Applying a UHPLC HRAM MS-MS/MS method to assess host cell protein clearance during the purification process development of therapeutic mAbs

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Abstract

Purpose: Developing a platform HRAM HPLC MS-MS/MS method for monitoring of HCPs across multiple downstream purification steps to support bioprocess development and optimization.

Methods: Two trastuzumab samples collected from Protein A and polish purification pools of the in-house trastuzumab product, respectively were used for the case study. A commercially available NISTmAb sample was used for method development and performance evaluation. The mAb samples were digested with trypsin under non-denaturing conditions and the generated digest mixtures were separated using a 25 cm long C18 column at 300 µL/min flow rate over a 90 min linear gradient. For data detection, a data dependent MS/MS approach was used, in which a HRAM full MS scan was followed by top 15 data dependent HRAM MS/MS scans on a Thermo Scientific™ Orbitrap™ Ascend BioPharma Tribrid™ mass spectrometer. Thermo Scientific™ Proteome Discoverer™ 3.1 software was used for data processing.

Results: The developed HRAM HPLC MS-MS/MS method was successfully applied for the monitoring of the HCPs from the two trastuzumab samples (385 HCPs from the Protein A purification pool and 91 HCPs from the polish purification pool). From the NISTmAb sample, 235 HCPs were identified over a wide dynamic range. Very low abundant HCPs (down to 0.007 ppm[1]) were detected from the NISTmAb sample.

Introduction

Host cell protein (HCP) is one of the process-related impurities that needs to be well characterized and controlled throughout biomanufacturing processes to assure the quality, safety and efficacy of the monoclonal antibodies (mAbs). Although ELISA remains the gold standard method for quantification of total HCPs, it lacks the specificity and coverage to identify and quantify individual HCPs. As a complementary method to the ELISA, HPLC MS-MS/MS method has emerged as a powerful tool to identify and profile large amounts of HCPs during the downstream purification process. In this study, we developed a platform HPLC MS-MS/MS method for HCP analysis and applied it to the in-house trastuzumab samples to assess HCPs clearance using the newly introduced Thermo Scientific™ POROS™ Caprylate Mixed-Mode Cation Exchange Chromatography Resin (POROS Caprylate mixed mode resin) for polishing purification.

Materials and methods

Sample preparation

Two in-house trastuzumab samples (one was from the pool of Protein A affinity chromatography; another was from the pool of POROS Caprylate mixed mode resin chromatography) were used for the case study. A commercially available NISTmAb reference was used for the method development and the method performance evaluation for HCP analysis.

All the mAb samples were digested with trypsin under non-denaturing conditions following the protocol published by Huang et al.[2] Briefly, about 1 mg of each mAb sample was buffer exchanged to 50 mM Tris-HCl using 3k Amicon ™ Ultra-0.5 Centrifugal Filter Unit (≈ 85 µL) and digested at 37 ºC for two hours using a solution of Trypsin enzyme at a 1:800 enzyme/protein ratio. The digest mixture was reduced with 2 µL of 0.5M TCEP for 10 min at 95 ºC . The supernatant was acidified with 2 µL of 10%FA H_2O and used for the HPLC MS-MS/MS analysis.

HPLC conditions

For all experiments, chromatographic separations were carried out using a Thermo Scientific™ Acclaim™ VANQUISH™ C18 column (2.1 x 250 mm, 2.2 µm) on the Thermo Scientific™ Vanquish™ Flex UHPLC system. The solvent A was water with 0.1% formic acid and the solvent B was acetonitrile with 0.1% formic acid. The column temperature was set to 60 ºC. The flow rate was 300 µL/min. The gradient condition used is listed in Table 1.

Table 1. HPLC gradient condition

Time (min)	%B
0.0	3
1.0	3
90.0	35
95.0	85
100.0	85
105.0	3
110.0	85
115.0	85
115.1	3
135.0	3

Table 2. ESI and MS settings

MS source setting	Value
Sheath gas	40
Auxiliary gas	10
Spray voltage (v)	3400
Capillary Temp. (°C)	320
Vaporizer Temp. (°C)	250
General	
Application mode	Peptide
Pressure mode	Standard
RF lens (%)	40
Full MS	
Scan rage (m/z)	350-1200
Resolution	12,000 at 200 m/z
AGC target value (%)	300
Max inject time (ms)	50
dd MS/MS (top 15)	
Resolution	30,000 at 200 m/z
Isolation window (m/z)	2
AGC target value (%)	100
Max inject time (ms)	150
HCD collision energy (v)	28

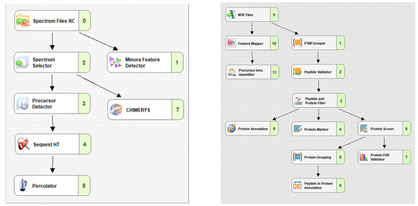
Mass spectrometer

The Orbitrap Ascend BioPharma Tribrid mass spectrometer was used for data collection. A data dependent MS/MS method was used. For data acquisition, a full MS scan was collected with very high resolving power (120k at 200 *m/z*), followed by 15 MS/MS scans of the most intense precursor ions with high resolving power (30k at 200 *m/z*). The detail instrument parameter settings for the ESI and the mass spectrometer are shown in Table 2.

Data processing

Thermo Scientific Proteome Discoverer 3.1 software was used for HCP characterization and relative quantification. For improving the HCP identification coverage, two search algorithms (Sequest™ HT and CHIMERYS™ (MSAID GmbH)) were used. A database containing all Mus musculus entries (17,728 entries, TaxID = 10090, 2024/03/11) extracted from UniProtKB/Swiss-Prot was searched for NISTmAb sample and a database containing all *Cricetulus griseus* entries (89,053 entries, Tax ID = 10029, 2024/03/15) extracted from UniProtKB/TrEMBL was searched for tratuzumab samples. Methionine oxidation and N-term modification of Met-loss and Acetylation were set as variable modifications. Mass tolerance was set to 5 ppm for precursor ion masses and 0.02 Da for fragment masses. Inferys_3.0.0_fragmentation prediction mode was used for CHIMERYS. The false discovery rate (FDR) was calculated using the Percolator node set to 1% at PSM, peptide and protein levels. Figure 1 shows the software workflow notes settings for data processing.

Figure 1. Proteome Discoverer 3.1 workflow nodes



Results

HPLC MS-MS/MS method development and evaluation using NISTmAb

The NISTmAb digest mixture was used for the optimization of the Orbitrap Ascend Tribrid mass spectrometer parameter set ups. The parameters evaluated include Vaporizer temperature for ESI, full scam mass range, numbers of top precursor ions for triggering MS/MS scans and maximum ion injection time for the MS/MS data acquisition (data not shown). The optimized parameter set ups are shown in the Table 2. For optimizing the database search workflow, we tried to use a single search algorithm (Sequest HT) approach and two search algorithms (Sequest HT & CHIMERYS) approach. For evaluating the performance of the established HPLC MS MS/MS method for HCP analysis, 15 µL of the NISTmAb digest mixture was loaded on column and analyzed in triplicate. Figure 2 shows the total ion chromatograms of the triplicate LC MS-MS/MS runs. Good separation efficiency and retention time reproducibility were observed. Multiple filters were applied to the Proteome Discoverer 3.1 software search results and only the proteins which are 1) representative unique protein in each protein group; 2) 1% false discovery rate FDR at protein level; 3) at least two unique peptides identified were identified as positive HCPs. On the total, 191 HCPs was identified using the single Sequest HT search algorithm for database search. The total HCP id numbers increased to 235 by using both Sequest HT and CHIMERY for database search (Figure 3).

Figure 2. TIC of triplicate LC-MS runs of NISTmAb digest mixture

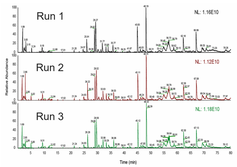
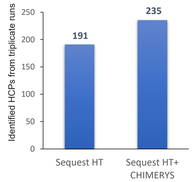
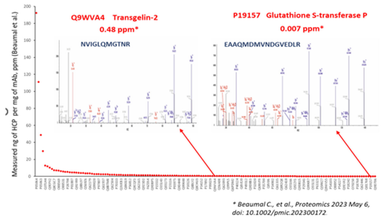


Figure 3. HCP id number comparison using one and two search engines



To evaluate if the developed HPLC MS-MS/MS method can detect low abundant HCPs, we used the reported NIST mAb HCP concentrations[1] as a reference. Figure 4 shows the representative identified HCPs which matched the HCPs with quantification results (measured ng of HCP per mg of mAb, ppm) shown in the reference paper. Most matched HCPs had less than 1 ppm concentrations. Great quality of MS/MS data were observed event down to 7 ppb low concentration level, allowing confident HCP identification.

Figure 4. Identified HCPs with the concentration information shown in the reference. Most of them (>60%) were less than 1 ppm.



Applying the developed HPLC MS-MS/MS method for HCP monitoring across the mAb purification processes

Two in-house trastuzumab samples. were analyzed in triplicate, respectively using the developed HPLC MS-MS/MS method. Figure 5 shows the detail information of the samples. Sample 1 was collected from the Protein A chromatography pool. Sample 2 was collected from the Polish chromatography pool. The POROS Caprylate mixed mode resin used for the polish purification has been developed to remove high aggregate efficiently from the targeted mAbs.[3] The object of this case study is to evaluate if The POROS Caprylate mixed mode resin can also remove the HCPs efficiently by monitoring the individual HCP clearance during the polish purification process. Roughly 1 mg of each trastuzumab sample was used for the trypsin digestion and 15 µL of the digested mixtures was injected per LC MS-MS/MS run. Figure 6 shows the total HCPs identification number comparison between the two samples. The subset of "high risk" HCPs[4] and "difficult to remove HCPs"[5] identification number comparisons are shown in Figures 7 and 8, respectively. The POROS Caprylate mixed mode resin was able to clear most HCPs from the trastuzumab product efficiently.

Figure 5. Information of the Trastuzumab samples

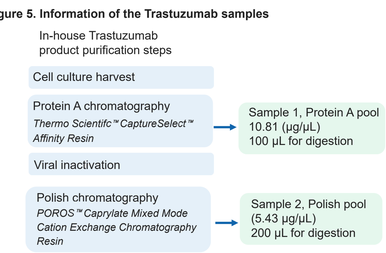


Figure 6. The total HCP id numbers comparison between the two samples

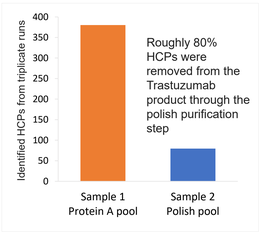
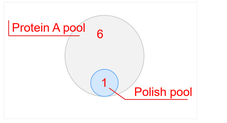


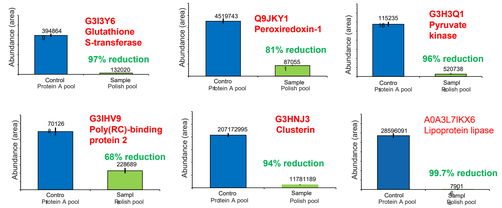
Figure 7. The numbers of identified "high risk" HCPs from the two samples

Figure 8. The numbers of identified "difficult to remove" HCPs from the two samples



For the HCP relative quantification, the average of top 3 peptide abundances (peak areas) were used without normalization. The data from the triplicate runs/sample were grouped and defined as the "control group" for the Protein A pool sample and the "sample group" for the Polish pool sample. The abundance ratios of identified HCPs were calculated based on "grouped abundances of sample group/grouped abundances of control group." Excellent quantitative repeatability was observed. 80% of the identified HCPs showed ≤15% CVs (n=3) in at least one of the sample groups. Figure 9 shows the HCP abundance change trends for the representative "high risk" and "difficult remove" HCPs remained in the Polish pool sample.

Figure 9. "High risk/difficult to remove" HCP abundance changes after the polish purification



Conclusions

- A platform HRAM HPLC MS-MS/MS method which uses a UHPLC system for peptide mixture separation was developed for identifying and monitoring HCPs from the therapeutic protein products in different purification process steps, including the final therapeutic protein products.
- The HPLC MS-MS/MS method was approved to offer sufficient HCP identification coverage and excellent sensitivity for detecting sub-ppm HCPs. When applying the method to the well studied NISTmAb sample, 235 HCPs were identified over a wide concentration ranges, down to 7 ppb.
- The HPLC MS-MS/MS method was successfully applied to monitor the HCPs from the in-house Trasturzumab product during the polish purification step.
- The POROS Caprylate Mixed Mode Cation Exchange Chromatography Resin used for the polish purification was approved to be able to remove the HCPs very efficiently. Roughly 80% HCPs including most "high risk" HCPs and "difficult to remove" HCPs were completed removed from the Trasturzumab product after the polish purification. Of the HCPs that remained in the POROS Caprylate purified sample, most showed significant decrease in abundances.

References

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