 25 1.5 35 1.51 35 3.0 60 4.0 60
Post time	2 minutes
Injection volume	1 µL
Flow rate	1.0 mL/min
TCC	80 °C
UV detection	220 and 280 nm

Reagents, samples, and materials

Innovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instruction. PBS and *tris*(2-carboxyethyl) phosphine (TCEP) were purchased from Sigma-Aldrich. All chemicals and solvents were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile and 2-propanol were purchased from Lab-Scan (Bangkok, Thailand). For intact and reduced analysis, rituximab samples were diluted to 2 mg/mL using PBS.

Sample preparation

Reduction of mAbs

For the separation of the light and heavy chains, an aliquot of 0.5 M TCEP stock was added to the mAb samples to obtain a final concentration of 10 mM. The mixture was held at 60 °C for 30 minutes.

Results and Discussion

Intact mAb analysis

The AdvanceBio RP-mAb column with superficially porous particles and wide pores delivers higher resolution and faster run times to provide accurate, reproducible results when analyzing monoclonal antibodies for biopharma discovery, development, and QA/QC applications. Combined with the Agilent 1260 Infinity Bio-inert Quaternary LC System with a power range up to 600 bar, it can be used for mAb separation. The mobile phase was a combination of isopropanol (IPA), acetonitrile (ACN), water, and trifluoroacetic acid (TFA). Figures 1 and 2 depict the optimized RP HPLC elution profile of intact innovator and biosimilar rituximab on an AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm and AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column, respectively, demonstrating excellent peak shape and fast separation in 4 minutes. Comparing Figures 1 and 2 demonstrates that different selectivity can be obtained through the use of different bonded phases using the same chromatographic conditions, with the diphenyl phase resolving in finer detail.

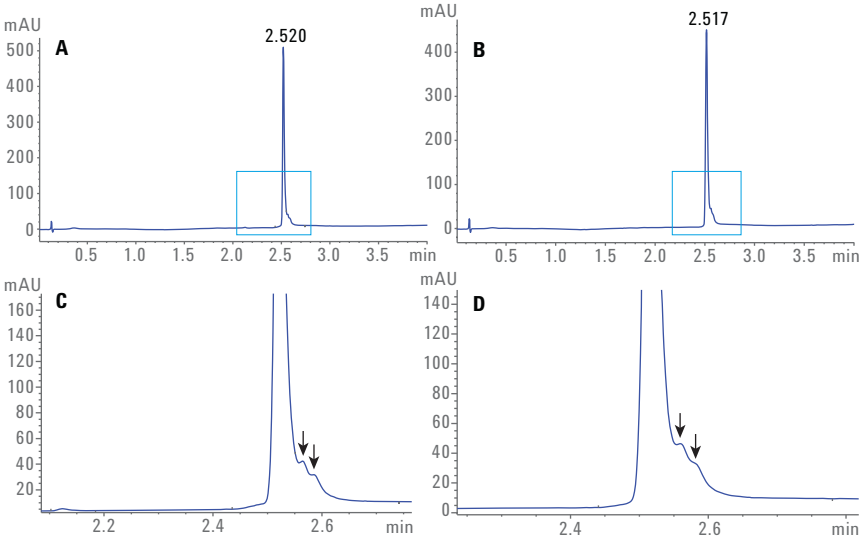

Figure 1. RP-HPLC analysis of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

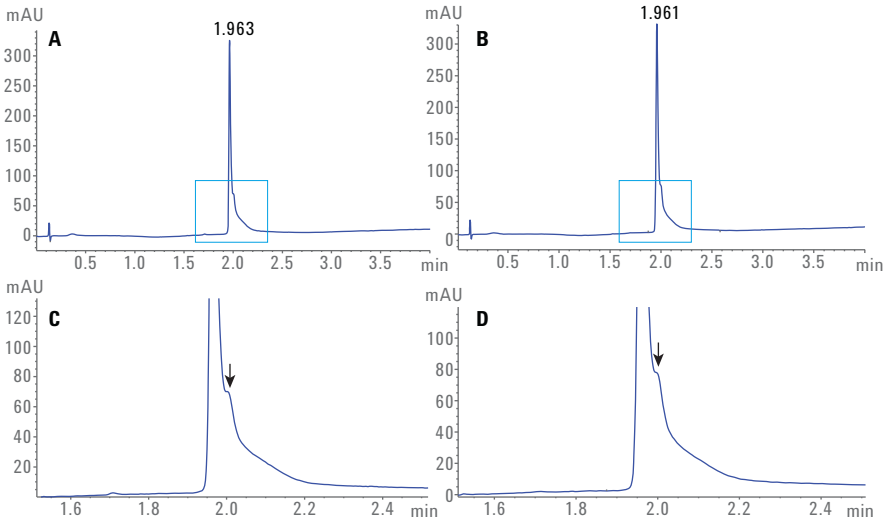

Figure 2. RP-HPLC profile of intact innovator rituximab (A) and biosimilar rituximab (B) on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

Reduced mAb analysis

TCEP was used to separate free antibody light and heavy chains. AdvanceBio RP-mAb columns are very effective in providing fast and high-resolution separations of antibody fragments. The profiles in Figures 3 and 4 show a rapid reversed phase analysis optimized for the separation of antibody fragments in approximately 4 minutes using C4 and diphenyl phases, respectively. In both cases, due to reduction of the disulfide bonds, mAbs eluted as distinct light chain (LC) and heavy chain (HC) separations with high efficiency. The same gradient used for the intact analysis was able to resolve the LC and HC for the reduced samples. As we have seen with intact mAb analysis, the LC and HC show different selectivities with diphenyl and C4 columns.

RP HPLC analysis of intact and reduced innovator and biosimilar using AdvanceBio RP-mAb diphenyl and C4 columns indicates that the mAb pair are highly similar.

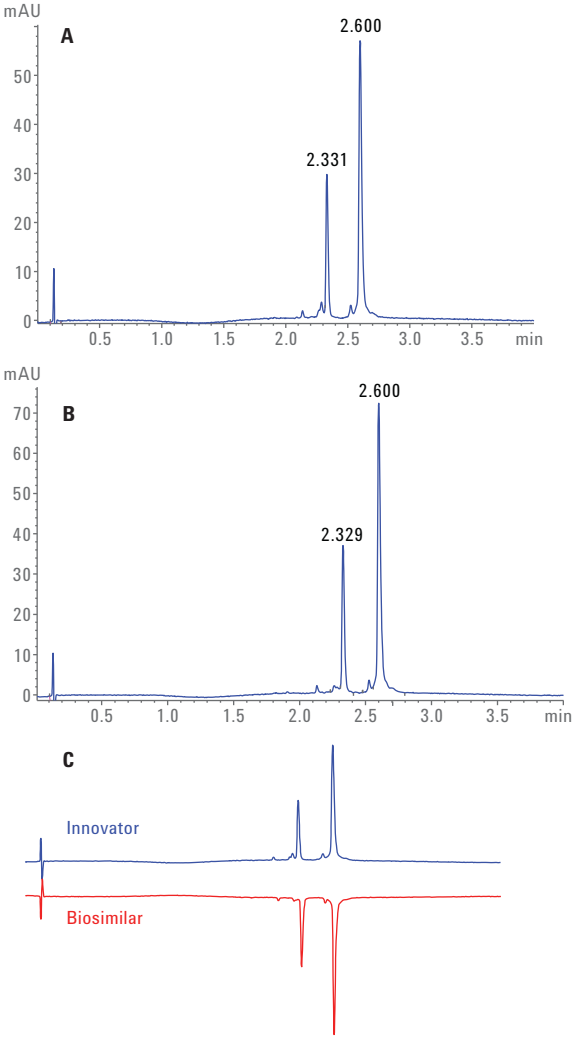

Figure 3. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

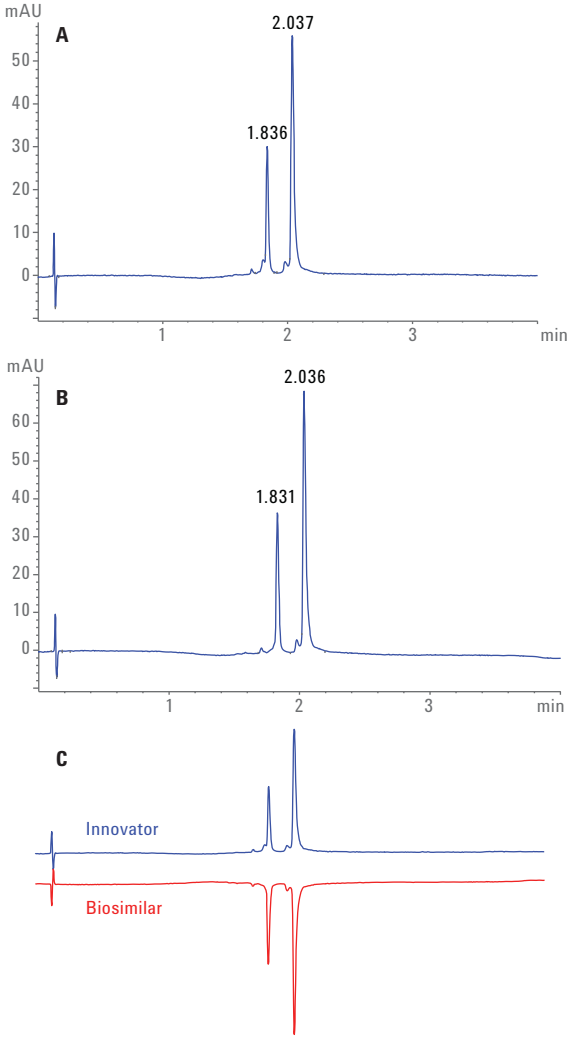

Figure 4. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

Precision of retention time and area

Tables 2 and 3 present the average retention times and area RSDs from six replicates of intact and TCEP reduced innovator, and biosimilar rituximab for the diphenyl and C4 phases. The results show that both columns provide precision of RT and area within the acceptable limit of ±3 % and ±5 %, respectively.

Conclusion

In this application note, we have demonstrated a simple LC-UV-based approach to define the molecular similarity between a biosimilar and its innovator reference. We first used the Agilent 1260 Bio-inert Quaternary LC system with Agilent AdvanceBio RP-mAb Diphenyl and C4 columns to develop a high-resolution and rapid separation of intact mAbs. Using the same method, we were also able to show the separation of light chain and heavy chain after TCEP reduction. Area and RT precision of intact and reduced analysis using AdvanceBio RP-mAb columns were excellent, and show the reliability of the method. Such fast, simple, and reproducible methods for intact and reduced analysis of mAbs, coupled with bio-inertness of the system makes this solution suitable for the comparability analysis of mAbs for the biopharma industry.

Table 2. Retention time and peak area RSD (%), n = 6 for intact analysis

	Retention time		Peak area	
Samples	Mean (min)	RSD	Mean (mAU/min)	RSD
Agilent AdvanceBio RP-mAb, C4, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab	1.96	0	71.61	1.98
Biosimilar rituximab	1.95	0.26	77.3	0.47
Agilent AdvanceBio RP-mAb, Diphenyl, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab	2.51	0.20	66.7	0.458
Biosimilar rituximab	2.51	0	73.3	1.86

Table 3. Retention time and peak area RSD (%), n = 6 for reduced analysis

	Retention time		Peak area	
Samples	Mean (min)	RSD	Mean (mAU/min)	RSD
Agilent AdvanceBio RP-mAb, Diphenyl, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab LC	2.32	0.60	19.71	4.24
Innovator rituximab HC	2.58	1.52	57.33	1.57
Biosimilar rituximab LC	2.32	0.07	23.56	3.25
Biosimilar rituximab HC	2.60	0.05	58.40	5.61
Agilent AdvanceBio RP-mAb, C4, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab LC	1.83	0	21.5	1.4
Innovator rituximab HC	2.03	0.04	51.2	2.25
Biosimilar rituximab LC	1.83	0.03	24.47	3.84
Biosimilar rituximab HC	2.03	0.06	52.66	0.84

Reference

1. Navas, N; *et al.*, *Anal. Bioanal. Chem.* **2013**, *405*, pp 9351-9363.

www.agilent.com/chem

Published in the USA, November 2, 2017
5991-6274EN