Utilizing hydrophobic interaction chromatography to detect product-related impurities in a monoclonal antibody

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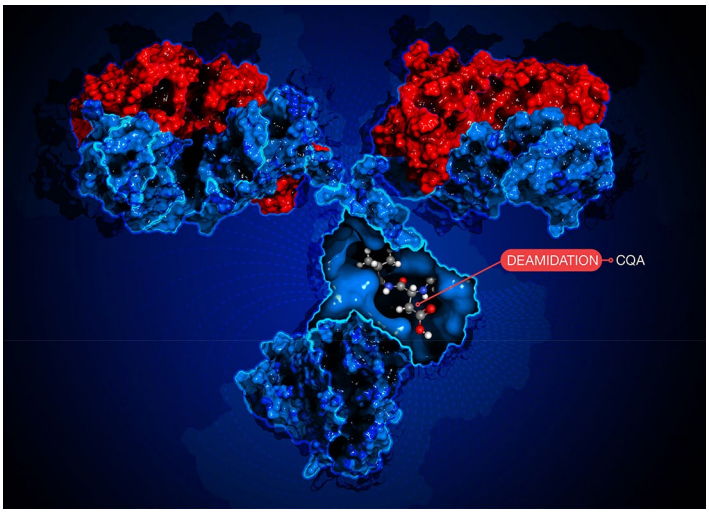

Goal

Showcase the ability of the Thermo Scientific™ MAbPac™ HIC-20 column for oxidized and deamidated impurities separation. The column is also capable of separating fragments of mAb that are generated post papain digestion.

Introduction

Pertuzumab is a recombinant humanized monoclonal antibody that targets the extracellular dimerization domain (Subdomain II) of the human epidermal growth factor receptor 2 protein (HER2). It consists of two heavy chains and two light chains that have 448 and 214 residues, respectively.

Recombinant mAbs can be highly heterogeneous due to various biochemical modifications, such as sialylation, oxidation, deamidation, and C-terminal lysine truncation. Some of these modifications may reduce the stability and efficacy of the drug. Therefore, it is critical to detect, characterize, and quantify impurities, as well as biochemical variants, during production of mAb therapeutics.

Among various HPLC methods, hydrophobic interaction chromatography (HIC) often provides high-resolution separation of FAb and Fc fragments, and is generally used for mAb variant analysis. HIC separates proteins in order of increasing hydrophobicity under non-denaturing conditions. Analytes bind to the weakly hydrophobic stationary phase in the presence of high salt concentration and elute off the column as the salt concentration decreases.

Unlike reversed-phase liquid chromatography that denatures the protein and effectively destroys the native conformation, HIC preserves the native structure and bioactivity of the protein, which is useful for downstream functional analysis, such as binding and cell-based potency assays. It also allows for the separation based on conformational changes occurring in the native form. In addition, HIC typically provides separation with little or no carryover. Therefore, HIC is not only useful for separation of mAb variants but is also valuable as a purification method for mAb products by biopharmaceutical companies.

In this study, a pertuzumab sample was analyzed using the MAbPac HIC-20 column. The pertuzumab sample was degraded using a condition that is known to generate oxidation and deamidation in protein. Molecules after post degradation treatment are analyzed using the same chromatographic condition used for control sample analysis.

To further separate iso-forms and impurities that are difficult to separate at the intact level, molecules were fragmented into FAb and Fc using papain digestion and were analyzed under the same chromatographic conditions. This study also gives primary information on positions of modification. Papain digested sample was oxidized with peroxide to further localize the position of oxidation in the entire structure of the mAb. Both sample types were analyzed with same chromatographic condition.

Experimental

Recommended consumables

- UHPLC-MS grade water, Thermo Scientific™ (P/N W8-1)
- Deionized water, 18.2 MΩ·cm resistivity
- MAbPac HIC-20 column (P/N 088554)
- Convenience kit, 2 mL clear screw glass vial with AVCS technology (P/N C5000-95W)
- Hydrogen peroxide, 30% w/v, Qualigens (P/N 15465)
- Sodium dihydrogen orthophosphate, Qualigens (P/N 14115)
- Ammonium sulfate (P/N 15915)
- Papain, Sigma-Aldrich (P/N P4762-1G)
- Tris HCl, Sigma-Aldrich (P/N 1185-53-1)
- EDTA, disodium salt, Sigma-Aldrich (P/N 6381-92-6

Sample pretreatment

Control sample preparation

The pertuzumab sample was diluted to 2.0 mg/mL using deionized water and then further diluted to 1.0 mg/mL using mobile phase A.

Preparation of post degraded sample

Condition 1 - Oxidized sample preparation

Sample was diluted to 2 mg/mL in lab grade water. 100 µL of diluted sample mixed with 10 µL of H_2O_2 and sample was incubated in the dark at room temperature for 3.5 hrs. After incubation sample was diluted further to 1:1 in mobile phase A and injected.

Condition 2 - Deamidated sample preparation

The sample was diluted to 2 mg/mL with 1% ammonium bicarbonate solution and incubated at 37 °C for different time intervals. After incubation the sample was further diluted to 1:1 with mobile phase A and injected into the chromatographic system.

Preparation of sample for fragment analysis

Papain digested sample

A system was prepared containing 6.7 µL of sample, 50 µL of digestion buffer (refer below for preparation), 10 µL of papain enzyme (refer below for preparation), and 33.4 µL of water. The sample system was then incubated at 37 °C for 7 hours.

Oxidized sample

50 µL of control papain digested sample was aliquoted and mixed with 2 µL of H_2O_2 (30% H_2O_2). The sample was incubated for 20 minutes.

Papain digestion buffer preparation

2.43 g of Tris HCL and 0.3 g of EDTA buffer salt were dissolved in 100 mL water and the pH was adjusted to 7.6 using HCl.

Papain preparation

2 mg/mL papain was prepared by weighing 2 mg of papain and dissolving in 1 mL of water.

Compound, standard

- Pertuzumab

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with:

- Thermo Scientific™ Dionex™ SRD-3600 solvent racks with degasser (P/N 5035.9230)
- Thermo Scientific™ Dionex™ UltiMate™ DGP-3600RS rapid separation pump (P/N 5040.0066)
- Thermo Scientific™ Dionex™ UltiMate™ WPS-3000TRS rapid separation thermostatted autosampler (P/N 5841.0020)
- Thermo Scientific™ Dionex™ UltiMate™ TCC-3000RS rapid separation thermostatted column compartment (P/N 5730.0000)
- Thermo Scientific™ Dionex™ UltiMate™ DAD-3000RS rapid separation diode array detector (P/N 5082.0020)

Separation conditions

Table 1. Separation details

Parameter	Value
Column	MAbPac HIC-20, 4.6 × 250 mm, 5 µm (P/N 088554)
Mobile phase A	2 M ammonium sulfate and 100 mM sodium phosphate pH 7.0
Mobile phase B	100 mM sodium phosphate pH 7.0
LC gradient conditions	Time (min) / A% / B%: 0.0 / 63.0 / 37.0; 45.0 / 0.0 / 100.0; 50.0 / 0.0 / 100.0; 52.0 / 63.0 / 37.0; 72.0 / 63.0 / 37.0
Flow rate	0.5 mL/min
Column temperature	30 °C
Injection details	25 µL for pertuzumab sample
Injection wash solvent	50% acetonitrile in water
Wavelength	214 nm

Data processing

The Thermo Scientific™ Chromeleon™ 7.0 Chromatography Data System (CDS), version 7.2.2.6394 was used for data acquisition and analysis.

Result and discussion

The analysis and separation of pertuzumab with the MAbPac HIC-20 column shows seven peaks for pertuzumab (Figure 1). The chromatographic profile for post degraded pertuzumab sample with condition 2 shows an increase in the % area for pre-peaks, and corresponding decrease in main peak area when compared to the control sample (Figure 3 and Figure 4, zoomed). The post degradation change in % area of pre-peaks and main peaks increases correspondingly with the increase in time of incubation. The similarities in retention time of pre-peaks and main peaks in the degraded and control samples indicates that pre-peaks in the control sample are likely to be deamidated impurities, which are well separated from the main peak. The selected degradation conditions are known to generate deamidation in molecules.

The chromatographic profile for the post degraded pertuzumab sample in condition 1 also shows that the major peak retention time shifts lower compared to the principal peak of the pertuzumab control sample (Figure 2). Peroxide treatment in mAbs is likely to generate oxidation in molecules. The oxidized impurities generated in the pertuzumab sample were not well resolved from the main peak of the control sample. This could be due to lower hydrophobicity difference between oxidized and main form of the protein.

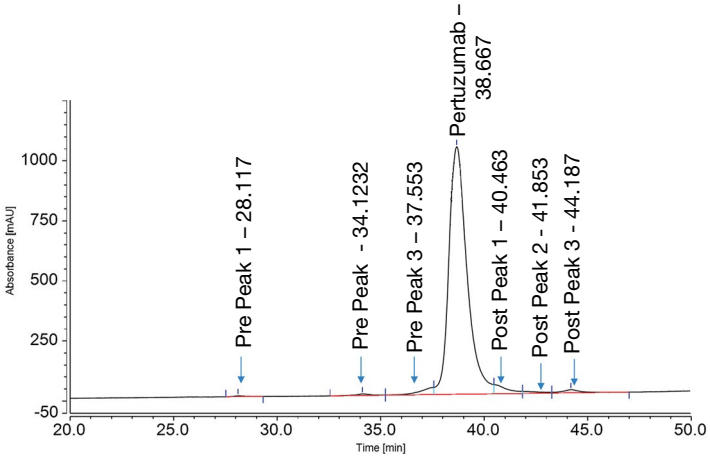

Figure 1. Chromatogram of the pertuzumab control sample

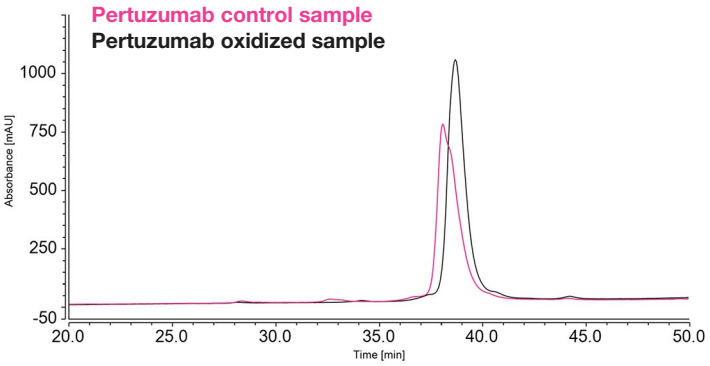

Figure 2. Overlay of oxidized and control samples of pertuzumab

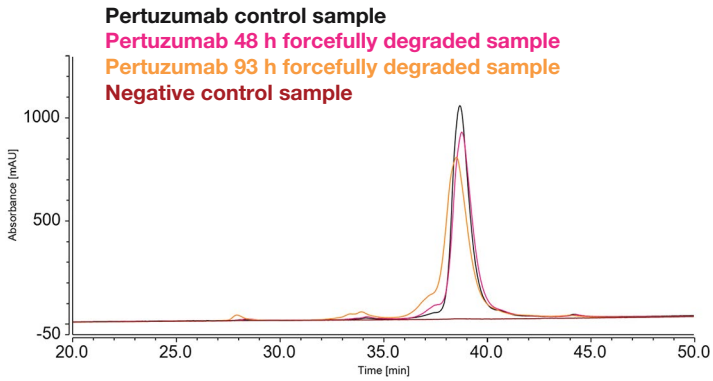

Figure 3. Overlay of control, placebo, and deamidated samples of pertuzumab

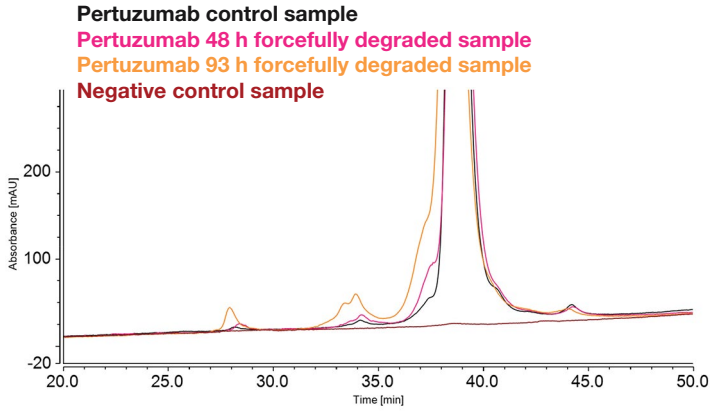

Figure 4. Zoom overlay of control, placebo, and deamidated samples of pertuzumab

A sample of pertuzumab was digested with papain enzyme to generate FAb and Fc fragments. Separated on the MAbPac HIC-20 column, the papain digested sample showed both FAb and Fc fragments are well resolved (Figure 5). Comparing the retention time for the main peak of the control and papain digested sample indicates that there was complete digestion of the pertuzumab sample under these conditions. Peak assignment was based on peak area comparisons showing two FAb and one Fc fragment. Confirmation of the peak assignments was carried out using the MAbPac Protein A column (data not shown here) that retains Fc portion but does not bind FAb fragments. After papain digestion, minor variant peaks in front of both FAb and Fc fragments were observed.

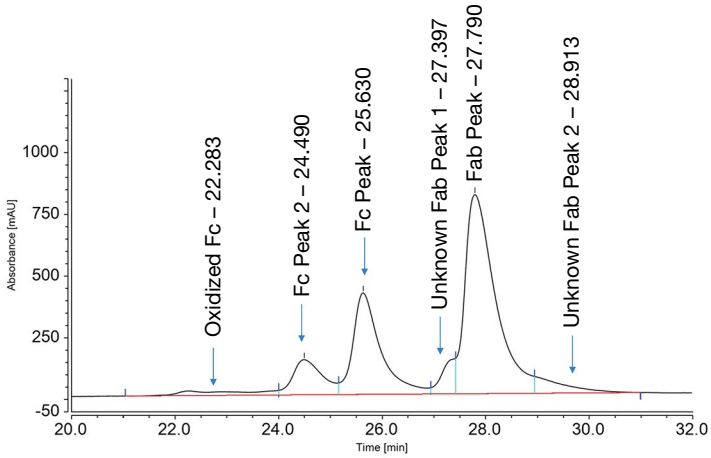

Figure 5. Chromatogram of papain digested pertuzumab sample

Comparison of the chromatographic profiles for the peroxide treated papain digested sample and the control papain digested sample indicates that the Fc peak 1 and 2 retention time shifts after peroxide treatment. The peaks elute as a single major Fc peak and have a lower retention time compared to the Fc peak in the control papain digested sample (Figure 6). There was no detectable change for retention of FAb related peaks. This suggests that the site for oxidation is likely to be present in Fc portion and not with FAb portion of the mAb.

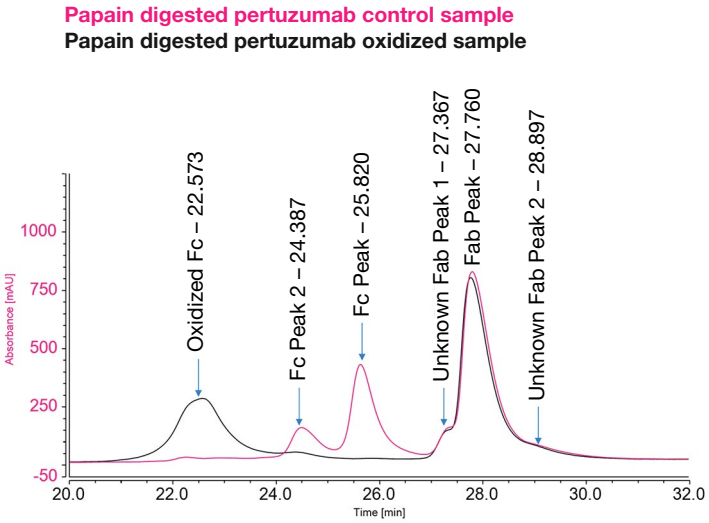

Figure 6. Chromatogram of papain digested control and papain digested oxidized sample

Conclusion

In this study, we have demonstrated an orthogonal approach to ion exchange methods for separation of deamidated impurities at intact level for monoclonal antibodies. Hydrophobic interaction chromatography is a valuable approach when modifications do not produce a charged variant, or for uncovering protein folding variations. The method also can separate FAb and Fc fragment of mAbs that are generated post papain digestion. Fragments of oxidized mAbs impurities separated from the sample and provided primary information on the localization of the impurities in this 150 kDa large protein.

References

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