Biopharmaceuticals

Performance evaluation of a new reversed-phase column for peptide mapping and monitoring of monoclonal antibodies

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Abstract

Purpose: Demonstrating the benefits of the newly introduced Thermo Scientific™ Hypersil GOLD™ Peptide C18 column with 1.9 µm particle and 175 Å average pore size, in the dimension of 150 x 2.1 mm (L x ID) for the analysis of tryptic mAb digest samples.

Methods: UHPLC-MS/MS peptide mapping and UHPLC-UV-MS peptide monitoring.

Results: The Hypersil GOLD Peptide column enables to uniquely meet the demands of peptide level analysis where consistent lot-to-lot and column-to-column performance is required.

Introduction

Peptide mapping analysis is routinely used in biotherapeutic characterization for the confirmation of the primary sequence and to investigate the presence and relative levels of post-translational modifications (PTMs) such as deamidation, oxidation, glycosylation, and more. For this purpose, high-resolution accurate mass (HRAM) mass spectrometry (MS) and MS/MS analysis are applied to localize and quantify these PTMs on the peptide level. Once characterized, monitoring can be transferred to a comprehensive MS1-only approach. Peptide mapping and peptide monitoring experiments are performed first by reduction and alkylation of the disulfide bonds followed by enzymatic digestion using proteases. The most commonly applied protease for monoclonal antibody (mAb) peptide mapping and monitoring is trypsin, which cleaves specifically at the C-terminal of arginine (R) and lysine (K) residues.[1] Peptides are then separated by reversed-phase (RP) liquid chromatography, most commonly applying a C18 column, coupled to ultraviolet detection (UV) and mass spectrometry.

In the presented studies, a newly introduced RP column, the Thermo Scientific™ Hypersil GOLD™ Peptide column, was evaluated in terms of peak shape, peak area, chromatographic resolution, and retention of hydrophobic as well as hydrophilic peptides in the analysis of the monoclonal antibodies NISTmAb (RM 8671), rituximab, and adalimumab digests. Column conditioning is a critical step in achieving consistent and reliable performance. Thus, prior to peptide mapping analysis, column conditioning was performed by multiple injections of tryptic BSA digest (n =6). BSA is used as a robust and well-established system performance evaluation test (SET) to assess the performance of the chromatography system and the mass spectrometer. NISTmAb digests were injected at the start and the end of the sequence as a system suitability test (SST) while six technical replicates of rituximab digest were investigated.[2] In addition, digested adalimumab sample was analyzed to evaluate the column-to-column and lot-to-lot reproducibility.[3]

Materials and methods

Sample Preparation

The following samples have been prepared :

1. SET sample: BSA tryptic digest
2. SST sample: NISTmAb RM 8671 (reference material), tryptic digest
3. Peptide mapping sample: rituximab tryptic digest
4. Peptide monitoring sample: adalimumab tryptic digest

Test Method(s)

UHPLC-MS/MS peptide mapping analysis with Hypersil GOLD peptide column

Solvent A / solvent B: water / acetonitrile + 0.1% formic acid, step gradient: 1% to 10% B in 1 min, 10% to 35% B in 64 min, 35% to 90% B in 2 min, total run time 115 min.

UHPLC-UV-MS peptide monitoring analysis with Hypersil GOLD peptide column
Solvent A / solvent B: water / acetonitrile + 0.1% formic, acid step gradient: 2% to 9% B in 0.5 min, 9% to 35% B in 21 min, 35% to 90% B in 1 min, total run time 45 min.

Data Analysis

Thermo Scientific™ BioPharma Finder™ software, version 5.1 and Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3.2.

For further details refer to Reference 2 and Reference 3.

Results

System performance evaluation (SET) and system suitability test (SST) for peptide mapping analysis

Tryptic BSA digests were injected six times prior to peptide mapping analysis to evaluate the performance of the chromatography system and the mass spectrometer (Figure 1a and 1b).

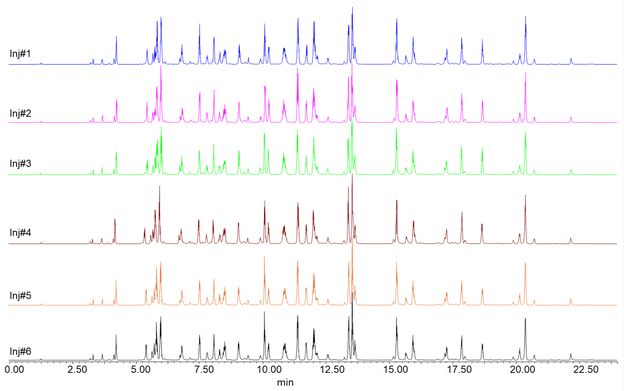

Figure 1a. Stacked total ion chromatograms (TICs) obtained for six replicate injections of BSA tryptic digest on the Hypersil GOLD peptide column, separated over a 24 minute gradient.

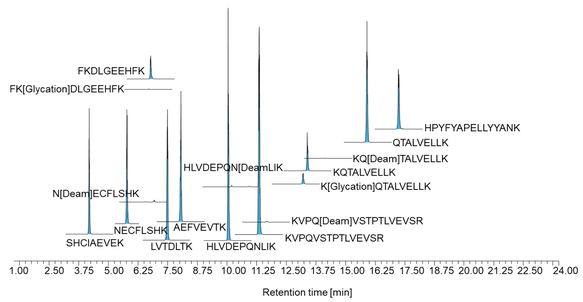

Figure 1b. Extracted ion chromatograms (XICs) of monitored peptides from BSA tryptic digest.

Retention time precision was determined for both the SET runs (24-min gradient) and SST runs (115 min gradient) with %RSD values between 0.04 and 0.63% and thus easily meet the criteria of ≤ 1%.
Furthermore, a peak area precision of ≤ 10% was observed, which is also below the established limit.

Assessment of chromatographic resolution

Chromatographic resolution was evaluated as this parameter helps to assess the separation capability of the column according to the Ph. Eur formula.[4] A specific focus was placed on the ability to separate the various deamidated forms of the PENNYK peptide in NISTmAb reference standard as it is considered a well-known deamidation hot spot of the mAb Fc region. Results showed that the Hypersil GOLD Peptide column provided good resolution, with values between 1.4 and 1.9 for peak 2 and between 2.0 and 4.3 for peak 3 (see Figure 2).

Performance of the new Hypersil GOLD peptide column

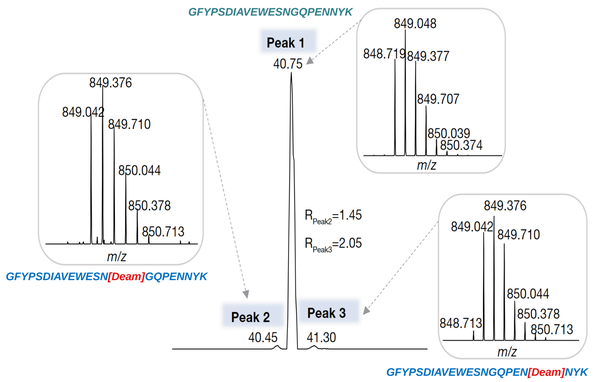

Figure 2. Extracted ion chromatogram for the PENNYK peptide located in the Fc region, representing the sum of all detected charge states with 5 ppm mass tolerance. RT for all identified peaks are indicated. Inserts represent the isotope pattern with mass labels of the non-deamidated (peak 1) and deamidated isoforms (peaks 2 and 3).

In addition, the Hypersil GOLD peptide column shows good chromatographic resolution for early eluting hydrophilic peaks and in general good distribution of the peptides along the full chromatographic gradient.

Evaluation of chromatographic performance for the rituximab peptide mapping analysis

Chromatographic performance was evaluated on Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Thermo Scientific™ Orbitrap™ Exploris™ 240 MS with Hypersil GOLD peptide column (150 x 2.1 mm, 1.9 µm, 175 Å).

Table 1. Peak area and retention time (RT) reproducibility and peak asymmetry results (n=6 columns). Peptides are listed in ascending order of retention time ranging from 1.64 min to 64.48 min.

Peptides	%CV (Area)	%CV (RT)	Max RT shift (min)	Asymmetry
VSNK	4.71	0.53	0.02	1.01
ADYEK	2.12	0.60	0.12	0.03
SLSLSPG[Lys]	1.45	0.07	0.03	0.92
QVQLQQPGAELVKPGASVK	4.48	0.03	0.01	1.05
FNWYVDGVEVHNAK	5.29	0.06	0.04	1.76
GFYPSDIAVEWESNGQPENNYK	3.52	0.03	0.03	1.22
GLEWIGAIYPGNGDTSYNQK	5.26	0.02	0.02	1.53
VVSVLTVLHQDWLNGK	3.37	0.04	0.05	1.54
DYFPEPVTV_NVNHKPSNTK	9.30	0.02	0.05	1.24

Column lot-to-lot and column-to-column reproducibility for adalimumab peptide monitoring analysis

Chromatographic performance was evaluated with a Hypersil GOLD peptide column (150 x 2.1 mm, 1.9 µm, 175 Å) placed in a Thermo Scientific™ Vanquish™ Flex UHPLC system coupled to a Thermo Scientific™ ISQ™ EM single quadrupole MS.

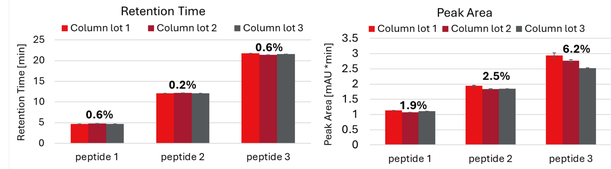

Figure 3. Column lot-to-lot reproducibility for retention time, and absolute peak area with error bars indicating the standard deviation for five consecutive injections for each column of each lot. The numbers above the bars represent %RSD values obtained when comparing the three column lots.

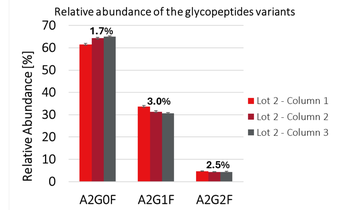

Figure 4. Column-to-column comparison for three columns each from same lot with error bars indicating the standard deviation for five consecutive injections on each column. The numbers above the bars represent % RSD values obtained when comparing the three columns from the same lot.

Conclusions

The two studies of peptide mapping and peptide monitoring demonstrate the benefits of the Hypersil GOLD Peptide column for peptide level biopharma analysis based on a comprehensive dataset obtained from rituximab and adalimumab digest samples.

- SET and SST criteria of retention time and peak area precision are easily met when using the Hypersil GOLD Peptide column.
- The Hypersil GOLD Peptide column provides
  - peaks with good asymmetry values, strong retention time and peak area reproducibility.
  - retains and separates hydrophilic peptides and has very good chromatographic resolution values for deamidated species in the PENNYK region.
  - excellent column lot-to-lot reproducibility with % RSD RT ≤ 0.6, and % RSD peak area ≤ 6.2 for peptides across the entire elution window.
  - consistent column-to-column results for the quantification of glycopeptides with % RSD of relative abundance ≤ 3.0.

References

1. Millán-Martín, S., et al. Comprehensive multi-attribute method workflow for biotherapeutic characterization and current good manufacturing practices testing. Nat. Protoc. **2023**, 18(4), 1056–1089.
2. Thermo Fisher Scientific Application Note 002735, Performance comparison of reversed-phase C18 columns for peptide mapping of monoclonal antibodies, **2024**.
3. Thermo Fisher Scientific Application Note 002776, Hypersil GOLD Peptide column for reliable monitoring of glycopeptide variants in monoclonal antibodies by LC-UV-MS analysis, **2024**.
4. EDQM, Ph. Eur. Commission adopts harmonised general chapter 2.2.46. Chromatographic separation techniques.

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