Application Note

Biotherapeutics and Biosimilars

Ultrasensitive Quantification of Monoclonal Antibody

Using nano LC and the Agilent 6495D triple quadrupole LC/TQ system

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Abstract

This application note describes a method for ultrasensitive quantification of monoclonal antibodies (mAbs), demonstrated with trastuzumab. Surrogate peptides generated by enzyme digestion were analyzed using the Evosep Whisper method, an IonOpticks nano-LC column, a Newomics UniESI ion source, and an Agilent 6495D triple quadrupole LC/MS system. The optimized workflow achieved a lower limit of quantification of 0.02 ng/mL and a linear dynamic range up to 10 ng/mL, providing superior sensitivity compared to standard analytical LC/MS. Delivering robust, ultrasensitive quantitation with minimal method development, this approach enables rapid adoption for IgG-based therapeutic analysis.

Introduction

The monoclonal antibodies (mAbs) therapy market has been experiencing robust growth over the past two decades, with market trend data indicating that this market could grow to $919 billion by 2033.[1]

Traditionally, these large molecules are analyzed using the ligand binding assay (LBA), an analytical approach that is sensitive, high throughput, low cost, and easily automated. However, during the last two decades, liquid chromatography mass spectrometry (LC/MS) has emerged as an attractive alternative to LBA due to its high specificity, sensitivity, wide dynamic range, and ability to provide structural information. LC/MS also enables direct measurement of intact antibodies or signature peptides, offering enhance accuracy and reducing dependency on reagent/antigen availability.[2]

This application note presents a nanoflow LC/MS method for monoclonal antibody quantitation using surrogate peptides released by enzyme digestion, demonstrated with trastuzumab.[3] The results show that this new workflow can achieve sensitivity 50 times lower than traditional UHPLC/MS methods, making it highly valuable in biomarker research and drug development.

Experimental

Materials and methods

Formulated Herceptin (trastuzumab) was acquired from Genentech (South San Francisco, CA). Formic acid (FA), dithiothreitol, and iodoacetamide were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing-grade trypsin was purchased from Promega (Madison, WI). The 96-well LoBind plates were purchased from Eppendorf USA (Hauppauge, NY).

Instrumentation

- Evosep One LC system
- Newomics UniESI ion source for Agilent MS
- Agilent 6495D triple quadrupole LC/MS system
- IonOpticks column/heater

Software

- Agilent MassHunter acquisition software for LC/MS systems (version 12.0)
- Agilent MassHunter Quant software (version 12.0)

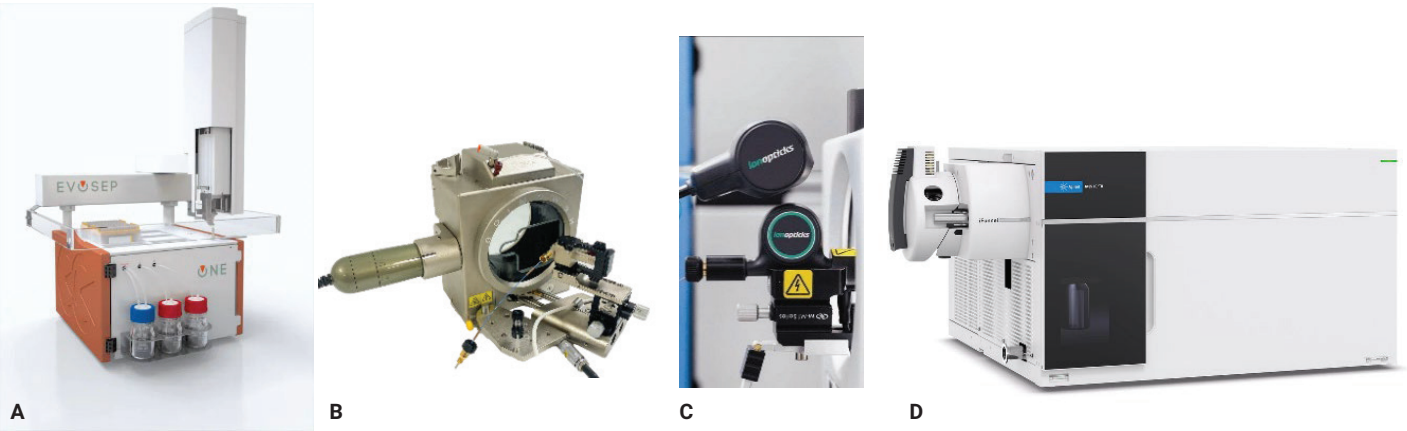

Figure 1. (A) Evosep One, (B) Newomics source, (C) IonOpticks column/heater, and (D) Agilent 6495D triple quadrupole LC/TQ system.

Sample preparation

Trypsin digestion of trastuzumab: Trastuzumab (10 µg) was diluted in 200 µL of 50 mM ammonium bicarbonate buffer. Next, dithiothreitol solution was added to each sample (10 mM final concentration), followed by incubation at 60 °C for one hour. After cooling, iodoacetamide was added to each sample (10 mM final concentration), and the samples were incubated at room temperature in the dark for 30 minutes. Lastly, 0.5 µg of trypsin was added to each sample, followed by incubation at 60 °C for two hours with shaking. The digestion was stopped by adding 250 µL of 1% formic acid (FA) solution to achieve a final concentration of 20 µg/mL.

Evotips loading

The trastuzumab digestion was serially diluted to 0.02 ng/mL in preparation for loading onto Evotips. Briefly, Evotips were conditioned with 20 µL acetonitrile (0.1% FA) and spun down for one minute at 700 × g. Next, the Evotips were placed in isopropanol until they turned to pale white color, then equilibrated with 20 µL water (0.1% FA) and spun down for one minute at 700 × g. After conditioning, the different concentrations of trastuzumab digest were loaded onto the Evotips and spun down for one minute at 700 × g. The Evotips were then washed by adding 20 µL of water (0.1% FA) and spinning down for one minute at 700 × g. Finally, 100 µL of water (0.1% FA) was added to each Evotip, followed by a 10-second spin-down at 700 × g. Evotips were placed on the Evosep One for LC/MS peptide analysis.

LC/MS analysis

Data acquisition was performed using an Evosep One LC coupled to an Agilent 6495D triple quadrupole LC/MS system with Newomics UniESI ion source. Separation was obtained with an IonOpticks Aurora Elite XT C18 (15 cm × 75 µm id, 1.7 µm) column. Tables 1 and 2 list the LC and MS parameters used in this workflow. Positive electrospray ionization was used for trastuzumab surrogate peptide analysis. Multiple reaction monitoring (MRM) transitions of the ALPAPIEK peptide, along with optimal collision energies, are listed in Table 3. Specifically, peptide ALPAPIEK is a conserved peptide derived from human IgG, which is shared among different human IgG isoforms.

Table 1. Liquid chromatography parameters.

LC Parameters	
Column	IonOpticks Aurora Elite XT C18 column (15 cm × 75 µm id, 1.7 µm)
Method	Whisper Zoom 40 SPD
Mobile Phase	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Flow Rate	200 nL/min
Stop Time	33.0 min

Table 2. MS acquisition parameters.

MS Parameters	
Ion Source	Newomics UniESI ion source
Ion Mode	Positive
Gas Temperature	120 °C
Drying Gas Flow	11 L/min
Capillary Voltage	1,800 V
Ion Funnel	Large molecule

Table 3. Surrogate peptide MRM transitions.

Peptide	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy
ALPAPIEK	419.8	654.4	10
ALPAPIEK	419.8	486.3	15
ALPAPIEK	419.8	327.6	10

Data processing

All MS data were processed using MassHunter Qualitative Analysis software (version 12.0) and MassHunter Quantitative Analysis software (version 12.0).

Results and discussion

Method optimization for surrogate peptide quantitative analysis

To improve the sensitivity and reproducibility of peptide quantitative analysis, sample preparation and MS conditions were optimized. The peptide MRM transitions and collision energies were also optimized to achieve the best MS sensitivity. These parameters are listed in Table 2.

Quantitative analysis of trastuzumab

The Evosep Whisper method, combined with IonOpticks nano-LC column, Newomics UniESI ion source, and the 6495D LC/TQ system, can achieve excellent peptide sensitivity. Figure 2 shows the MRM chromatogram of the trastuzumab surrogate peptide at 0.02 ng/mL after trypsin digestion, demonstrating a sensitivity 50 times greater than that of analytical LC/MS analysis reported in the literature. Figure 3 shows 20 µL of 1 ng/mL trastuzumab digest analyzed using this nano LC/MS workflow compared to a standard analytical LC/MS workflow. These data show that the peak area of the surrogate peptide in the nano LC/MS workflow is 68 times larger than the peak area in the standard analytical LC/MS workflow, indicating superior sensitivity for peptide quantification.

MassHunter Quant software (version 12.0) was used to perform quantitative analysis of trastuzumab surrogate peptides. As shown in Figure 4, the lower limit of quantification (LLOQ) for trastuzumab was 0.02 ng/mL, and the calibration curve was linear up to 10 ng/mL with linear fit and $1/x^2$ weighting.

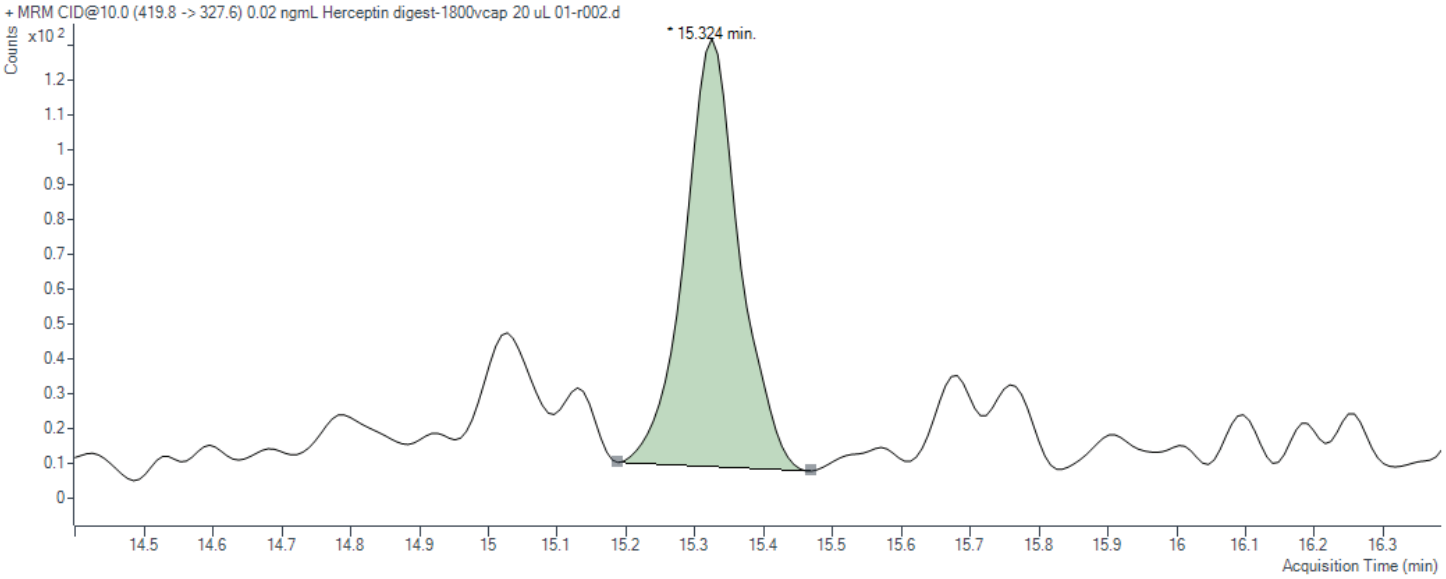

Figure 2. Trastuzumab surrogate peptide ALPAPIEK quantifier (*m/z* 419.8 → 327.6) MRM chromatogram at 0.02 ng/mL.

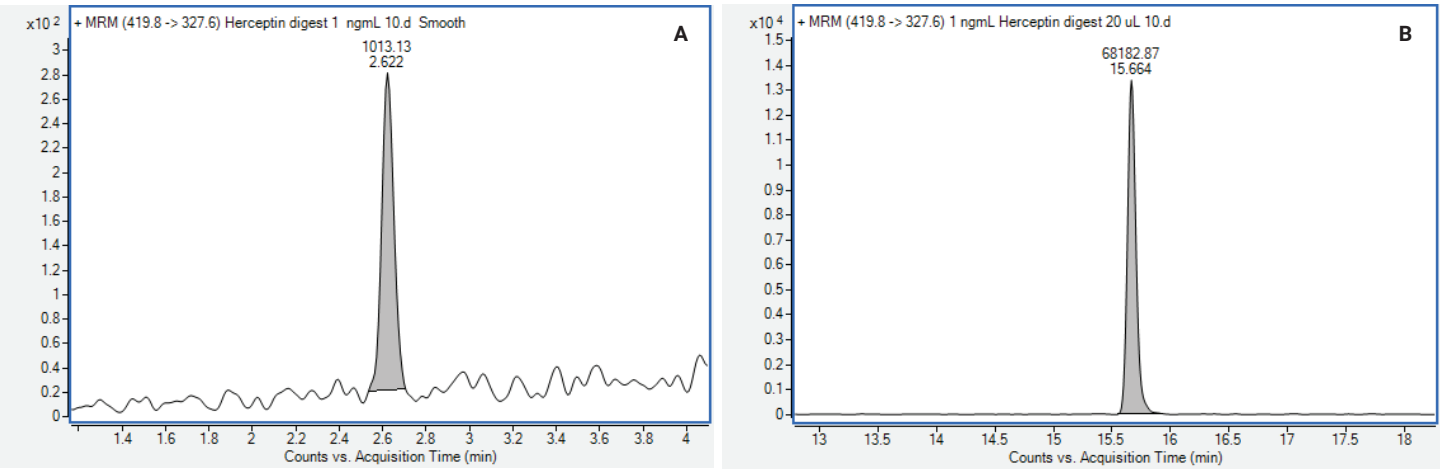

Figure 3. Trastuzumab surrogate peptide ALPAPIEK quantifier (*m/z* 419.8 → 327.6) MRM chromatograms at 1 ng/mL, showing the result from analytical LC/MS (A) and the result from nano LC/MS workflow (B).

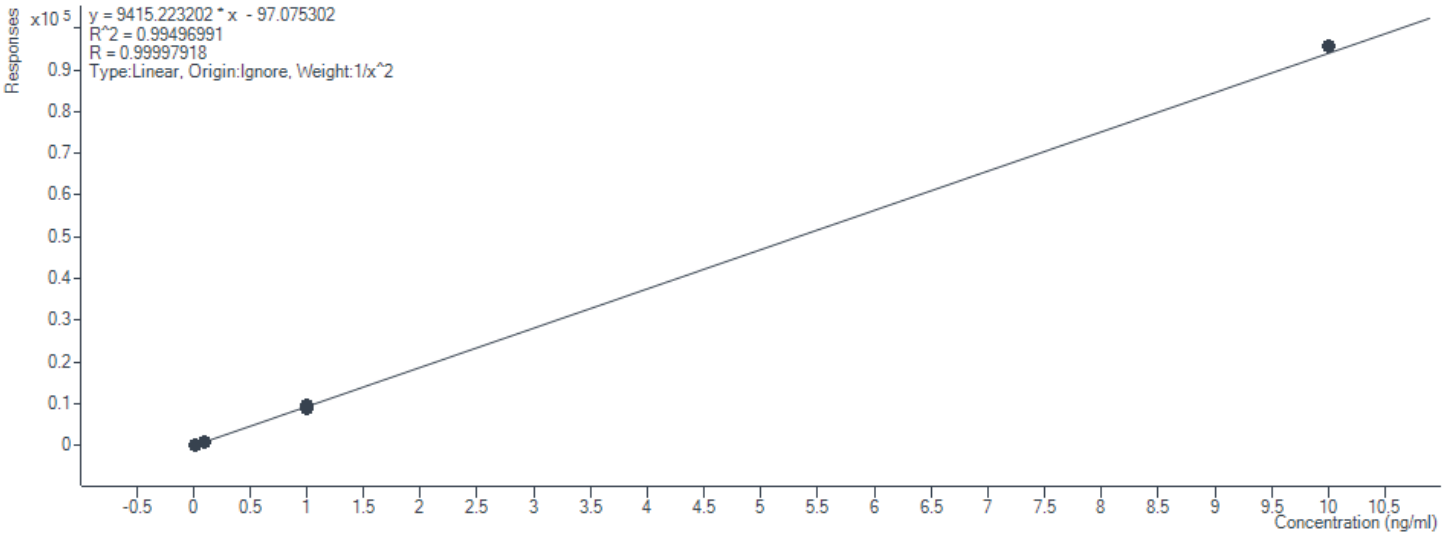

Figure 4. Calibration curve of trastuzumab digest from 0.02 to 10 ng/mL.

Conclusion

The Evosep Whisper method, with an IonOpticks nano-LC column, the Newomics UniESI ion source, and the Agilent 6495D triple quadrupole LC/MS system, constitutes an optimized platform for quantitative analysis at ultralow analyte concentrations. Using a 20 µL mAb digestion sample, this workflow achieved a lower limit of quantification of 0.02 ng/mL with a linear dynamic range up to 10 ng/mL, corresponding to an effective on column quantification limit of 0.4 pg. An additional advantage of this workflow is its broad applicability. It can be readily extended to other human IgG based monoclonal antibody therapeutics with minimal method development, facilitating rapid implementation in drug discovery and development workflows.

References

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