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LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

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Introduction

Herceptin is approved for the treatment of early-stage breast cancer that is Human Epidermal growth factor Receptor 2-positive (HER2+) and has spread into the lymph nodes, or is HER2-positive and has not spread into the lymph nodes. With the wide use of Herceptin in clinic, the requirement is increasing for precision and accuracy in quantitative analysis of Herceptin in human plasma.
With the development of high performance liquid phase, mass spectrometry and separation technology in biological samples, LC-MS/MS technology has been used for protein quantity study. Relative to conventional analytical technology ELISA, LC-MS/MS quantitative method improved the precision and accuracy of protein analysis. For the close combination of protein quantitative technology and drug development, Shimadzu combined LC-MS/MS analysis platform and proteomics software "Skyline". And we also developed nSMOL pretreatment technology for selective enzymolysis of Fab zone in monoclonal antibody. Thus, LC-MS/MS combining with Skyline software and nSMOL technology can provide useful tool for the accurate quantity of monoclonal antibody drugs.

Methods and Materials

Pretreatment: 1, peptide screening, 10 µg/mL Herceptin standard sample was hydrolyzed according to nSMOL technology, and the hydrolysis product was analyzed by LC-MS/MS; 2, quantitative analysis of Herceptin in plasma, Herceptin standards with 0.122, 0.244, 0.488, 0.975, 1.95, 3.90, 7.80, 15.6, 31.3, 62.5, 125 µg/mL in plasma were prepared, and then pretreated with nSMOL kit according to the operation manual, and then the hydrolysis products were analyzed by LCMS-8060.

Instrument : As an LC-MS/MS system, UHPLC was coupled to triple quadrupole mass spectrometer (Nexera MP with LCMS-8060, Shimadzu Corporation, Kyoto, Japan).
LC-MS/MS with electrospray ionization was operated in multiple-reaction-monitoring (MRM) mode.
Separation: A BEH Peptide C18 column (2.0 mm I.D.× 150 mm L., 1.7 µm) was used for method developing and an Inertsil Sustain Swift C18 column (2.1 mm I.D.×50 mm L., 1.9 µm) was used for quantitative analysis.

Table 1 MRM parameters of Herceptin

Peptides	MRM [m/z]	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)	Peptides function
GLEWVAR	415.70 → 660.30*	-16.0	-15.0	-34.0	Qualitative peptide
	415.70 → 531.30	-16.0	-16.0	-40.0	
	415.70 → 345.20	-16.0	-20.0	-24.0	
FTISADTSK	485.15 → 721.30*	-19.0	-18.0	-38.0	Qualitative peptide
	485.15 → 608.30	-19.0	-20.0	-32.0	
	485.15 → 521.20	-19.0	-21.0	-26.0	
IYPTNGYTR	542.80 → 404.70*	-20.0	-18.0	-30.0	Quantitative peptide
	542.80 → 808.40	-20.0	-18.0	-28.0	
	542.80 → 610.30	-20.0	-25.0	-22.0	
P14R (IS)	512.10 → 292.30*	-38.0	-20.0	-20.0	IS
	512.10 → 389.30	-38.0	-16.0	-28.0	
	512.10 → 757.50	-38.0	-19.0	-38.0	
	512.10 → 660.40	-38.0	-17.0	-24.0	
	512.10 → 563.30	-38.0	-17.0	-40.0	

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High Speed Mass Spectrometer

Ultra Fast Polarity Switching
- 5 msec

Ultra Fast MRM
- Max. 555 transition /sec

Figure 1 LCMS-8060 triple quadrupole mass spectrometer

Result

Characteristic peptides screening of Herceptin

The nSMOL technology is designed as solid–solid proteolysis on the nanoparticle surface with retaining protease activity. Antibody Fc was immobilized onto the resin via protein A/G, such that the Fab is oriented outward to the solution, and Fab-selective limited proteolysis was occurred. In this work, firstly, Herceptin was selectively hydrolyzed using nSMOL technology, and then characteristic peptides of Herceptin were predicted by Skyline software, and analyzed by LC-MS/MS (Figure2). There were 10 peptides which had good sensitivity and good shape after deleting the peptides with no signal and weak response (Figure 3). Among these 10 peptides, 8 peptides belonged to Fab zone, which indicated that nSMOL technology can selectively hydrolyzed Fab zone of monoclonal antibodies.

UHPLC conditions (Nexera MP system)	
Column	: BEH Peptide C18 column (2.0 mm I.D.× 150 mm L., 1.7 µm)
Mobile phase A	: 0.1% formic acid in H_2O
B	: 0.1% formic acid in acetonitrile
Flow rate	: 0.4 mL/min
Time program	: B conc. 0.0-3.0 min, 5% ; 3.0-35.0 min, 5%~40% ; 35.0-37.5 min, 40%~95% ; 37.5~45.0 min, 95% ; 45.5-50.0 min, 5%
Injection vol.	: 10 uL
Column temperature	: 40°C
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive MRM mode

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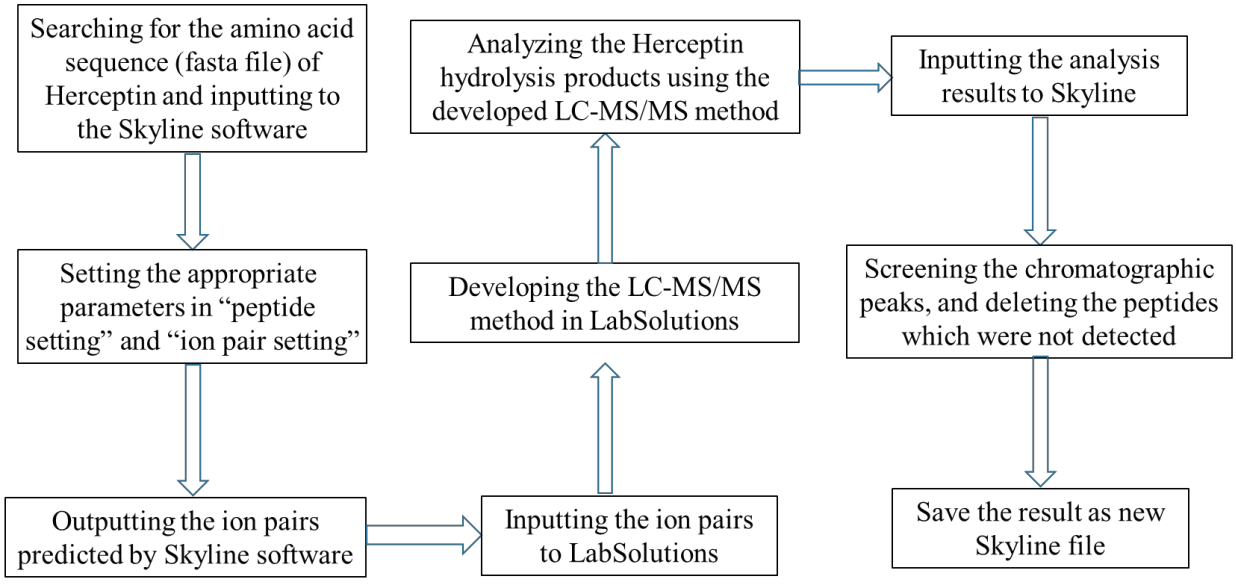

Figure 2　Operational procedure of characteristic peptide screening

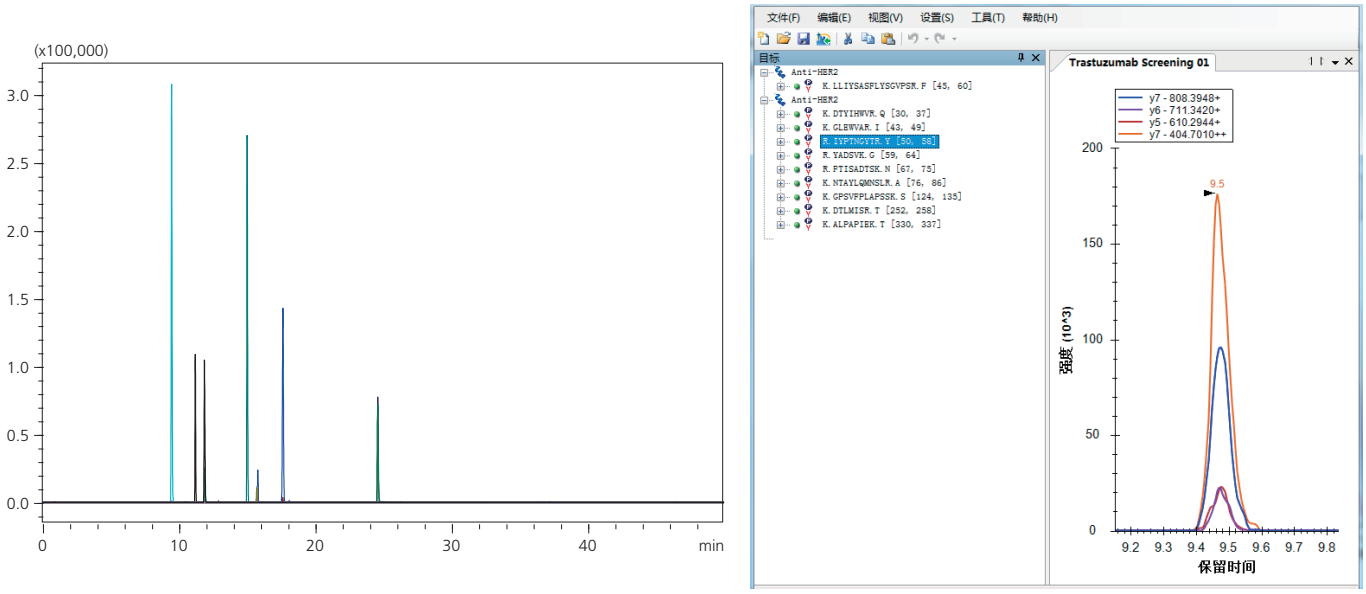

Figure 3　Screened characteristic peptide chromatogram and the corresponding result in Skyline

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Characteristic peptides screening of Herceptin

For establishing good quantitative method, MRM collision energies for 8 peptides (LLIYSASFLYSGVPSR, DTYIHWVR, IYPTNGYTR, GLEWVAR, YADSVK, FTISADTSK, NTAYLQMNSLR, GPSVFPLAPSSK) which belonged to Fab zone were synchronously optimized based on the high speed scan of LCMS-8060. An optimizing LC-MS/MS method for 8 peptides was developed by exporting ion pairs setting in Skyline, and then the optimized results by LC-MS/MS were imported to Skyline for getting the optimization result (Figure 4). According to the optimized result, three peptides GLEWVAR, FTISADTSK, IYPTNGYTR which belonged to CDR zone were selected as characteristic peptides for quantitative and qualitative analysis.

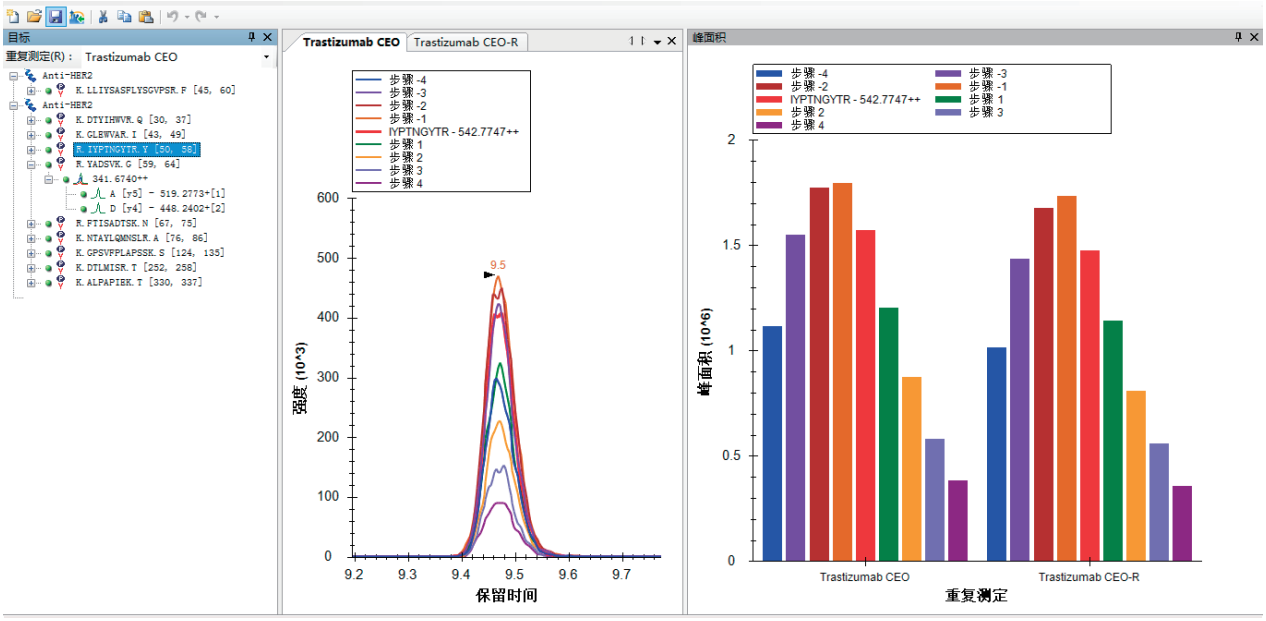

Figure 4 The optimized results by LC-MS/MS which were imported to Skyline

Quantitative analysis of Herceptin in plasma

Based on the optimized CE energies for the characteristic peptides, a complete LC-MS/MS method was established. The peptide IYPTNGYTR (542.80>404.70) which have high intensity and no matrix interference was selected as quantitative characteristic peptide, and the peptides GLEWVAR and FTISADTSK were selected as qualitative characteristic peptides.

For establishing LC-MS/MS quantitative method, firstly, different concentrations of Herceptin standards (0.122-125 μg/mL) in plasm were prepared, and then these standard samples were pretreated with nSMOL kit to get selectively hydrolyzed products for LC-MS/MS analysis. As a result, a calibration curve (Figure 5) was obtained with correlation coeffient 0.9995 and accuracy 91.5-114.0% (Table 2).

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UHPLC conditions (Nexera MP system)	
Column	: Inertsil Sustain Swift C18 Column (2.1 mm I.D.×50 mm L., 1.9 µm)
Mobile phase A	: 0.1% formic acid in H_2O
B	: 0.1% formic acid in acetonitrile
Flow rate	: 0.4 mL/min
Time program	: B conc. 0.00-1.50 min, 1% ; 1.50-5.00 min, 1%~30% ; 5.02-5.83 min, 95% ; 5.85~7.00 min, 1%
Injection vol.	: 10 uL
Column temperature	: 40°C
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive MRM mode

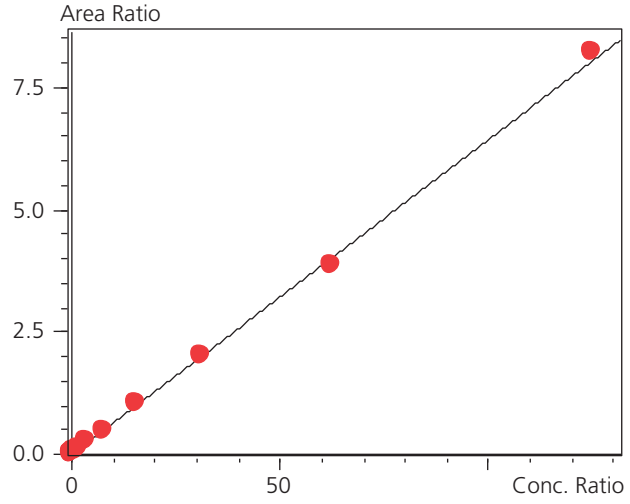

Figure 5 The calibration curve of the characteristic peptide IYPTNGYTR

Table 2 The related information about the calibration curve

Drug name	Peptide for quantitative	Calibration curve	Linear (µg/mL)	Correlation coefficient	Accuracy (%)
Herceptin	IYPTNGYTR	Y = (0.0644864)X + (-0.00317900)	0.122~125	0.9995	91.5~114.0

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Conclusions

There were 8 peptides belonging to Fab zone among the 10 peptides which screened by Skyline and LC-MS/MS, which indicated that the peptides in Fab zone were selectively hydrolyzed by nSMOL technology. For establishing good quantitative method, MRM collision energies were optimized. Based on these results, the peptide IYPTNGYTR (542.80>404.70) was selected as characteristic peptide for quantity. The calibration curve range was from 0.122 to 125 mg/L and the correlation coefficient was 0.9995.

Disclaimer: Shimadzu LCMS-8060 CL is registered in the U.S. as a Class I device and is not specifically cleared for TDM. Nexera X2 UHPLC system is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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