Technical Note 173

Influence of Column Temperature on Reversed-Phase Chromatography of an Intact Antibody

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Key Words
Monoclonal Antibodies, MabPac RP Column, Biocompatible UHPLC, Biotherapeutics Characterization, Biopharma, Vanquish Flex UHPLC System

Goal
Optimize peak shape and recovery for reversed-phase analysis of an intact antibody by tuning the column temperature.

Introduction
Monoclonal antibodies (mAbs) are highly complex biomolecules with masses of approximately 150 kDa. MAbs are used as therapeutic proteins for the treatment of certain types of cancer and autoimmune diseases. Therapeutic antibodies must be characterized through the development process of the drug to ensure that the protein attributes meet the requirements.

Most analytical tools to characterize the mAb are based on liquid chromatography. Several liquid chromatographic methods target the intact molecule. Hydrophobic interaction (HIC), size exclusion, and ion exchange chromatography are methods of choice for intact antibody characterization. Thanks to the high chromatographic efficiency in comparison to HIC, reversed-phase chromatography is used as a complementary technique. Additionally, reversed-phase chromatography is often used when hyphenation to electrospray ionization mass spectrometry is required.

Reversed-phase chromatography of intact antibodies, and large proteins in general, usually requires high temperature in order to obtain peaks with satisfactory shape.[1] The high temperature ensures fast mass transfer of the large molecule and reduces the effects of secondary interactions with the stationary phase.[2] Additional problems often encountered in mAbs reversed-phase analysis are poor recovery and carryover effects.

The Thermo Scientific™ MAbPac™ RP column was designed for intact antibody characterization. The packing material consists of polymeric particles structured with ultra-wide-pores (1500 Å) to ensure the accessibility for very large proteins such as antibodies. The polymeric packing material has a significant benefit over silica because of the temperature stability, low carryover, and wider pH range stability.

In this work we show separation examples of an intact commercial antibody with a generic gradient. Chromatographic performance, as well as carryover, were evaluated for different column temperatures.

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Experimental

Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater, 0.1 × 380 mm, VH-C1 (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- Light Pipe, Flow Cell, 10 mm (P/N 6083.0100)

Default Thermo Scientific™ Viper™ capillary fittings were used for flow connections of the devices.

Chromatographic Conditions

Column	MAbPac RP Column (2.1 × 100 mm, P/N 088647)
Mobile Phase A	0.05:90:10 TFA/Water/Acetonitrile (v/v/v) (P/N TFA 85183)
Mobile Phase B	0.05:10:90 TFA/Water/Acetonitrile (v/v/v) (P/N Acetonitrile TS-51101)
Gradient	0–30 min: 0–70% B; 30–31 min: 70–100% B; 31–45 min: 100% B; 45–46 min: 100–0% B; 46–55 min: 0% B
Flow Rate	300 µL/min
Temperature	50–110 °C (Forced Air)
Maximal Pressure	186 bar (2697 psi) at 50 °C
Sample	Avastin® (bevacizumab) (0.5 µg/µL)
Injection Volume	1 µL
Detection	214 nm
	Data Collection Rate: 10 Hz
	Response Time: 0.4 s
	Slit Width: 4 nm slit

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2 SR3, was used for data analysis.

Results and Discussion

Elution of bevacizumab in the MAbPAc-RP column was carried out at six column temperatures between 50 and 110 °C. Peak shapes and areas at different temperatures were compared. The chromatograms of Figure 1 show that retention decreases with temperature. Excellent peak shape is found at 70 and 80 °C. In order to maintain the thermal balance between the mobile phase and the column, and to avoid undesired band broadening and retention effects caused by thermal mismatch, the use of a mobile phase pre-heater is extremely important. The requirements of the pre-heating device are fast eluent thermostatting and low internal volume to minimize extra-column dispersion. Both of these characteristics are met by the active pre-column mobile phase heater of the Vanquish Flex UHPLC.

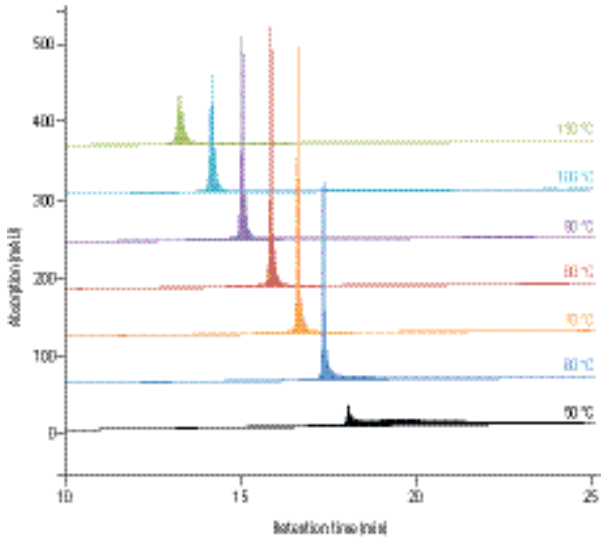

Figure 1. Overlay of intact bevacizumab injections at different temperatures.

Table 1. Results of integration of sample and blank injections at different temperatures.

Temperature [°C]	Retention Time [min]	Peak Area [mAU × min]	Peak Width at Half Height [s]	Blank Area [mAU × min]	Carryover [%]
50	18.02	20.75	4.86	0.139	0.66
60	17.76	23.51	2.64	0.145	0.61
70	17.48	24.23	2.58	0.092	0.37
80	17.16	23.58	2.70	0.076	0.32
90	16.79	21.55	2.94	0.067	0.31
100	16.38	17.66	3.84	–	–
110	15.90	11.41	9.42	–	–

Results of peak analysis for the run at different temperatures are collected in Table 1. Peak area increases when temperature increases from 50 to 70 °C. This observation can be interpreted as improved protein recovery. When temperature is raised further, the area decreases. This may be ascribed to degradation of proteins triggered by high temperature.

Carryover was estimated from the UV trace of a run without injection (blank run), recorded after two consecutive sample runs. The peak of the blank run was integrated and the area relative to the sample injection peak calculated. Carryover was low at all tested temperatures, and the values decreased with temperature as expected, with no carryover detected at the highest temperatures. At 70 °C, the estimated carryover was 0.37%.

Conclusion

This work provides an example of temperature optimization in reversed-phase separations of intact antibodies. In the case of bevacizumab, the optimum conditions in terms of peak shape and recovery were found at 70 °C. This temperature should be interpreted as a compromise between the best chromatographic conditions and preservation of sample integrity. This optimal condition is nonetheless sample dependent, and temperature optimization is recommended for the development of a reversed-phase method for any new intact protein. Although the carryover reported here at 70 and 80 °C is considered more than acceptable for most analytical requirements, it should be considered that this is also sample specific; its dependence to column temperature should be assessed whenever deemed important.

References

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