High Temperature, High Throughput Reversed Phase Separation of Intact Monoclonal Antibodies

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Overview

Purpose: Demonstrate reversed phase chromatography of intact IgG with a novel UHPLC system and a novel dedicated column

Methods: In this work, the Thermo Scientific™ MAbPac™ RP column was operated with the Thermo Scientific™ Vanquish™ Flex UHPLC system. To assess the suitability of the approach for stability studies, the separation of a reference and a stressed mAb chromatogram is compared.

Results: The novel reversed phase column for intact protein allowed fast and efficient elution of monoclonal antibodies with a generic gradient. The suitability of the method for stability-indicating studies was demonstrated by comparing the profile of a reference and stressed antibody

Introduction

In the biopharmaceutical early analytical development, characterization of monoclonal antibody is required to support process development. Reversed Phase Chromatography (RPC) is routinely applied to profile the therapeutic protein during this stage of development. RPC can be run with MS-compatible mobile phase, hence the method can be easily transferred to the MS characterization laboratories when required. RPC is an excellent tool for protein quantitation of main compound and minor variants. RPC of intact proteins is typically run at high temperature to improve peak shape and recovery. Thus, high resolution columns, packed with temperature-stable material are required. The method should be sufficiently fast, in order to allow the processing of a large number of samples in a reasonable time.

A typical example of early-stage analytical development is the early stability evaluation of the new biological entities. The analytical methods for the stability assessment need to be able to indicate, and approximately quantify, sample degradation.

Methods

Sample Preparation

All antibody samples were dissolved in aqueous buffers. The concentration of the samples were the following: trastuzumab 21 mg/mL; bevacizumab 25 mg/mL; cetuximab 5 mg/mL; rituximab 1 mg/mL. The antibody referred as mAb-C (reference and stressed) was donated by a customer; the original solution was diluted 10 times.

Liquid Chromatography (or more generically Separations)

Chromatography systems:1) Vanquish Flex UHPLC System equipped with low pressure mixing quaternary pump. 2) Thermo Scientific Vanquish UHPLC system equipped with high pressure mixing binary pump

Mobile Phases: **A**- 0.1/100 = TFA/water (v/v); **B**- 0.1/9/1 TFA/acetonitrile/water (v/v/v)

Flow rate= 0.3 mL/min

LightPipe™ DAD detector setting: wavelength: 280 nm, Data Acquisition Rate: 10Hz, Response Time: 0.4sec

Thermostatted Column Compartment: still air mode at temperature 70-120 °C; active pre-column heater set at same temperature as column compartment. Post-colum cooler at 50 °C

Column: MAbPac-RP 4 µm, 2.1 × 50 mm

Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2

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Results

Reversed Phase of intact antibodies

Five intact antibodies were eluted with a generic gradient 0→100 %B in 10 minutes at 80 °C Sharp peaks were obtained for all samples (Figure 1). Active mobile phase pre-column heating prevented extra band broadening effects related to thermal mismatch between column and in-coming mobile phase.

The generic gradient is suitable to identify and quantify degradation products in early stability studies without the need to optimize for each antibody. Figure 2 shows the overlay between a reference and a stressed sample. The main peak is broader for the stressed protein, and degradation products are detected in front of the main peak. By using the same gradient and directly comparing the results, the 12 minute assay allows a fast confirmation of antibody purity.
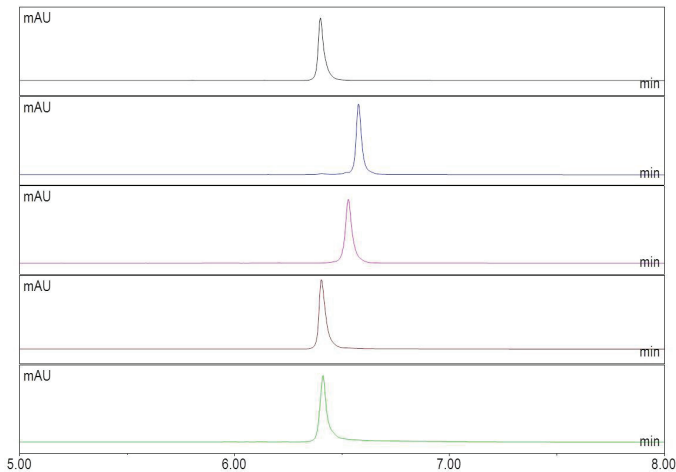
FIGURE 1. Elution of 5 intact mAb with generic gradient 0→ 100 %B in 10 minutes., at 80 °C From top to bottom panel: trastuzumab (0.2 µL), cetuximab (1 µL), mAb-C (0.25 µL), rituximab (1 µL), infliximab (1 µL).
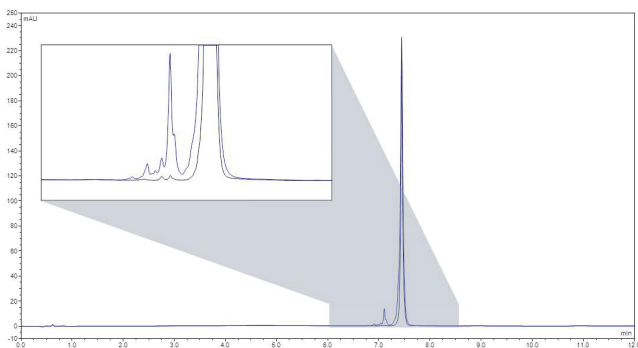

FIGURE 2. Comparison between the reference (black trace) and stressed (blue trace) antibody mAb-C. Gradient 0-→ 100 %B in 10 minutes, 80 °C. Degradation products elute in front of the main peak.

Influence of Column Temperature

High temperature column thermostatting is used for reversed phase of intact proteins. Normally temperature above 60 °C are the rule, with the range 70-90 °C as the preferred option.. One of the reasons temperatures above 90 °C are not common, is that the majority of silica-based columns lack the required thermal stability. In fact many columns are stable up to 60 °C. The other reason is to avoid the risk of thermally degrading the protein.

The exceptional thermal stability of the MabPac RP column, combined with the thermostatting capabilities of the Vanquish UHPLC system allowed to experiment with temperatures normally not accessible. Even though sharp peaks were obtained at 80 °C, in some cases we observed that higher temperatures were required to obtain satisfactory peak shape and recovery. Figure 3 for instance, shows the comparison between the same IgG sample eluted at 80 and 120 °C.
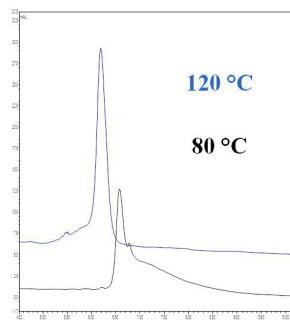

FIGURE 3. Injection of 1 µL rabbit IgG. At 80 °C the peak is affected by large tailing whereas at 120 °C the tailing has disappeared. Peak area was increased with temperature.

The chromatographic behavior of two antibodies eluted at different temperatures was studied in detail. Height and width of the main peak, and the sum area of main peak and impurities were evaluated. Peak width should decrease with temperature for pure peaks; increase of peak width at higher temperature indicates an increase of protein heterogeneity that may indicate that a degradation process is occurring. Peak area is an indicator of the recovery. Peak height is influenced both by degradation and recovery.

In Figure 4 and 5, these parameters recorded at 70,90 and 100 °C for Cetuximab and mAb-C are observed. The main peak shape is in all cases excellent, however small but important variations of peak parameters can be observed.. Firstly, the peak height, hence the sensitivity of the method, decreased with temperature for both antibodies.

Cetuximab shows a slight increase of peak width from 70 to 100 C. Recovery estimated by the sum area of all peaks improve from 70 to 90 °C, but drops at 100 °C. The behavior of mAb-C at different temperatures was more homogeneous, and the parameters relative variation less pronounced. Unlike cetuximab, peak width decrease from 70 to 90 °C. Ramping up of the width at 100 °C indicates likely degradation at this temperature.
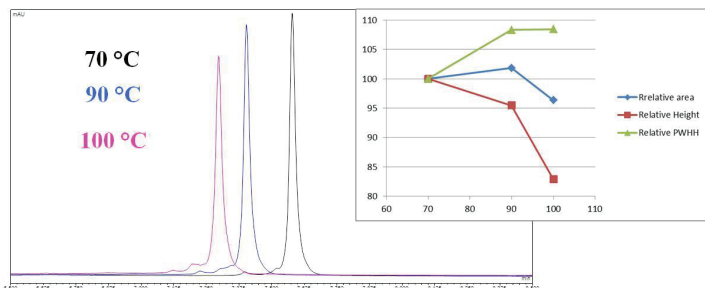

FIGURE 3. Injections of 0.25 µL cetuximab at different temperatures.

The graph shows some peak parameters of the chromatograms at different temperature: area (sum of main peak and impurities), height of the main peak, and Peak Width at Half Height of the main. Values are expressed as relative to those at 70 °C
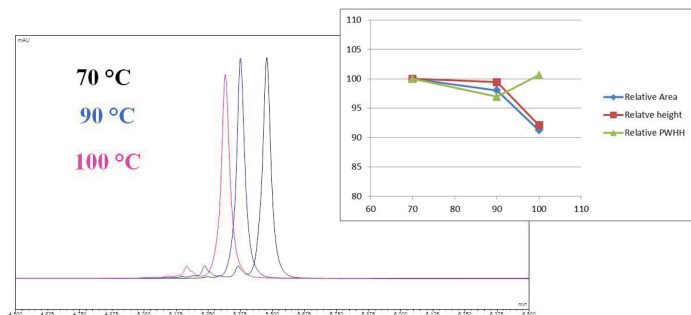
FIGURE 4. Injections of 0.25 µL mAb-C at different temperatures.

The graph shows some peak parameters of the chromatograms at different temperature: area (sum of main peak and impurities), height of the main peak, and Peak Width at Half Height of the main. Values are expressed as relative to those at 70 °C

High throughput separation of intact antibodies from degradation products can be obtained by running 5 minutes gradient (Figure 6). For high-throughput separation the Vanquish UHPLC system with binary high-pressure mixing pump is preferred, because of the low gradient delay volume (? µL)
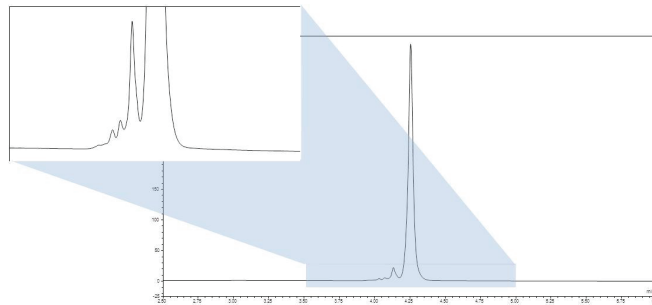

FIGURE 6. High-throughput separation of intact mAb-C and degradation products. Injection volume 0.25 µL. Gradient 0→ 100 %B in 5 minutes at 80 °C

Conclusion

- All tested mAb were eluted at 80 °C as sharp peaks with the same general purpose gradient

- Satisfactory peak shape for the rabbit IgG required column thermostatting at 120 °C

- Significant effects of temperature in the range 70-100 °C are sample-dependent

- The MabPac RP column with the Vanquish Flex system allows for rapid assessment of sample integrity in stability-indicating assays