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Development of a Mass Spectrometric Method for Pharmacokinetic Study of Trastuzumab

Nam Young Hong, Jin Nyoung Choi, Jeong Won Kang, Do Young Choi, Gyu-Tae Park,[†] and Kwang Pyo Kim[*]

Department of Applied Chemistry, College of Applied Sciences, Kyung Hee University, Yongin 446-701, Korea
[*]E-mail: kimkp@khu.ac.kr
[†]CKD Research Institute, Yongin 446-916, Korea
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The therapeutic monoclonal antibody drug Trastuzumab (INN; trade name Herceptin) is widely used for treating metastatic breast cancer patients with overexpression of HER2 on the tumor. Trastuzumab is a representative target therapeutics as a monoclonal antibody that selectively binds with high affinity to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2.[1] HER2 is amplified or overexpressed in about 20% to 30% of patients with breast cancer and is associated with aggressive disease.[2-4] Trastuzumab has an inhibitory effect on the overexpression of HER2 receptor, thereby it has been used in treating breast cancer,[5] and also gastric cancer.[6,7] To date, the quantification of the plasma level of monoclonal antibody drugs, including trastuzumab, is performed with the antibody based immunological assays such as ELISA.[8,9] However, the availability of the specific antibody for the quantification is not always guaranteed. Also the specificity of the antibody for quantification is not enough. Recently, quantitative method based on isotope-dilution mass spectrometric method has been developed.[10] A method of absolute quantitation (also terms AQUA) that is based on multiple reaction monitoring (MRM) is performed by spiking complex samples with stable isotope-labeled synthetic peptides that act as internal standards for specific peptides. To develop the quantitative assay based on the isotope-dilution mass spectrometric method, the synthesis of the reference peptide called AQUA peptide is necessary.[11] The synthesis of the reference peptides is cheap and fast compared to the development of antibody for the quantitative analysis. This study describes a robust trastuzumab assay using the MRM with AQUA peptides by LC-MS/MS.

Trastuzumab and serum samples of ICR mice received trastuzumab (10 mg/kg) were prepared by CKD Research Institute (Yongin, Korea). HPLC-grade acetonitrile and water were purchased from Burdick and Jackson (Muskegon, MI, USA).

Trastuzumab were separated by SDS-PAGE. Expected trastuzumab light chain bands were excised and subject to in-gel digestion. Sequencing grade modified trypsin (Promega, Madison, WI, USA) was added to the samples at a 50:1 substrate:enzyme ratio and incubated overnight at 37 ^{o}C. The digested trastuzumab was dried completely using a Speed-Vac and re-suspended in 200 μL of 0.1% formic acid in HPLC water for LC-MS/MS analysis to get the reference MS/MS spectra for trastuzumab. The tryptic peptides were subjected to linear trap quadrupole mass spectrometer (LTQ-XL, Thermo Fisher, Waltham, MA) coupled with an Eksigent-Nano-UPLC system (Eksigent Technologies, CA). The tryptic peptides were applied to a homemade column (75 μm × 12 cm) packed with Alltima C18 5 μm sized resin (Grace, Breda, The Netherlands).A linear gradient was achieved from 97% solvent A (0.1% formic acid in H_2O) to 50% solvent B (0.1% formic acid in acetonitrile) for 35 min with a flow rate of 0.3 μL/min. The dynamic exclusion time was set at 30s, *m/z* range was 300-2000 *m/z*, number of microscan was 1, and electrospray voltage was 1.85 kV. All of obtained MS/MS spectra were searched against forward and reversed IPI human database (version 3.78) to identify peptides corresponding to the trastuzumab using SEQUEST (rev. 3.3.1) search engine (Thermo Fisher scientific Inc, MA, USA). On the basis of intensity of mass spectrum and peptide sequence uniqueness to trastuzumab, the AQUA peptide for MRM was selected.

AQUA peptide with a unique peptide sequence corresponding to trastuzumab generated from LC-MS/MS analysis was synthesized by AnyGen (Gwangju, Korea). After synthesis, the AQUA peptide is analyzed by LC-MS/MS to confirm its chromatographic behavior and fragmentation spectrum for MRM assay. The quantification of trastuzumab and a corresponding AQUA peptide were performed by using the optimized MRM transitions of precursor ions at 664.8 and 665.8 *m/z* (z = 3), fragment ions 852.9 (y15) and 854.1 *m/z* (y15), respectively.

Blood samples of ICR mice received trastuzumab (10 mg/kg) *via* intravenous (IV) bolus were obtained at 1, 2, 4, 8 h, 1, 2, 4, 6, 12, 15 and 30 day after administration. 3 mice blood samples were obtained from each time point as a biological replication. Then, all samples were centrifuged immediately and stored at −80 ^{o}C until use. For internal standard, 250 fmol of AQUA peptide was spiked into the ICR mouse serum samples before tryptic digestion. With the digested samples, quantitative analysis was performed on LTQ-XL with injection of 100 nL plasma equivalent volume. Briefly, the LC-MS/MS system consisted of Eksigent LC

Peptide sequence	Parent Ion	Product Ion	Ion	Charge state	
				Parent Ion	Product Ion
R.ASQDVNTAVAWYQQKPG*K.A	**665.8**	**854.1**	y15	3	2
		918.2	y16		2
		747.1	y13		2

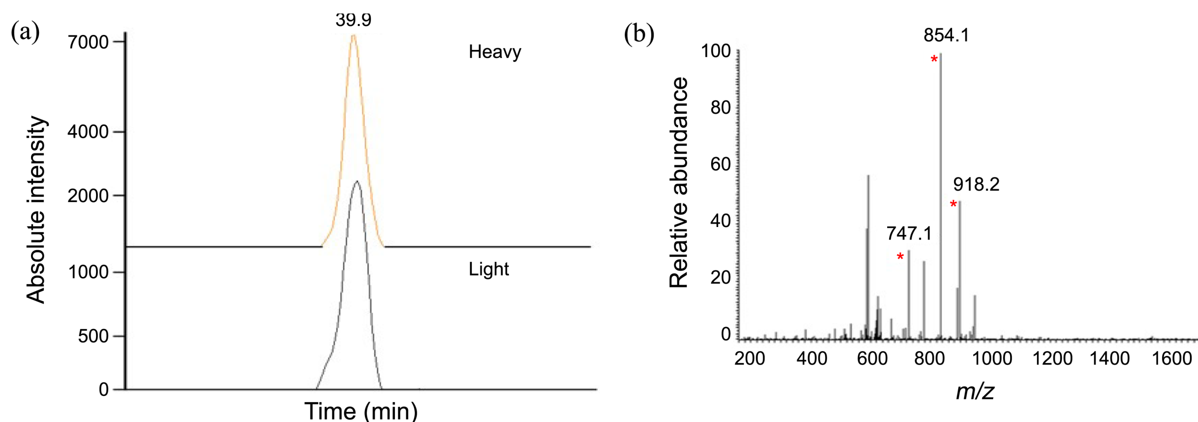

Figure 1. Optimization of MRM transition of AQUA peptide of trastuzumab. (a) MRM spectra of heavy and light peptide (3+). (b) Representative MS/MS spectra. The most intense fragment ions, 854.1 (y15), 918.2 (y16) and 747.1 (y13) *m/z* were chosen for MRM transition.

with custom analytical columns (75 μm × 110 mm, 100 Å C18, 5 μm) and LTQ-XL linear ion trap mass spectrometer with a nanospray ionization.

Enzyme-linked immunosorbent assay (ELISA) was carried out to validate MRM analysis. 96-well flat-bottom Maxisorp plate was coated with rabbit anti-idiotype antibodies. Each PK serum sample and standard was diluted with HPE buffer containing 1% NRS (normal rabbit serum) and added to wells in duplicate. The Goat anti-human igG Fc specific antibody was also added as a second antibody. After incubation at 37 °C and washing with 0.05% Tween in TBS, TMB substrate was added to start color development. Trastuzumab level in ICR mouse serum was determined by referring to a standard curve.

After refining of MRM candidate peptides, the ASQDVNTAVAWYQQKPGK peptide was selected for MRM analysis. Following optimization of MRM analysis with the synthesized reference peptide corresponding to the selected peptide, ASQDVNTAVAWYQQKPG[$^{13}C_2$, ^{15}N]K, the MRM transition (the parent-product ion transition) was selected as *m/z* 665.8→854.1, *m/z* 665.8→918.2, and *m/z* 665.8→747.1 for MRM (Fig. 1).

The calibration curve for quantification of trastuzumab in ICR mice serum showed good linearity in the concentration range from 10 pg to 1 μg ($R^2 > 0.99$) for trastuzumab. A typical equation for the curve is $y = 6.8923x + 69949$. The

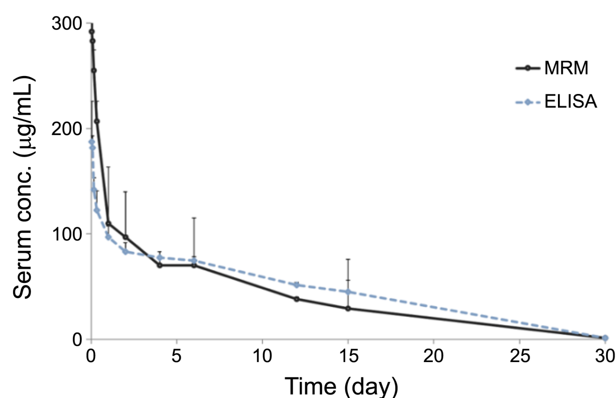

Figure 2. Mean serum concentration-time profiles of Trastuzumab in male ICR mice determined by LC-MRM method (black solid line) and ELISA (blue dotted line) following intravenous (10 mg/kg) administration. Each point represents mean ± SD. (n = 3 each in 3 technical replicates).

Table 1. The main pharmacokinetic parameters after IV administration of single dosage of 10 mg/kg trastuzumab in 32 mice

Pharmacokinetic parameters	IV bolus (10 mg/kg)	
	ELISA	MRM
C_{max} (μg/mL)	187.3	292.0
C_0 (μg/mL)	193.0	194.5
AUC_{last} (μg.hr/mL)	33509.4	29235.1
AUC_{inf} (μg.hr/mL)	33635.1	29498.6
Cl (mL/day/kg)	32.2	47.0
V_z (mL/kg)	7.14	8.14
$t_{1/2}$ (day)	3.13	4.01

Values are expressed as mean ± SD of 32 mice. (Origin value from Peak area: Include 3 transition, n = 3). C_{max}: maximum serum concentration; AUC_{last}: area under the concentration–time curve from time 0 to the time of the last quantifiable concentration; AUC_{inf}: area under the plasma concentration–time curve from time 0 extrapolated to infinity; Cl: clearance; Vz; $t_{1/2}$: half-life

LOQ for trastuzumab was determined as 10 pg/mL in mouse serum. The standard MRM curve generated using isotopically labeled synthetic peptide (250 to 1000 fmol/mL) that is determined the linearity of the assay ($R^2 = 0.992$). The typical equation for the standard curve is $y = 0.0076x-1.37$.

The results of MRM that represent time profiles of trastuzumab (black solid line in Fig. 2) showed that after intravenous administration, the concentration of trastuzumab in serum declined rapidly in the initial phase followed by a relatively slow elimination phase. The experimental values were obtained from 3 mice each in 3 technical replicate experiments. The serum concentration time curve from the ELISA (blue dotted line in Fig. 2) showed similar tendency to that of the MRM. The main pharmacokinetic parameters of both MRM and ELISA are summarized in Table 1. The maximum serum concentration of trastuzumab from MRM and ELISA was 187.3 μg/mL and 292.0 μg/mL; the distribution half-life ($t_{1/2}$) was 3.13 day, 4.01 day respectively. These results indicated the applicability of this method to pharmacokinetic study of trastuzumab.

In conclusion, the HPLC-MS/MS method described here is a simple, rapid and specific method for determining the concentration of biologics in mouse serum. This method was validated for the quantification trastuzumab in ICR mice serum. Although the ELISA method is available for the pharmacokinetics study for trastuzumab, but these methods require development of the target specific antibodies. Generally, the development of the target specific antibodies for quantification is time consuming and expensive. In contrast, MRM-based method does not need antibodies for quantification.

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