Application Note

Biotherapeutics & Biosimilars

Quantification of Monoclonal Antibody Trastuzumab in Mouse Plasma with the Agilent 1290 Infinity II Bio LC and Agilent 6495 Triple Quadrupole LC/MS Systems

Author

Xi Qiu
Agilent Technologies, Inc.

Introduction

Over the past two decades, monoclonal antibody (mAbs) therapy has emerged as an important therapeutic for many diseases. The market trend has indicated that the fast-growing mAb therapeutics market is set to grow to $390.6 billion by 2030.[1] The traditional analytical approach of these large molecules is via the ligand binding assay (LBA) due to its sensitivity, high throughput, low cost, and ease-of-automation. Over the past two decades, liquid chromatography/mass spectrometry (LC/MS) has become an alternative method for analyzing these large molecules due to their high specificity, sensitivity, wide dynamic range, and fast method development. At the same time, LC/MS can avoid cross-reactivity, improve productivity, and reduce costs and delays related to reagent/antigen availability.[2]

Trastuzumab, sold under the brand name Herceptin, is an mAb used to treat breast and stomach cancers with HER2-positive patients.[3] Traditionally, the plasma concentration of trastuzumab was determined by the ligand binding assay, which needs time to develop the antibody, and lacks selectivity and specificity. We demonstrate a hybrid LBA/LC/MS workflow, which combines LBA and LC/MS technologies, to quantify the mAb drug in mouse plasma using an Agilent 1290 Infinity II bio LC and an Agilent 6495 triple quadrupole LC/MS system (Figure 1). Our results show that this hybrid LBA/LC/MS workflow can be used for quantitative analysis of mAb therapeutics without the need for a specific antibody, while providing excellent sensitivity, high specificity, and fast method development. These factors will play an important role in drug discovery and development.

Experimental

Materials and methods

Formulated Herceptin (trastuzumab) was obtained from Genentech (South San Francisco, CA). Formic acid (FA), bovine serum albumin (BSA), PBS buffer, and biotinylated antihuman Fc antibody were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing-grade trypsin was purchased from Promega (Madison, WI). 96-well LoBind plates were purchased from Eppendorf USA (Hauppauge, NY) and LodeStars Streptavidin magnetic beads were obtained from Agilent Technologies (Santa Clara, CA).

Instrumentation

- Agilent 1290 Infinity II bio LC system including:
  - Agilent 1290 Infinity II bio High Speed Pump (G7132A)
  - Agilent 1290 Infinity II bio multisampler (G7137A)
  - Agilent 1290 Infinity II thermostat column compartment (G7116A) equipped with a Standard Flow Quick Connect bio heat exchanger (G7116-60071)
- Agilent 6495 triple quadrupole LC/MS system

Software

- Agilent MassHunter Acquisition software
- Agilent MassHunter Quantitative Analysis software

Sample preparation

Immunoaffinity purification: First, biotinylated antihuman Fc antibody was conjugated onto LodeStars streptavidin magnetic beads in PBS buffer with shaking at room temperature for one hour. The conjugated beads were then washed twice with PBS buffer and resuspended in PBS buffer to their original concentration. Then, 50 µL of mouse plasma aliquots, fortified with different concentrations of trastuzumab, 50 µL of conjugated magnetic beads, and 300 µL of PBS buffer were mixed and incubated at room temperature for one hour with shaking. After incubation, the magnetic beads were pulled to the side by a magnetic stand, and the supernatant was discarded. Then, the magnetic beads were washed three times with PBS buffer. The final release step was carried out by incubating the magnetic beads with 100 µL of 0.1% trifluoroacetic acid buffer at room temperature for 10 minutes with shaking.

Trypsin digestion of trastuzumab: Release buffer was transferred to another tube by pulling magnetic beads to the side, and adding 10 µL of 1 M ammonium bicarbonate buffer to neutralize. Dithiothreitol solution was then added to each sample at 10 mM final concentration and incubated at 60 °C for 1 hour. After cooling down, iodoacetamide was added to each sample at 10 mM final concentration and incubated at room temperature in darkness. Lastly, 0.5 µg of trypsin was added to each sample and incubated at 37 °C overnight with shaking. Digestion was stopped by adding 10 µL 1% formic acid solution. Then, 20 µL was injected into LC/MS for peptide analysis.

Figure 1. Agilent 1290 Infinity II bio LC system and Agilent 6495 triple quadrupole LC/MS system.

LC/MS analysis

Data acquisition was performed using an Agilent 1290 Infinity II bio UHPLC coupled to an Agilent 6495 triple quadrupole LC/MS system with Agilent Jet Stream source. Separation was obtained with an Agilent Poroshell EC-C18 column (2.1 × 50 mm, 120 Å, 2.7 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow. Positive electrospray ionization was used for trastuzumab surrogate peptides analysis. MRM transitions of peptides are listed in Table 3 with optimal collision energy. Peptides IYPTNGYTR and FTISADTSK are unique peptides for trastuzumab, which do not share a sequence with any other proteins. Peptide GPSVFPLAPSSK is a conserved peptide from human IgG, which is shared between different human IgG isoforms.

Data processing

All MS data were processed using MassHunter Quantitative Analysis software.

Table 1. Liquid chromatography parameters.

LC Conditions	
Column	Agilent Poroshell EC-C18, 2.1 × 50 mm, 2.7 µM
Column Temperature	50 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Needle Wash	3 seconds in wash port (50/50 water/methanol)
Mobile Phase	A: water + 0.1% formic acid B: acetonitrile + 0.1% formic acid
Flow Rate	0.4 mL/min
Gradient Program	Time %B 0 5 1.0 5 4.0 50 4.1 90 5.0 90 5.1 5
Stop Time	6.0 min

Table 2. MS acquisition parameters.

MS Conditions	
Ion Mode	Positive
Gas Temperature	180 °C
Drying Gas Flow	16 L/min
Nebulizer Gas	20 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Ion Funnel	200/110 V

Table 3. Surrogate peptides MRM transitions.

Peptide	Precursor Ion	Product Ion	Collision Energy
IYPTNGYTR	542.8	808.4	16
IYPTNGYTR	542.8	405.1	15
FTISADTSK	485.2	721.3	14
FTISADTSK	485.2	608.2	16
GPSVFPLAPSSK*	593.8	846.5	19
GPSVFPLAPSSK*	593.8	699.4	19

* Indicates universal peptide conserved in human IgG

Results and discussion

Method optimization for surrogate peptide quantitative analysis

To improve the sensitivity and reproducibility for peptide quantitative analysis, sample preparation, LC, and MS conditions were all optimized. Peptide MRM transitions and collision energy were also optimized to achieve the best MS sensitivity for peptide quantitative analysis. The optimized source parameters are listed in Table 2.

Quantitative analysis of trastuzumab in mouse plasma

The hybrid LBA/LC/MS workflow combines the advantages of two technologies for bioanalysis of large molecules in biological matrix, which decreased sample complexity and achieved great assay sensitivity. Figure 2 shows the MRM chromatograms for the three surrogate peptides of trastuzumab purified from mouse plasma at 5 ng/mL.

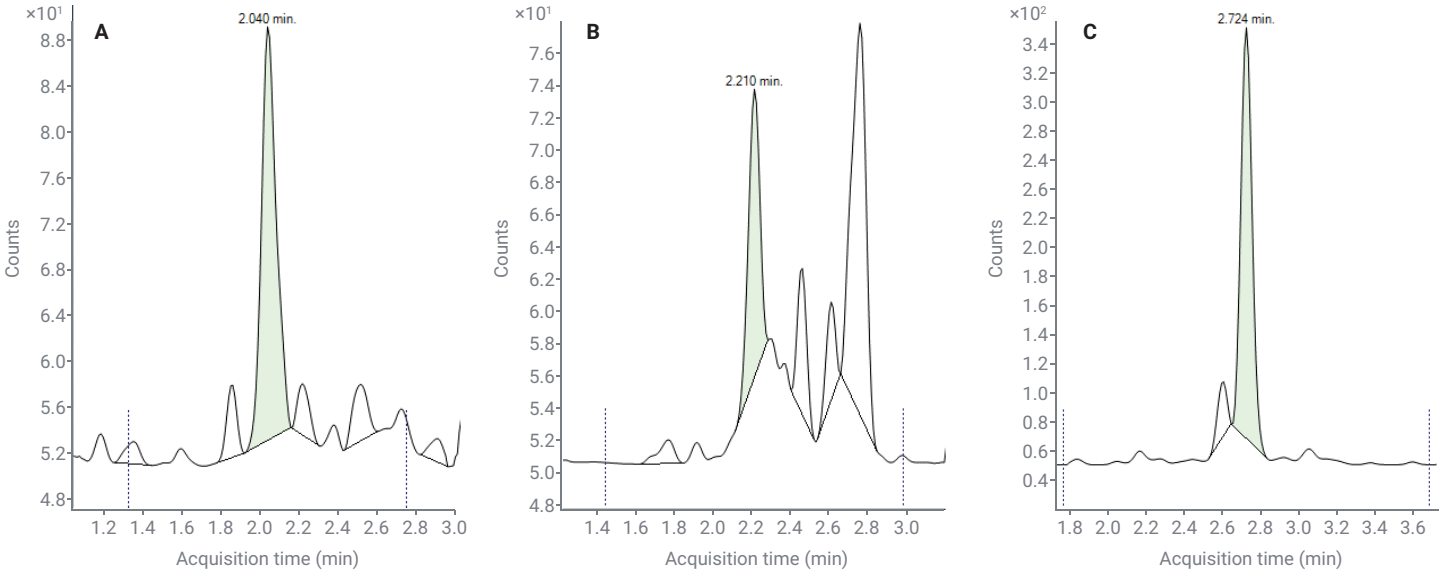

Figure 2. Trastuzumab surrogate peptide MRM chromatograms purified from mouse plasma. (A) IYPTNGYTR quantifier (542.8 → 405.1) MRM chromatogram at 5 ng/mL; (B) FTISADTSK quantifier (485.2 → 721.3) MRM chromatogram at 5 ng/mL; (C) GPSVFPLAPSSK quantifier (593.8 → 699.4) MRM chromatogram at 5 ng/mL.

Agilent MassHunter Quantitative Analysis software was used to perform trastuzumab surrogate peptide quantitative analysis. As shown in Figure 2, the lower limit of quantification for trastuzumab in mouse plasma was 5.0 ng/mL for all three surrogate peptides. By using 50 µL of plasma as a starting material, the calibration curve was from 5 to 2,000 ng/mL with quadratic fit and $1/x^2$ weight (Figure 3). The bending at the top of the curve was probably caused by immunoaffinity saturation.

Table 4. Trastuzumab surrogate peptides calibration curve result summary.

Calibration (ng/mL)	5	10	50	100	200	2,000
GPSVFPLAPSSK	5.195	9.278	48.478	96.051	217.202	2,034.420
IYPTNGYTR	4.533	11.960	51.757	82.037	207.609	2,008.332
FTISADTSK	4.695	11.414	45.494	96.201	209.850	1,996.972
Mean	4.807	10.884	48.576	91.430	211.553	2,013.241
% Bias	-3.85	8.84	–2.85	–8.57	5.78	0.66
% CV	7.18	13.02	6.45	8.90	2.37	0.95

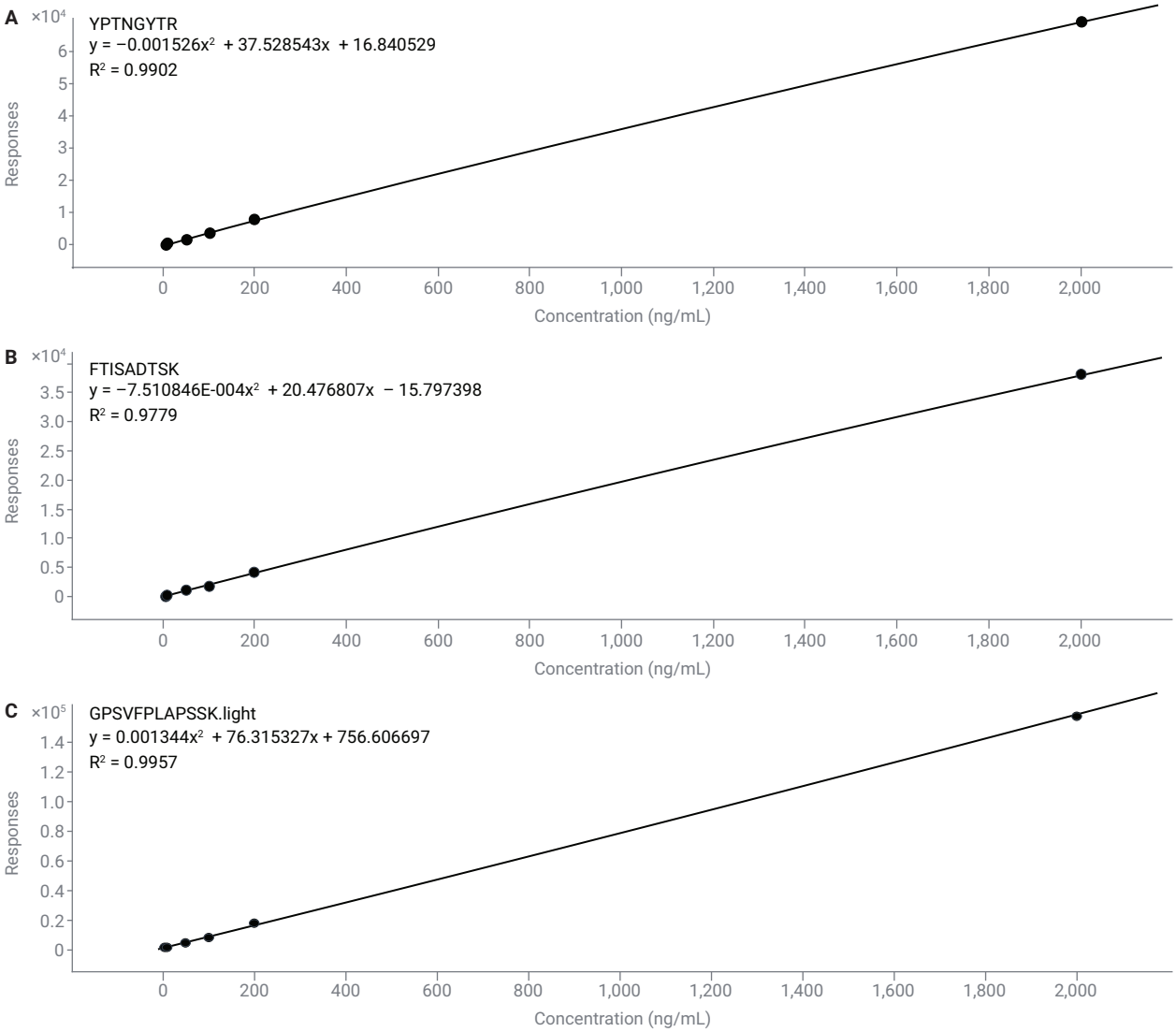

Figure 3. Calibration curve of trastuzumab surrogate peptides from 5 to 2,000 ng/mL in mouse plasma. (A) Unique surrogate peptide IYPTNGYTR calibration curve. (B) Unique surrogate peptide FTISADTSK calibration curve. (C) Universal surrogate peptide GPSVFPLAPSSK calibration curve.

Conclusion

The Agilent 1290 Infinity II bio LC and Agilent 6495 triple quadrupole LC/MS system are ideal platforms for peptide quantitative analysis. The hybrid LBA/LC/MS workflow combines the advantages of both technologies, and provides excellent assay sensitivity and reproducibility. This application note demonstrates a sensitive hybrid LBA/LC/MS workflow for the quantitative analysis of trastuzumab surrogate peptides from biological matrix. The workflow achieved a lower limit of quantification of 5 ng/mL by using 50 µL of biological sample, and the calibration curve was from 5 to 2,000 ng/mL, with excellent accuracy and precision across all three surrogate peptides, including two unique peptides from trastuzumab and one conserved universal peptide from human IgG. Another advantage of this workflow is that it is universal and specific, meaning that it can be applied to many other human IgG-based mAb therapeutics. It also requires minimum method development time, which will greatly support drug discovery and development.

References

1. Allied Market Research Latest Reports.
2. Jenkins, R. *et al.* Recommendations for Validation of LC–MS/MS Bioanalytical Methods for Protein Biotherapeutics. *AAPS J*. **2015**, *17(1)*, 1–16.
3. Boekhout, A. H.; Beijnen, J. H.; Schellens J. H. M. Trastuzumab. *Oncologist* **2011**, *16(6)*, 800–810.

www.agilent.com

RA44818.6240972222

Printed in the USA, October 18, 2022
5994-5225EN

Agilent
Trusted Answers