Physicochemical characterization of a therapeutic protein by peptide mapping, SEC and IEX using the Agilent 1260 Infinity Bio-inert Quaternary LC system

Application Note

Biopharmaceuticals

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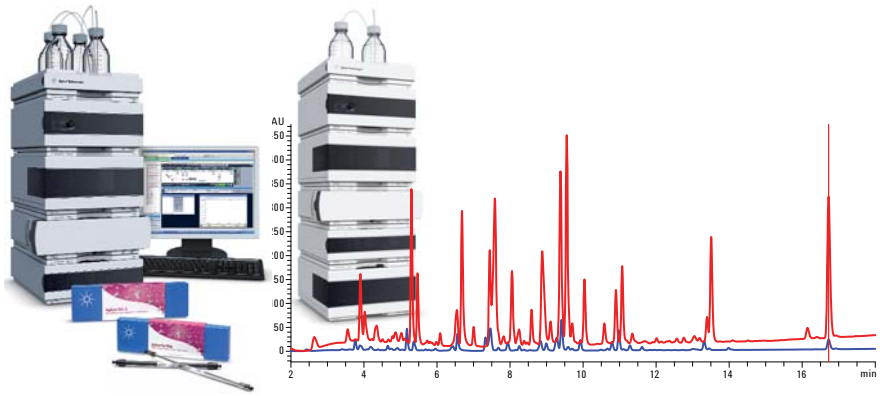

Abstract

Sensitive and rapid LC methods were developed on the new Agilent 1260 Bio-inert Quaternary LC system to characterize the therapeutic protein, P128. Optimized peptide maps were constructed using sub-2-µm column technology to decrease analysis speed for this protein. Size exclusion chromatography (SEC) and ion exchange chromatography (IEX) were performed using the new Agilent Bio-HPLC columns to detect potential impurities or isoforms of the protein preparation. The bio-inertness and corrosion resistance of the instrument coupled with the simple and reproducible methods make this solution suitable for routine use in QA/QC labs.

Agilent Technologies

Introduction

In-depth physicochemical characterization of therapeutic proteins is required during all phases of drug development to ensure drug safety and efficacy. Providing proof of protein drug identity and integrity is usually demonstrated by performing several (LC-based) assays such as peptide mapping, SEC and IEX. P128 is an antistaphylococcal protein with a nominal mass of 28 kDa. It is recombinantly expressed in *E coli*. In this Application Note, the suitability of the Agilent 1260 Bio-inert Quaternary LC System for resolving the peptides generated by tryptic digestion of P128 by RP chromatography and for identifying the structural variants by IEX and SEC using the new bio-LC columns is demonstrated.

Experimental

The completely bioinert, biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC system with a maximum pressure of 600 bar consisted of the following modules:

- Agilent 1260 Bio-inert Quaternary LC pump (G5611A)
- Agilent 1260 Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Series Thermostat (G1330B)
- Agilent 1260 Thermostatted column Compartment containing bioinert click-in heating elements (G1316C option 19)
- Agilent 1260 DAD with Max-Light 60 mm, high sensitivity flow cell (G4212B option 33)
- Software: ChemStation B.04.02

The complete sample flow path is free of any metal components such that the sample never touches metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Chemicals and reagents

P128 was provided by GangaGen Biotechnologies Pvt. Ltd., trizma base, urea, DTT and Iodoacetamide were purchased from Sigma Aldrich and trifluoroacetic acid, disodium hydrogen phosphate, monosodium hydrogen phosphate, sodium chloride from Fluka. Acetonitrile (LiChrosolv) and Millipore water were used to prepare mobile phases.

Peptide mapping

Sample preparation

The protein was reduced with DTT in the presence of urea and alkylated with Iodoacetamide. Prior to overnight digestion with trypsin in tris buffer (pH 7.6) the diluted sample mix was subjected to ultrafiltration. The enzyme to substrate ratio was maintained at 1:20 for digestion. The enzymatic activity was quenched by adding 1 µL of 10% formic acid solution. The sample was dried by vacuum concentration and the residue redissolved in 100 µL of 0.25 M tris buffer (pH 7.6).

Chromatographic conditions

Columns

Column 1:	Agilent ZORBAX Eclipse Plus C18 3.0 mm × 100 mm, 1.8 µm (p/n 959758-302)
Column 2:	AdvanceBio Peptide Mapping, 3.0 mm × 150 mm, 2.7 µm, (p/n 653950-302)

LC Methods

Injection volume:	2 µL
Mobile phase A:	Water containing 2% ACN with 0.1% TFA
Mobile phase B:	100% ACN with 0.08% TFA

Flow Rate:

0.2 mL/min		**0.4 mL/min**	
Minute	%B	Minute	%B
0	2	0	2
9	15	4.5	15
60	45	30	45
63 – 64.5	80	31.5 – 32.25	80
66 – 81	2	33 – 40.5	2

0.6 mL/min		**0.8 mL/min**	
Minute	%B	Minute	%B
0	2	0	2
3	15	2.25	15
20	45	15	45
21 – 21.5	80	15.75 – 16.12	80
22 – 27	2	16.5 – 20.25	2

UV detection: 230 nm/4 nm, Reference: 400/60 nm (data also acquired at 214, 240, 260 and 280 nm)

Size exclusion chromatography

Columns

Column 1:	Agilent Bio-SEC-3, 3 µm, 300 Å, 7.8 mm × 300 mm, (p/n 5190-2511)
Column 2:	Agilent Bio-SEC-5, 5 µm, 300 Å, 7.8 mm × 300 mm, (p/n 5190-2526)
Column 3:	Brand A SEC-5, 5 µm, 300 Å, 7.8 mm × 300 mm

LC Method

Injection volume:	1 µL
Mobile phase A:	150 mM sodium phosphate (pH = 7.0) containing 150 mM sodium chloride
Flow rate:	1 mL/min
UV detection:	220 nm/4 nm Reference: Off (data also acquired at 230, and 280 nm)

Ion exchange chromatography

Sample was desalted by ultrafiltration and extracted into 20 mM sodium phosphate (pH = 6).

Columns

Column 1:	Agilent Bio MAb NP 5, PK, 4.6 mm × 250 mm, 5 µm, (p/n 5190-2407)
Column 2:	Agilent Bio MAb NP 10, PK, 4.6 mm × 250 mm, 10 µm, (p/n 5190-2415)
Column 3:	Brand B WCX-10, 4.0 mm × 250 mm, 10 µm

LC Method

Injection volume:	2 µL
Mobile phase A:	20 mM sodium phosphate (pH = 6.0)
Mobile phase B:	20 mM sodium phosphate (pH = 6.0) containing 1.0 M sodium chloride
Flow rate:	0.5 mL/min

Minute	%B
0	10
35	35
36	10
45	10

UV detection:	220 nm/4 nm, Reference: Off (data also acquired at 220, 230, 240, and 280 nm)

Results and discussion

Peptide mapping

Proof of protein drug identity is usually demonstrated by peptide mapping. Peptide maps are constructed by proteolytic digests of the therapeutic protein, followed by high resolution reversed phase HPLC. The goal of the analysis is to chromatographically resolve and detect all protein fragments and achieve complete sequence coverage. Due to the complexity of the analyzed sample containing hundreds of different compounds, peptide mapping requires very long analytical runs. In early phases of drug development, mass spectrometry is the detection technique of choice to identify all peptide fragments. During later phases and in QA/QC, confirmation of peptide profiles is most commonly done by UV detection. Bio-inert instrumentation has been beneficial especially for hydrophobic peptides and proteins that tend to stick to metal surfaces.

In this study, we present data for a 26 kDa recombinantly expressed new biological entity (NBE), a bacteriophage protein P128 under development at GangaGen Biotechnologies for human therapy. Peptide mapping, SEC and ion-exchange chromatography are performed for this protein using Agilent Bio HPLC columns in combination with an Agilent 1260 Infinity Bio-inert Quaternary LC instrument developed for the analysis of large biomolecules.

UV-based peptide maps are often generated in a QA/QC NBE environment to detect any sequence abnormalities caused during the manufacturing or processing of a recombinant protein drug.

Figure 1 shows the peptide maps generated using the Agilent 1260 Infinity Bio-inert Quaternary LC with two reverse-phase columns:

- Agilent ZORBAX Eclipse Plus C18, 3.0 × 100 mm, 1.8 µm
- AdvanceBio Peptide Mapping, 3.0 × 150 mm, 2.7 µm

In this example, slightly better separation was obtained with the Agilent Eclipse Plus column. Therefore, this column was used for further investigation.

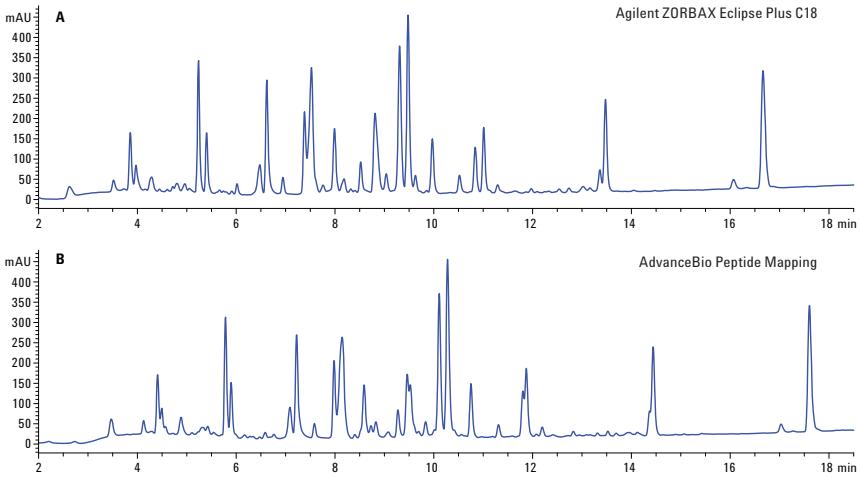

Figure 1. Analyses of P128 digest on columns A) Agilent ZORBAX Eclipse Plus C18, 3.0 mm × 100 mm, 1.8 µm and B) AdvanceBio Peptide Mapping, 3.0 mm × 150 mm, 2.7 µm at 0.6 mL/min flow rate with the corresponding gradient.

The highest possible resolution is required for a very complex mixture, which often results in excessively long analysis times, such as peptide maps. Sub-2-µm (STM) particle technology in combination with UHPLC instrumentation shortens analysis time significantly while preserving resolution (Figures 2a – 2d).

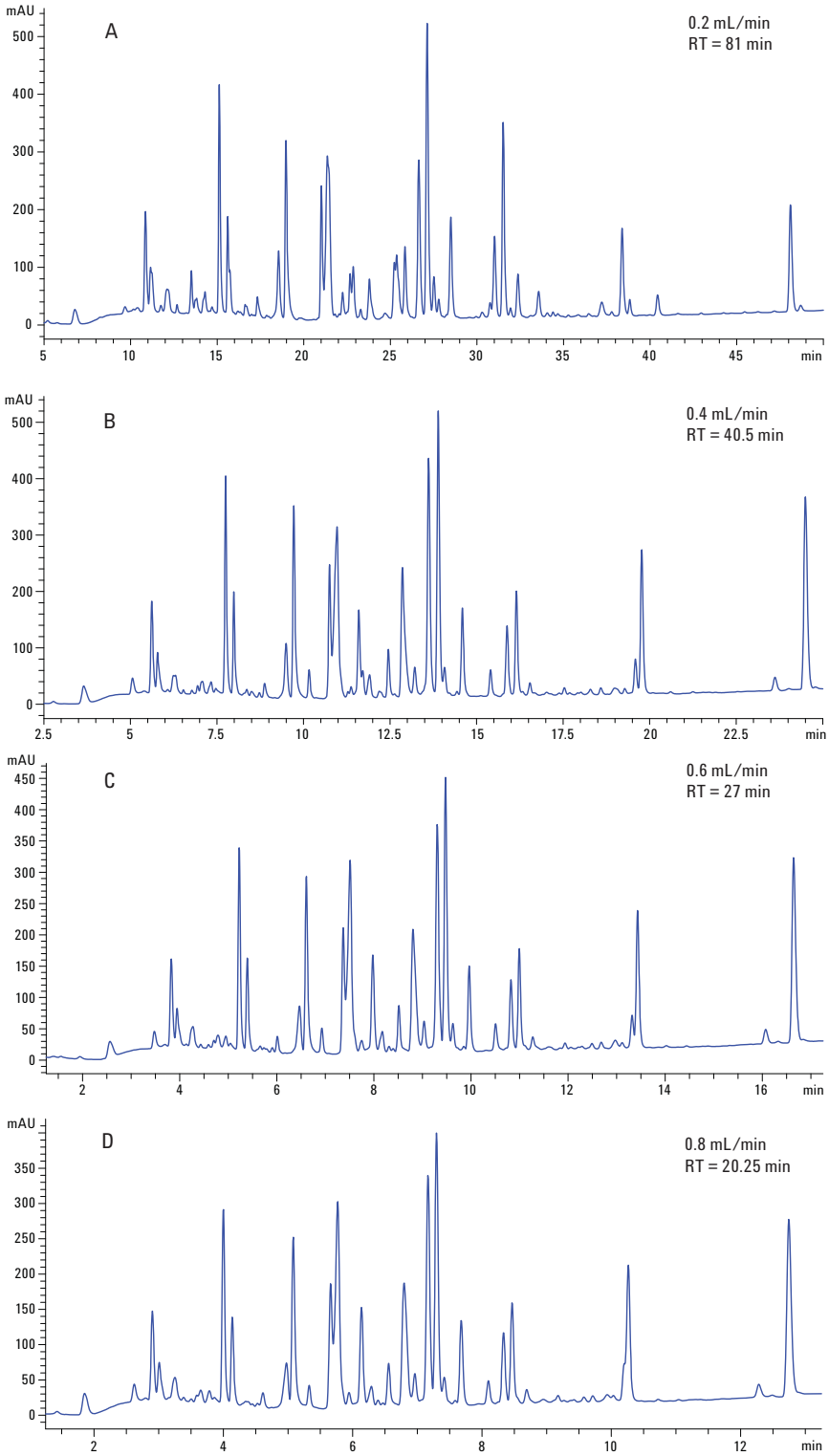

Figure 2a–2d. Analyses of P128 digest with different gradients and flow rates on Agilent ZORBAX Eclipse Plus C18 3.0 mm × 100 mm, 1.8 µm column.

Robustness and repeatability of analyses are the most important factors when characterizing therapeutic proteins for proof of integrity and identity of the selected NBE, and fulfilling regulatory requirements. Five replicate injections were performed on an Agilent ZORBAX Eclipse Plus C18 3.0 mm × 100 cm, 1.8 µm column with the Agilent 1260 Infinity Bio-Inert Quaternary LC were performed. (Figure 3 demonstrates excellent repeatability of retention time and peak areas).

High sensitivity analysis is required for the detection of minor impurities. The Agilent 1260 Infinity DAD with Max-Light flow cell technology achieves sensitivity that is ten times higher than the Agilent 1260 Infinity DAD VL Plus with the older flow cell design (Figure 4). This technology can detect modifications for example, from mutations in the expression clone. It also contributes to lower consumption of valuable samples. Using the Agilent 1260 Infinity DAD (G4212B) with 60 mm Max-Light flow cells, a ten times sensitivity increase was observed compared to the Agilent 1260 Infinity DAD VL Plus detector (G1315C).

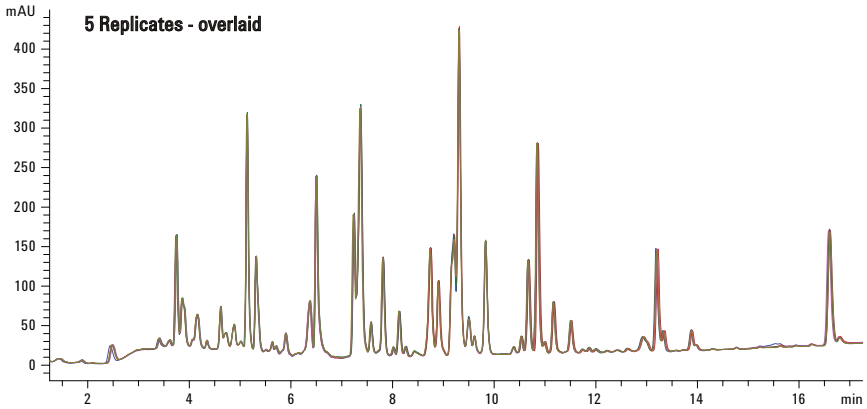

Figure 3. Overlay of five replicate runs of P128 digest on Agilent ZORBAX Eclipse Plus C18 3.0 mm × 100 mm, 1.8 µm column at 0.6 mL/min flow rate with the corresponding gradient.

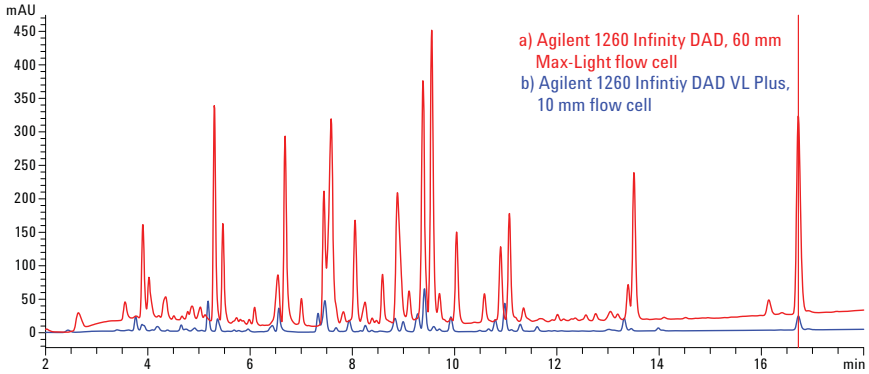

Figure 4. Overlay chromatograms of P128 digest obtained using Agilent ZORBAX Eclipse Plus C18 3.0 × 100 mm, 1.8 µm column at 0.6 mL/min flow rate with the corresponding gradient on the Agilent 1260 Infinity Bio-inert HPLC system equipped with A) Agilent 1260 Infinity DAD with 60 mm Max-Light flow cell or B) Agilent 1260 Infinity DAD VL Plus (G1315C).

Size exclusion chromatography

In order to demonstrate protein integrity or prove the absence of dimers or multimer formation in its native conformation, size exclusion chromatography (SEC) with UV detection is a commonly applied analytical technique. A disadvantage of size exclusion is its lack of resolution. Small particle SEC columns produce superior resolution with minimal secondary interaction, providing a powerful tool for detecting impurities or multimer formation. The same protein used for peptide mapping was used to perform the SEC experiment. Results were compared for two 5-µm particle columns and a 3-µm particle column. The second compound present in the mixture could only be detected with the Agilent Bio-SEC 3-µm column (Figure 5).

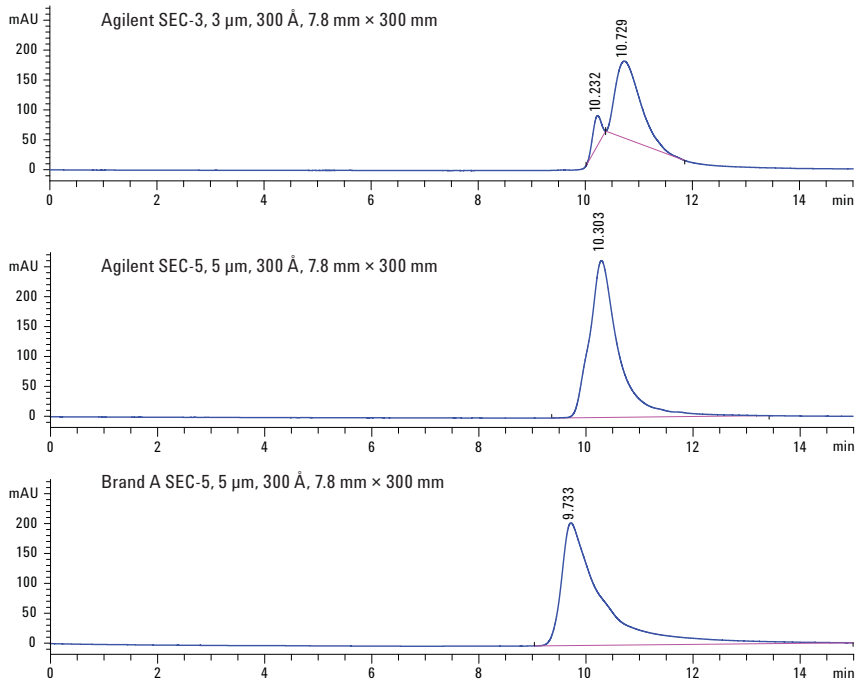

Figure 5. Size exclusion of a P128 therapeutic protein sample performed on the Agilent 1260 Infinity Bio-Inert Quaternary LC using different SEC columns.

Ion exchange chromatography

Ion exchange chromatography is applied to separate charge variants of NBEs that might be formed through oxidation, de-amidation or truncation of terminal lysine residues during manufacturing or processing steps. In order to separate basic and acidic variants from the main peak, high resolution and minimal secondary surface interaction are required. A desalted sample of P128 protein preparation was separated on the Agilent 1260 Infinity Bio-inert and corrosion resistant quaternary LC system using the Agilent Bio Mab 5 and Bio Mab 10 columns and a commercially available column (Brand B) of similar dimensions. The best peak shape and highest efficiency were achieved on the Agilent Bio Mab 5 column (Figure 6).

Conclusions

The Agilent 1260 Infinity Bio-inert Quaternary LC system with the Agilent Bio HPLC column portfolio provides a powerful and versatile tool for characterizing the physicochemical properties of the therapeutic protein drug P128. The Agilent solution for the analysis of therapeutic proteins achieves bio-inertness, superior resolution, corrosion resistance, high sensitivity and fast separation speed.

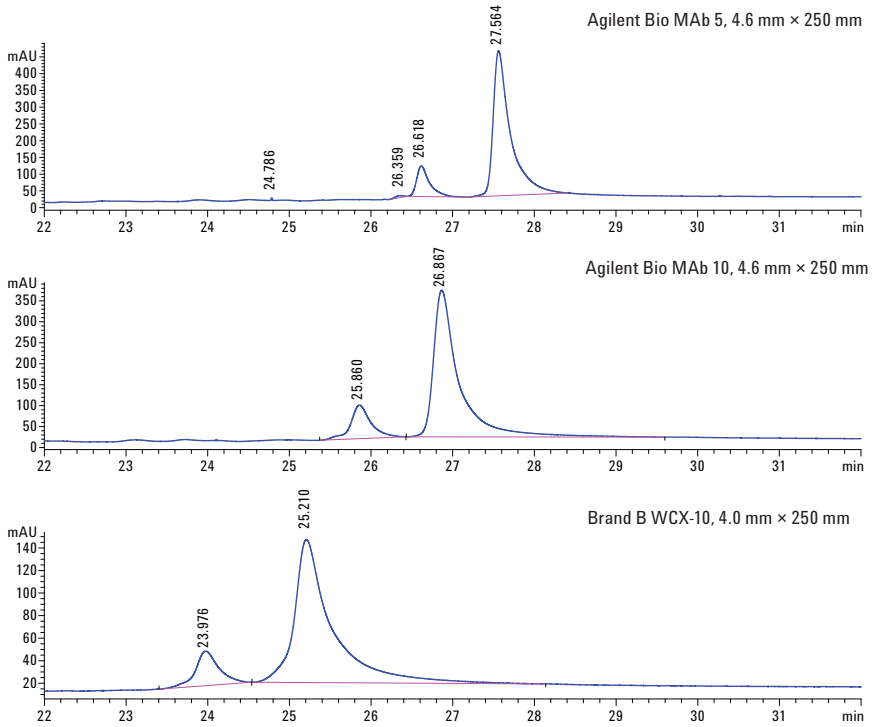

Figure 6. Weak cation-exchange chromatography for P128 therapeutic protein sample on the Agilent 1260 Bio-inert Quaternary LC system using different cation-exchange columns.

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