

Cite this: *Anal. Methods*, 2020, 12, 54

Quantitative LC-MS/MS method for nivolumab in human serum using IgG purification and immobilized tryptic digestion†

Kazuki Abe,^a Kaito Shibata,^a Takafumi Naito,^a *^a Masato Karayama,^b Etsuko Hamada,^c Masato Maekawa,^c Yasuhide Yamada,^b Takafumi Suda^d and Junichi Kawakami^a

A liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method is a novel approach for the quantification of therapeutic monoclonal antibody. However, this method has severe ionization suppression and time-consuming sample preparation. In the present study, we developed a simple and rapid LC-MS/MS method for quantifying serum nivolumab with good analytical performance using immunoglobulin G (IgG) purification and immobilized tryptic digestion. Surrogate peptide derived from nivolumab was identified by a hybrid quadrupole-Orbitrap mass spectrometer. IgG purification and immobilized tryptic digestion were completed within 25 minutes. The chromatographic separation was completed in 10 minutes with stepwise-gradient elution. Chromatographic peaks interfering with the surrogate peptide and its stable isotope-labeled peptide as an internal standard were not observed from serum digests. The dynamic range of the calibration curve was 2–200 $\mu\text{g mL}^{-1}$. The intra- and inter-assay accuracies and imprecisions were 92.2–104.5% and less than 10.0%, respectively. Serum nivolumab concentrations ranged from 12–112 $\mu\text{g mL}^{-1}$ in 14 cancer patients. The measured concentrations in the LC-MS/MS method were strongly correlated with those in the ELISA method ($r = 0.92$, $P < 0.01$). In conclusion, a simple and rapid LC-MS/MS method for quantifying serum nivolumab using immobilized tryptic digestion coupled to IgG purification was found to be acceptable for clinical settings.

Received 25th September 2019

Accepted 24th November 2019

DOI: 10.1039/c9ay02087j

rsc.li/methods

1. Introduction

Nivolumab, a fully human IgG4 monoclonal antibody (mAb), is used for the treatment of various cancers including malignant melanomas, non-small cell lung cancer (NSCLC), and renal cell cancer. Nivolumab binds programmed cell death-1 (PD-1) expressed on T cells and reactivates T cell antitumor immunity. The reactivation of T cells causes immune-related adverse events (irAEs) such as colitis, interstitial lung disease, and thyroid functional disorder.¹ Cancer patients treated with nivolumab can potentially have these irAEs at an unexpected time, and thus the nivolumab therapy must be interrupted or delayed. Although a large interindividual variation in serum

nivolumab concentration may affect the incidences of irAEs, their relationships are unclear.

The common quantitative method currently used for therapeutic mAb in human serum is an enzyme-linked immunosorbent assay (ELISA). This method performs a sensitive and high-throughput analysis of target mAb using antigen–antibody reaction. However, the ELISA method has potential problems including cross-reaction with endogenous substances and assay interference by different formats of antigen immobilization.^{2,3} Recently, a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) strategy has been applied to mAb quantification.^{4–9} This method determines surrogate peptide derived from target mAb and shows no cross-reaction, unlike ELISA methods.

Although an LC-MS/MS method can overcome ELISA problems, it has not been applied to clinical settings because of serious problems with respect to peptide-based analysis. One of the problems is how to select a surrogate peptide from among numerous mAb-derived peptides. Several earlier methods predicted surrogate peptides using amino acid sequence information of the target mAb *in silico* analysis.^{10,11} However, this *in silico* approach provides too many candidates for a surrogate peptide and cannot evaluate the uniqueness of a surrogate peptide. These candidate peptides potentially have the same

^aDepartment of Hospital Pharmacy, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. E-mail: naitou@hama-med.ac.jp; Fax: +81 53 435 2764; Tel: +81 53 435 2623

^bDepartment of Clinical Oncology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

^cDepartment of Laboratory Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

^dSecond Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ay02087j

amino acid sequences as endogenous protein-derived peptides. Confirmation of a surrogate peptide peak in LC-MS/MS is essential for quantifying target mAb in a biological sample.

The other problems of LC-MS/MS methods are the time-consuming sample preparation process and severe ionization suppression. Although trypsin solution is commonly used for protein digestion, this digestion takes a long time to be completed. In addition, trypsin solution undergoes self-digestion leading to a decrease in the digestive efficiency of target mAb.^{12,13} An immobilized enzyme has the potential to overcome these problems.^{14,15} A protein-removal approach including immunopurification was combined with the LC-MS/MS method to overcome severe ionization suppression caused by a large amount of digests derived from endogenous proteins.^{4,5,7,9} Sample preparation for mAb quantification using LC-MS/MS could be simplified and speeded up by using an immobilized enzyme and immunopurification.

In this study, a quantitative LC-MS/MS method for nivolumab in human serum using IgG purification and immobilized tryptic digestion was developed. This analytical method was applied to cancer patients treated with nivolumab.

2. Experimental

2.1. Chemicals and materials

Nivolumab was obtained from Ono Pharmaceutical Co. (Osaka, Japan). Three candidate peptides derived from nivolumab and a stable isotope labeled surrogate peptide as internal standard (IS) were synthesized by Scrum Inc. (Tokyo, Japan). RapiGest SF surfactant was provided by Waters (Milford, MA, USA). Dithiothreitol and iodoacetamide were purchased from Thermo Fisher Scientific K.K. (Waltham, MA) and Sigma Aldrich (St. Louis, MO, USA), respectively. HPLC-grade methanol, acetic acid, and hydrochloric acid were obtained from Fujifilm Wako Pure Chemical Industries (Osaka). Drug-free pooled normal human serum (catalog number, 12181201) was obtained from Kohjin-Bio Co., Ltd (Saitama, Japan). A Protein G column (MonoSpin ProG) and an immobilized trypsin column (MonoSpin Trypsin) were purchased from GL Science (Tokyo). The sizes of through pores and meso pores of the monolithic silica solid-phase were 2 μm and 60 nm for MonoSpin ProG and 5 μm and 10 nm for MonoSpin Trypsin, respectively. The disc size (i.d.) and length of the columns were 4.6 mm and 1.5 mm, respectively. The binding capacity of human IgG for MonoSpin ProG was 400 μg , and the trypsin amount of MonoSpin Trypsin was 100 μg . The columns were stored at 4 $^{\circ}\text{C}$ until use.

2.2. Surrogate peptide selection

Nivolumab (800 ng) was solubilized by RapiGest SF surfactant and then reduction and alkylation were performed by dithiothreitol and iodoacetamide, respectively. The reduced-alkylated nivolumab was digested by immobilized trypsin. The digestion products were extracted using a Pierce C18 Spin Column (Thermo Fisher Scientific K.K.) and then 40 μL of eluent was evaporated to dryness. The residues were reconstituted with 20 μL of 0.1% formic acid and a 15 μL aliquot of sample was

injected into a nano LC system connected to a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific K.K.). Candidate peptides for surrogate peptide were searched using Proteome Discoverer software version 1.4 (Thermo Fisher Scientific K.K.) based on the FASTA database including the amino acid sequences of nivolumab digested by trypsin. The raw data obtained from the mass spectrometer were compared to the sequence information of nivolumab from the FASTA database, and then the nivolumab-derived peptides were identified from the mass spectra of digested fragments. A surrogate peptide was selected from among the candidate peptides using the following criteria: (1) the candidate peptide is derived from the variable region of nivolumab, (2) the peak of the candidate peptide is separated from serum digests peaks, and (3) the surrogate peptide has the highest peak areas among the candidate peptides meeting criteria (1) and (2).

2.3. Optimization of IgG purification and immobilized tryptic digestion

Ionization suppression was examined by comparing the peak areas of surrogate peptide in serum digests between with and without a Protein G column. The recovery rate of IgG purification was evaluated by comparing the peak areas of surrogate peptide derived from serum nivolumab between with and without IgG purification. The number of passages through an immobilized trypsin column was optimized by comparing the peak areas of surrogate peptide passing through the column once, twice, and three times. The digestion efficacy rate was evaluated by comparing the peak areas of digested-nivolumab-derived surrogate peptide with those of spiked surrogate peptide in serum digests.

2.4. Sample preparation

A summary of the present sample preparation is presented in Fig. 1. The Protein G column was equilibrated by passing a mixture of 500 μL of 50 mmol L^{-1} sodium phosphate-150 mmol L^{-1} sodium chloride at pH 7.0 through the column. Two hundred microliters of 10-fold diluted human serum with 50 mmol L^{-1} sodium phosphate-150 mmol L^{-1} sodium chloride at pH 7.0 was loaded on the Protein G column. Next, the column was washed with 500 μL of 50 mM sodium phosphate-1 mol L^{-1} sodium chloride at pH 7.0. The IgG fraction was eluted with 350 μL of 100 mmol L^{-1} glycine-hydrochloric acid at pH 2.5. To the eluents, 50 μL of 1 mol L^{-1} Tris buffer at pH 8.5 and 100 μL of 50 mmol L^{-1} ammonium bicarbonate was added. Fifty microliters of 10 mg mL^{-1} RapiGest SF surfactant solution was added and the mixture incubated at 80 $^{\circ}\text{C}$ for 10 minutes. Thirty microliters of 500 mmol L^{-1} dithiothreitol was added and the mixture incubated at 80 $^{\circ}\text{C}$ for 1 hour. Next, 50 μL of 500 mmol L^{-1} iodoacetamide and 15 μL of 1 $\mu\text{g mL}^{-1}$ IS were added, followed by incubation at room temperature for 30 minutes under light shielding. Reduced-alkylated IgG solution was passed through an immobilized trypsin column by centrifugation at 100 \times g at 37 $^{\circ}\text{C}$ for 10 minutes twice. After a second centrifugal digestion, 300 μL of 50 mmol L^{-1} ammonium bicarbonate was passed through the column to

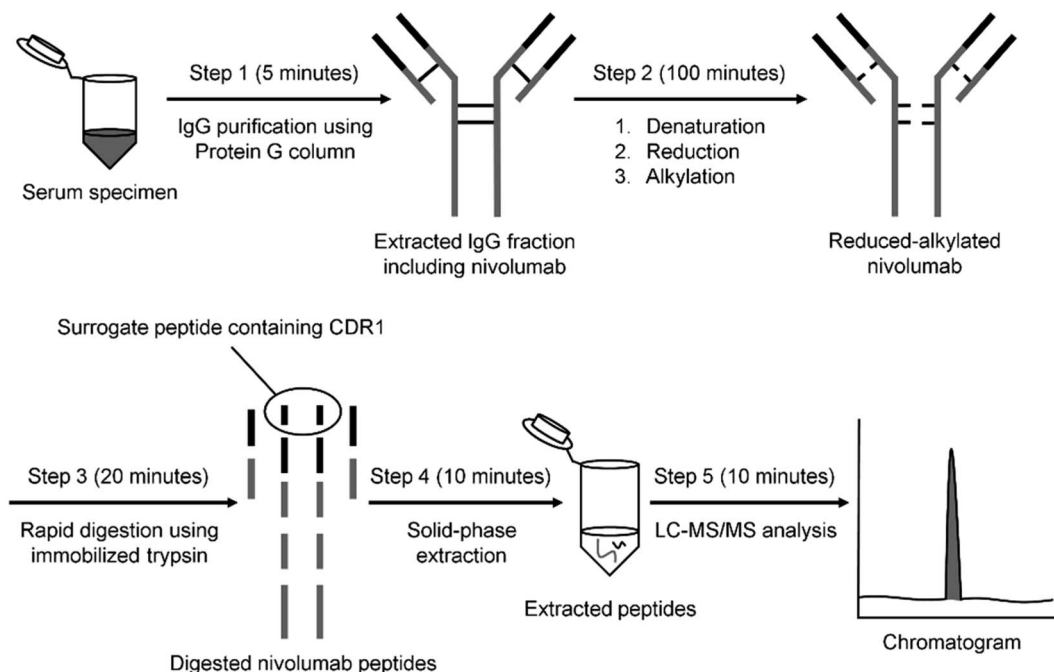


Fig. 1 Flow chart of sample preparation for nivolumab quantification.

collect the residual digests remaining in the column. To the products, 30 μL of 2 mol L^{-1} hydrochloric acid was added and the mixture centrifuged at 15 000 $\times g$ at 37 $^{\circ}\text{C}$ for 30 minutes. The supernatants were purified with a solid-phase extraction cartridge (Oasis HLB, 3 cc, 60 mg sorbent, 30 μm particle size, Waters). Polymeric solid-phases in the cartridge were activated using 1 mL of methanol and then equilibrated using 1 mL of water containing 4% acetic acid. The supernatants of serum digests were loaded on the cartridge, and then 1 mL of water containing 4% acetic acid was passed through the cartridge for washing. One milliliter of 80% methanol containing 0.5% acetic acid was passed through the cartridge and the eluents were evaporated to dryness at 35 $^{\circ}\text{C}$. The residues were reconstituted with 150 μL of 28% methanol containing 0.1% acetic acid and then centrifuged at 15 000 $\times g$ at 4 $^{\circ}\text{C}$ for 5 minutes. The supernatants were filtrated and a 10 μL aliquot of sample was injected into the LC-MS/MS system.

2.5. Instrument conditions

2.5.1. LC system. The UFLC_{XR} HPLC system consisted of a CBM-20A system controller, DGU-20A_{5R} degasser, LC-20AD_{XR} pumps, SIL-20AC_{XR} autoinjector, and a CTO-20AC column oven (Shimadzu Corporation, Kyoto, Japan). An Aeris Peptide C18 (3.0 mm i.d. \times 150 mm, 2.6 μm , Phenomenex Inc., Torrance, CA, USA) equipped with a SecurityGuard Ultra cartridge (3.0 mm i.d., Phenomenex Inc.) was used as an analytical column. The column was kept at 60 $^{\circ}\text{C}$, and a 10 μL aliquot of sample was injected into the LC system. The mobile phases consisted of 0.1% acetic acid in water (A solution) and 0.1% acetic acid in methanol (B solution). Initially, the mobile phase consisted of 28% B solution and 72% A solution for the first 2 minutes,

followed by 44% B solution/56% A solution from 2.1–4.5 minutes, 90% B solution/10% A solution from 4.6–7.0 minutes, and 28% B solution/72% A solution from 7.1–9.9 minutes. The flow rate was 0.4 mL min^{-1} from 0 to 4.5 and 9.1 to 9.9 minutes, and 0.5 mL min^{-1} from 4.6 to 9.0 minutes. The position of the switching valve was set to connect the MS/MS system from 4.0 to 5.0 and 6.5 to 9.9 minutes.

2.5.2. MS system. The column effluent was analyzed using a hybrid triple-quadrupole/linear ion-trap MS (3200 QTRAP, AB Sciex, Foster City, CA) equipped with an electrospray ionization interface in positive-ion multiple reaction monitoring mode. The interface heater was maintained at 600 $^{\circ}\text{C}$, and the ion spray voltage was 5500 V. Ion source gas 1, ion source gas 2, collision gas, and curtain gas, were set at 60 psi, 50 psi, 4 psi, and 40 psi, respectively. The amino acid sequence of surrogate peptide was ASGITFSNSGMHWVR and that of IS was ASGITFSNSGMHWVR [$\text{R}^{(13}\text{C}_6\text{ }^{15}\text{N}_4)$]. The triply protonated surrogate peptide and IS were detected as precursor ions. The monitoring ions were detected at m/z 550.8/661.4 for surrogate peptide and 554.2/666.4 for IS. Collision energies of surrogate peptide and IS were optimized at -30 V and -35 V, respectively. Dwell time was set at 500 milliseconds for surrogate peptide and IS. Data were analyzed using Analyst software version 1.6.1. (AB Sciex).

2.6. Method validation

Selectivity was confirmed by comparing the surrogate peptide peak with serum digest peaks derived from NSCLC patients without nivolumab treatment. Sensitivity was evaluated by the lower limit of quantification (LLOQ) of serum nivolumab and its accuracy and imprecision. Ten milligrams per milliliter of nivolumab prepared with saline was diluted with drug-free

pooled serum. The calibration standards were 2, 5, 10, 20, 50, 100, 150, and 200 $\mu\text{g mL}^{-1}$ in human serum. The calibration curve was constructed by plotting the peak area ratio of surrogate peptide to IS. The weighting coefficient of the calibration curve was the inverse of the concentration. Matrix factor and solid-phase extraction recovery rate were evaluated using five healthy individual sera. Matrix factor was calculated by comparing the peak areas of surrogate peptide in serum digests and in mobile phase. The solid-phase extraction recovery rate was observed by comparing the peak areas of surrogate peptide between with and without being passed through the solid-phase extraction cartridge. Carryover effect was investigated by comparing the peak areas of surrogate peptide in 2 $\mu\text{g mL}^{-1}$ nivolumab-spiked serum digests and in nivolumab-free mobile phase after injection of 200 $\mu\text{g mL}^{-1}$ nivolumab-spiked serum digests. The stability of nivolumab in human serum was investigated under three freeze–thaw cycles, storage at room temperature for 24 hours, and storage at $-80\text{ }^{\circ}\text{C}$ for 1 month. The stability of surrogate peptide in mobile phase was investigated at $4\text{ }^{\circ}\text{C}$ in an autoinjector system for 24 hours. These stabilities were evaluated by comparing the peak area ratios of surrogate peptide in the above conditions to that in the prepared condition. Intra- and inter-assay accuracies and imprecisions were determined using quality controls (QCs) of 4, 40, and 160 $\mu\text{g mL}^{-1}$. Accuracy was determined by comparing the calculated concentrations of QCs with the theoretical concentrations of QCs. Imprecision was evaluated by calculating the relative standard deviation (RSD).

2.7. Clinical application

2.7.1. Ethics and serum specimens. The present study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of Hamamatsu University School of Medicine. We obtained informed consent from all patients before collecting serum samples. Fourteen serum samples were collected from the NSCLC patients treated with nivolumab. These patients received intravenous nivolumab (Opdivo intravenous infusion,

Ono Pharmaceutical Co.) at 3 mg kg^{-1} every 2 weeks. Blood samples were gathered from a forearm vein into plain tubes just before nivolumab administration at the 2nd administration or later. The serum specimens were stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

2.7.2. Comparison between LC-MS/MS and ELISA. Quantification of serum nivolumab was performed by LC-MS/MS and ELISA (SHIKARI Q-NIVO, Matriks Biotechnology Co., Ankara, Turkey) in cancer patients treated with nivolumab. The Pearson correlation coefficient was used for evaluating the correlation between LC-MS/MS and ELISA. The regression equation was obtained by linear regression analysis using the ordinary least squares method. The statistical bias between LC-MS/MS and ELISA was evaluated by Bland–Altman analysis. All statistical analyses were performed using IBM SPSS 24 software (IBM Japan Ltd, Tokyo). Statistical significance was defined as a $P < 0.05$.

3. Results

3.1. Surrogate peptide selection

Fourteen nivolumab-derived peptides were identified using a hybrid quadrupole-Orbitrap mass spectrometer (Table 1). In three candidate peptides derived from variable regions of nivolumab, the ASGITFSNSGMHWVR peptide contains complementarity-determining region (CDR) 1. Peaks with the same retention time as ASGITFSNSGMHWVR or QVQLVESGGGVVQPGR peptide were not observed from nivolumab-free serum digests. In contrast, a peak with the same retention time as the EIVLTQSPATLSLPGER peptide peak was detected in nivolumab-free serum digests and was not separated from the EIVLTQSPATLSLPGER peptide peak. The peak areas of 1 $\mu\text{g mL}^{-1}$ ASGITFSNSGMHWVR peptide were approximately 8 times higher than those of 1 $\mu\text{g mL}^{-1}$ QVQLVESGGGVVQPGR peptide. Based on the above results, ASGITFSNSGMHWVR peptide was selected as a surrogate peptide of nivolumab.

3.2. Optimization of IgG purification and trypsin digestion

The surrogate peptide peak areas in serum digests prepared with a Protein G column were over 10 times higher than those in

Table 1 Characteristics of nivolumab-derived peptides identified by a hybrid quadrupole-Orbitrap mass spectrometer^a

Peptides	Region	Chain	Charge	Mass of [M + H] ⁺
ASGITFSNSGMHWVR	Variable region	Heavy	+3	1649.78666
QVQLVESGGGVVQPGR	Variable region	Heavy	+2	1609.86528
EIVLTQSPATLSLPGER	Variable region	Light	+2	1898.02483
WQEGNVFScVMHEALHNHYTQK	Constant region	Heavy	+4	2802.25258
GFYPSDIAVEWESNGQPENNYK	Constant region	Heavy	+2	2544.13384
VVSVLTVLHQDWLNGK	Constant region	Heavy	+3	1808.00626
TTPPVLDSDGSFFLYSR	Constant region	Heavy	+3	1901.92765
AEDTAVYYcATNDYWGQGLTVTVSSASTK	Constant region	Heavy	+3	3273.45517
ASQSVSSYLAWYQKPGQAPR	Constant region	Light	+3	2352.17447
SGTASVVeLLNNFYPR	Constant region	Light	+2	1797.89568
HKVYAcEVTHQGLSSPVTK	Constant region	Light	+4	2141.08217
VYAcEVTHQGLSSPVTK	Constant region	Light	+2	1875.92827
VDNALQSGNSQESVTEQDSK	Constant region	Light	+2	2135.97099
TVAAPSVFIFPPSDEQLK	Constant region	Light	+2	1946.02690

^a The c indicates cysteine with carbamidomethylation.

serum digests prepared without one (ESI Fig. 1†). The IgG purification recovery rate was calculated to be 90.1% ($n = 3$; RSD, 7.5%). Compared with the peak areas of surrogate peptide passing through an immobilized trypsin column twice ($n = 4$; RSD, 12.9%), the peak areas of surrogate peptide passing through once and three times were 75.9% ($n = 4$; RSD, 33.6%) and 82.4% ($n = 4$; RSD, 21.6%), respectively. The digestion efficacy rate when passing through the trypsin column twice was calculated to be 94.0% ($n = 5$; RSD, 9.2%). The peak area ratio of surrogate peptide to IS using the Protein G column and immobilized trypsin column showed slight variation (RSD, 8.1%) using serum from 5 cancer patients (the range of serum total globulin, 2.5–3.3 g dL⁻¹).

3.3. Chromatographic separation and selectivity

Fig. 2 shows the multiple reaction monitoring chromatograms of surrogate peptide and IS in human serum. A specific peak of surrogate peptide was detectable in serum digests spiked with 50 $\mu\text{g mL}^{-1}$ nivolumab. Surrogate peptide and IS peaks were observed at 4.62 minutes and were separated from serum-derived peaks. Peaks interfering with surrogate peptide and IS

were not detected from serum digests of 5 NSCLC patients without nivolumab treatment.

3.4. Method validation

3.4.1. Calibration curve and sensitivity. The correlation coefficient of the calibration curve (range, 2–200 $\mu\text{g mL}^{-1}$) was 0.996. The LLOQ for serum nivolumab was 2 $\mu\text{g mL}^{-1}$ in the present method. The intra- and inter-assay accuracies and imprecisions of LLOQ were 106.6% and 99.2%, and 11.8% and 10.5%, respectively ($n = 5$).

3.4.2. Matrix effect and solid-phase extraction recovery. The matrix factor of surrogate peptide and that corrected by IS in serum digests of five healthy subjects were 42.0% (RSD, 3.5%) and 104.0% (RSD, 3.1%), respectively. The solid-phase extraction recovery rate of surrogate peptide was 96.5% ($n = 5$; RSD, 7.5%).

3.4.3. Carryover effect. The peak of surrogate peptide was undetected in nivolumab-free mobile phase after injection of 200 $\mu\text{g mL}^{-1}$ nivolumab-spiked serum digests (ESI Fig. 2†). No carryover effect was observed in the present method.

3.4.4. Stability. The stability of nivolumab in human serum under three freeze–thaw cycles was calculated to be 104.4%

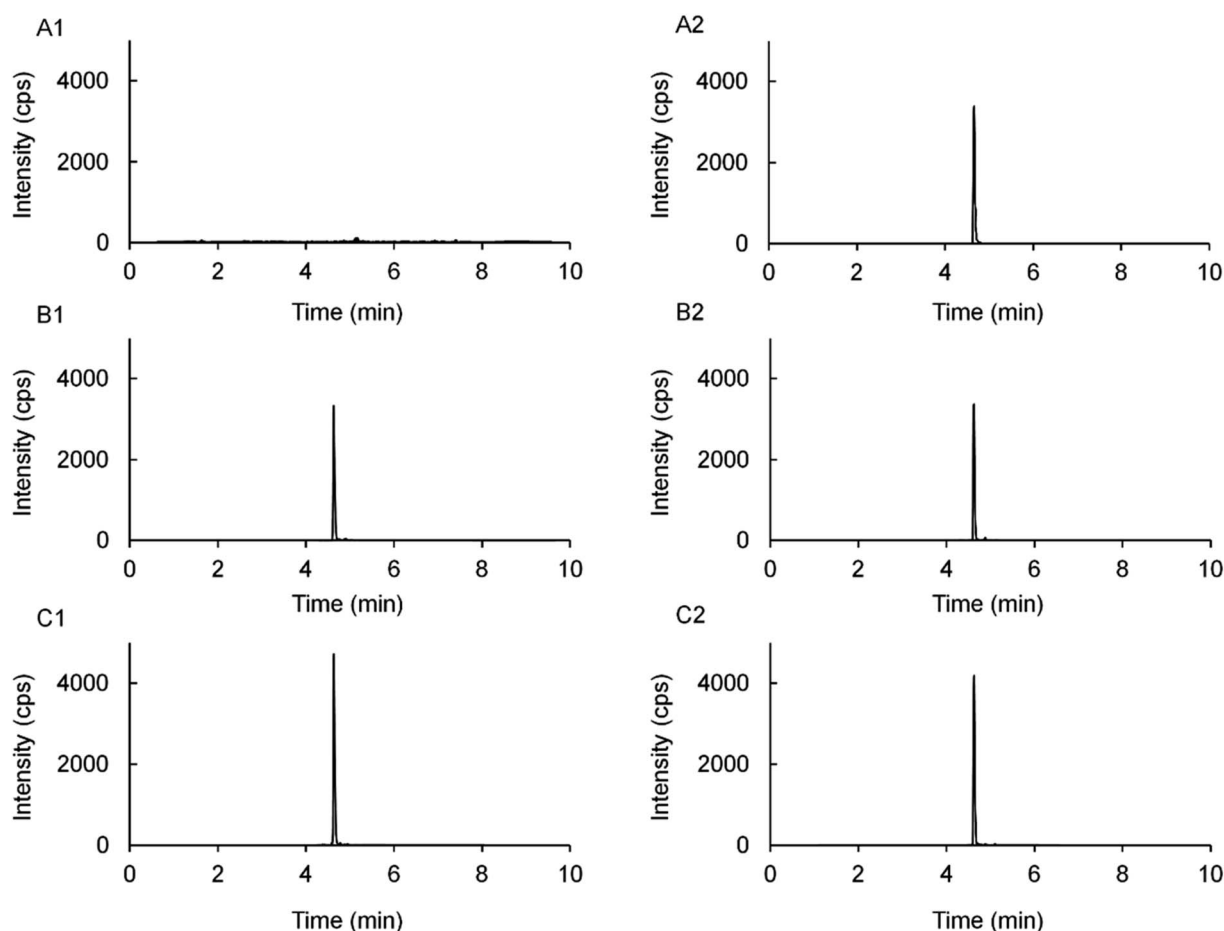


Fig. 2 Selective reaction monitoring chromatograms of surrogate peptide derived from nivolumab. (A) Nivolumab-free serum of NSCLC patient: surrogate peptide (A1) and 1 $\mu\text{g mL}^{-1}$ internal standard (IS) (A2). (B) Human nivolumab-free serum digests spiked with 50 $\mu\text{g mL}^{-1}$ nivolumab: surrogate peptide (B1) and IS (B2). (C) Serum of NSCLC patient treated with intravenous nivolumab: surrogate peptide (C1) and IS (C2). The concentration of IS corresponds to surrogate peptide concentration derived from approximately 50 $\mu\text{g mL}^{-1}$ nivolumab.

($n = 3$; RSD, 3.0%). The stabilities of nivolumab in human serum were 104.9% ($n = 3$; RSD, 6.0%) at room temperature for 24 hours and 94.3% ($n = 3$; RSD, 5.8%) at $-80\text{ }^{\circ}\text{C}$ for 1 month. The stability of surrogate peptide in mobile phase was calculated to be 104.9% at $4\text{ }^{\circ}\text{C}$ in an autoinjector system for 24 hours ($n = 3$; RSD, 6.0%).

3.4.5. Assay accuracy and imprecision. Table 2 shows the intra- and inter-assay accuracies and imprecisions for analyzing nivolumab in human serum. Intra-assay accuracy and imprecision were 92.2–104.5% and 6.8–10.0%, respectively. Inter-assay accuracy and imprecision were 96.9–103.4% and 0.9–5.0%, respectively.

3.5. Clinical application

3.5.1. Quantification of serum nivolumab in cancer patients. The enrolled patients received intravenous nivolumab once ($n = 2$), twice ($n = 4$), and more than six times ($n = 8$). Serum nivolumab concentrations in 14 cancer patients were measured within the calibration curve range and ranged from 12 to $112\text{ }\mu\text{g mL}^{-1}$ (interquartile range, 24 to $72\text{ }\mu\text{g mL}^{-1}$) (Fig. 3). The mean serum nivolumab concentration was $73\text{ }\mu\text{g mL}^{-1}$ in 8 cancer patients after the 6th nivolumab administration.

3.5.2. Comparison between LC-MS/MS and ELISA. The serum nivolumab concentrations measured by LC-MS/MS were compared with those measured by ELISA in 14 cancer patients treated with nivolumab (Fig. 4). The correlation between LC-MS/MS and ELISA was expressed by the following equation: $y = 0.88x - 0.53$ ($r = 0.92$, $P < 0.01$) (Fig. 4A). The statistical bias between LC-MS/MS and ELISA was evaluated by Bland–Altman analysis (Fig. 4B). The mean bias between LC-MS/MS and ELISA was 16.8% and its 95% confidence interval ranged from -1.5% to 35.1%.

4. Discussion

In this study, we developed a simple and rapid LC-MS/MS method for quantification of serum nivolumab using immobilized tryptic digestion coupled to IgG purification and applied it to clinical settings. The IgG purification and immobilized tryptic digestion were completed within 25 minutes. The calibration curve of serum nivolumab was linear over the concentration range of $2\text{--}200\text{ }\mu\text{g mL}^{-1}$. The serum nivolumab

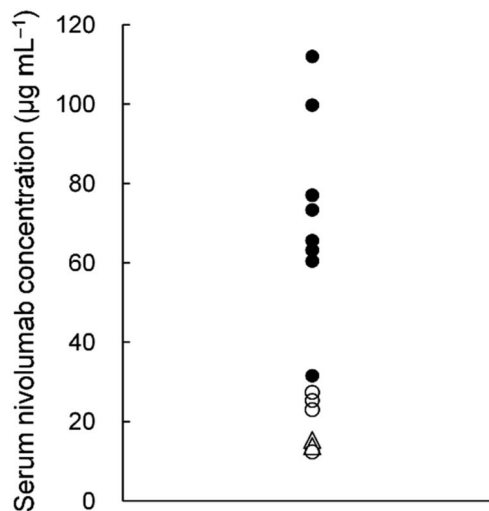


Fig. 3 Serum nivolumab concentrations in 14 non-small cell lung cancer patients. The patients were treated with intravenous nivolumab at 3 mg kg^{-1} every 2 weeks. Blood samples were collected just before nivolumab administration at the 2nd administration or later. Open triangle, open circle and filled circle represent patients administered nivolumab once, twice and more than six times, respectively.

concentrations measured by LC-MS/MS were similar to those measured by ELISA. The present method is the first report to determine human serum nivolumab using immobilized trypsin.

Surrogate peptide selection using a high-resolution mass spectrometer has an advantage in confirming the peptide uniqueness.^{16,17} The reliability of surrogate peptide could be confirmed by mass spectrometric evaluation with synthesized peptide. The present method identified 14 actual peptides using a hybrid quadrupole-Orbitrap mass spectrometer and synthesized 3 candidate peptides derived from variable regions of nivolumab. Surrogate peptide was determined according to the selection criteria.^{18,19} The ASGITFSNSGMHWVR peptide containing nivolumab CDR 1 was selected as a suitable surrogate peptide for the present quantification.

A large amount of digested endogenous proteins generates sample complexity. In an LC-MS/MS approach, pretreatment

Table 2 Intra- and inter-assay accuracies and imprecisions of nivolumab in human serum^a

Theoretical value ($\mu\text{g mL}^{-1}$)	Mean \pm SD ($\mu\text{g mL}^{-1}$)	Accuracy (%)	RSD (%)
Intra-assay (n = 5)			
4	3.69 ± 0.41	92.2	9.4
40	37.4 ± 3.8	93.5	10.0
160	167 ± 11	104.5	6.8
Inter-assay (n = 5)			
4	4.01 ± 0.04	99.8	0.9
40	38.7 ± 1.9	96.9	5.0
160	166 ± 8	103.4	4.6

^a SD, standard deviation and RSD, relative standard deviation.

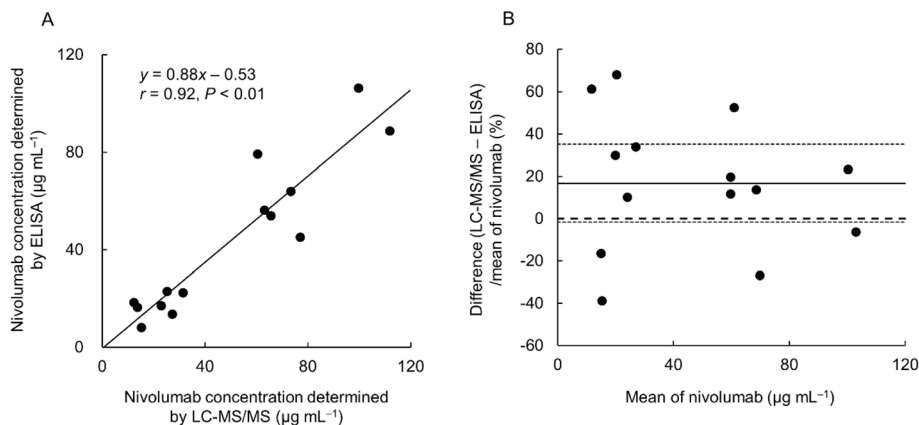


Fig. 4 Correlation between the nivolumab concentrations determined by LC-MS/MS and by ELISA using serum from 14 non-small cell lung cancer patients treated with nivolumab (A). Bland–Altman plot for the LC-MS/MS versus ELISA correlation data from 14 non-small cell lung cancer patients (B). Solid line, heavy dotted line, and dotted line in the Bland–Altman plot are bias, zero, and 95% confidence interval, respectively.

minimizing non-targeted serum digests is required to avoid ionization suppression. Immunopurification strategy using antigen–antibody reaction or IgG-specific immunocapture reduces ionization suppression by isolating target mAb. In these strategies, selective extraction of human IgGs using a spin-column type of Protein G is completed rapidly and simply in a few centrifugation steps. In the present method, extraction of IgG including nivolumab was achieved at a good recovery rate and small variation within 5 minutes using the Protein G column. Although endogenous IgGs in serum are not removed by Protein G purification, the serum nivolumab concentration was measurable with good analytical performance by reducing ionization suppression.

The digestion process commonly takes 3–7 hours using solution-phase trypsin or trypsin-binding nanoparticles.^{4,5,7} The time-consuming pretreatment is responsible for difficulty in applying LC-MS/MS to therapeutic mAb monitoring in clinical settings. In proteomics, an immobilized enzyme reactor is employed to increase digestion efficiency and minimize trypsin autolysis.^{14,15} The present method employed an immobilized trypsin spin column for rapid digestion. This trypsin column has solid-phases in which trypsin is densely immobilized on a monolithic silica surface. The density of trypsin in the solid-phase is approximately $3.3 \mu\text{g } \mu\text{L}^{-1}$, which is markedly higher than the concentration of conventional trypsin solution. This immobilized trypsin does not undergo self-digestion because solid-phase-fixed trypsin does not interact with itself.²⁰ Digestion conditions were optimized to maximize the peak areas of nivolumab-derived surrogate peptide. Digestion was completed within 20 minutes using the immobilized trypsin column. In the present method, up to 40 serum samples can be handled by each analysis on each day. The entire pretreatment procedure was completed within 5 hours.

The chromatographic analysis of surrogate peptide was finished within 10 minutes. The surrogate peptide peak was separated from the serum digest peaks by stepwise-gradient elution. Residual peptides in the analytical column were

washed out by elevating the ratio of methanol in the mobile phase, thereby minimizing non-target peptide carryover from repeated analyses. Column temperature was commonly kept at $40 \text{ }^\circ\text{C}$ for chromatographic separation for therapeutic mAb analysis.^{4,5,10} In the present method, a high temperature of $60 \text{ }^\circ\text{C}$ was selected to improve the analysis speed and separation efficiency by decreasing the mobile phase viscosity and accelerating the molecular motion of the peptide.

The calibration curve was linear over the range of 2 to $200 \mu\text{g mL}^{-1}$ and its LLOQ was $2 \mu\text{g mL}^{-1}$ in the present method. This LLOQ was sufficient to quantify serum nivolumab in NSCLC patients treated with nivolumab according to the earlier studies.^{21,22} Although a matrix effect of serum digests on surrogate peptide was observed, this effect was corrected by a stable isotope labeled peptide that had similar chromatographic and mass spectrometric profiles to the surrogate peptide. Any special consideration for serum sample storage is not required. Surrogate peptide in the mobile phase was stable at $4 \text{ }^\circ\text{C}$ for up to 24 hours. Accuracies and imprecisions for repeated analyses of intra- and inter-assay were acceptable according to the standards of an international guidance of bioanalytical method validation.²³

The present LC-MS/MS method was applied to NSCLC patients. Serum nivolumab is reported to reach a steady state after the 6th administration.²⁴ The present method was able to quantify serum nivolumab in 14 NSCLC patients within the calibration curve range, regardless of whether serum nivolumab reached the steady state. A Japanese phase II trial reported that the mean trough value of serum nivolumab at steady state was $73 \mu\text{g mL}^{-1}$ in NSCLC patients.²² Our result for serum nivolumab concentration was similar to this earlier report. The present method did not evaluate serum nivolumab in cancer patients treated with nivolumab at 240 mg every 2 weeks. However, an earlier study reported that the serum trough of nivolumab was $2\text{--}200 \mu\text{g mL}^{-1}$ in cancer patients receiving nivolumab at this fixed dose.²⁵ Additionally, serum nivolumab in the present cancer patient administered at approximately 240 mg nivolumab was measured within the calibration curve range.

The present study showed that serum nivolumab concentrations in LC-MS/MS were strongly correlated with those in ELISA in NSCLC patients. In Bland–Altman plots, the mean bias was 16.8% and its 95% confidence interval between LC-MS/MS and ELISA ranged from -1.5% to 35.1% . The 95% confidence interval including zero means that a systematic error between LC-MS/MS and ELISA was not observed, whereas the nivolumab concentrations in ELISA were slightly lower than those in LC-MS/MS. Soluble form of PD-1 was reported to be found in human serum and have the ability to bind to anti-PD-1 antibody.²⁶ Nivolumab bound to soluble PD-1 fails to bind to PD-1 on the ELISA assay plate and therefore cannot be detected by ELISA.

Our present method has several limitations. First, we did not evaluate serum IgG levels that could cause analytical variations in IgG purification and tryptic digestion. However, the present method was not affected by serum total globulin values over the range of $2.5\text{--}3.3\text{ g dL}^{-1}$ in these patients. This result demonstrates that the present method is applicable to cancer patients with normal levels of serum IgG. Second, the present method used a stable isotope labeled peptide that could not correct the pretreatment processes of IgG purification and tryptic digestion. In earlier reports, a stable isotope-labeled mAb or an animal origin IgG was used as an IS for therapeutic mAb quantification in human serum.^{27,28} In this method, the IgG extraction recovery rate and digestion efficiency were nearly 100%. The stable isotope labeled peptide could correct nonspecific adsorption of surrogate peptide to devices used for tryptic digestion.

5. Conclusions

In this study, we developed a quantitative LC-MS/MS method for nivolumab in human serum using immobilized tryptic digestion coupled to IgG purification and applied it to clinical settings. Our simple and rapid method with good analytical reliability can be helpful for the evaluation of serum nivolumab concentrations in cancer patients.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI grant number JP18H00408 and a Research Grant provided by the Japan Research Foundation for Clinical Pharmacology.

References

- 1 J. S. Lim and R. A. Soo, *Ther. Adv. Respir. Dis.*, 2016, **10**, 444–454.
- 2 N. Grebenchtchikov, A. J. Geurts-Moespot, L. Heijmen, H. W. van Laarhoven, C. M. van Herpen, A. M. Thijs, P. N. Span and F. C. Sweep, *Ther. Drug Monit.*, 2014, **36**, 765–770.
- 3 A. N. Hoofnagle and M. H. Wener, *J. Immunol. Methods*, 2009, **347**, 3–11.
- 4 M. Dubois, F. Fenaille, G. Clement, M. Lechmann, J. C. Tabet, E. Ezan and F. Becher, *Anal. Chem.*, 2008, **80**, 1737–1745.
- 5 N. Iwamoto, T. Shimada, H. Terakado and A. Hamada, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2016, **1023–1024**, 9–16.
- 6 I. van den Broek, W. M. Niessen and W. D. van Dongen, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2013, **929**, 161–179.
- 7 Q. Wang, J. Han, C. Sha, Y. Liang, Y. Sun, X. Shao, J. Sun and W. Liu, *Bioanalysis*, 2017, **9**, 1451–1463.
- 8 N. Iwamoto, A. Hamada and T. Shimada, *Anal. Biochem.*, 2018, **540–541**, 30–37.
- 9 T. T. T. N. Nguyen, U. H. Mistarz, N. Costa, A. Herbet, D. Boquet, F. Becher and K. D. Rand, *J. Pharm. Biomed. Anal.*, 2018, **159**, 384–392.
- 10 K. Irie, A. Okada, Y. Yamasaki, C. Kokan, A. Hata, R. Kaji, K. Fukushima, N. Sugioka, Y. Okada, N. Katakami and S. Fukushima, *Ther. Drug Monit.*, 2018, **40**, 716–724.
- 11 C. Hagman, D. Ricke, S. Ewert, S. Bek, R. Falchetto and F. Bitsch, *Anal. Chem.*, 2008, **80**, 1290–1296.
- 12 Z. Hu, L. Zhao, H. Zhang, Y. Zhang, R. Wu and H. Zou, *J. Chromatogr. A*, 2014, **1334**, 55–63.
- 13 M. Hu, Y. Liu, K. Yu and X. Liu, *J. Proteomics*, 2014, **109**, 16–25.
- 14 F. E. Regnier and J. Kim, *Bioanalysis*, 2014, **6**, 2685–2698.
- 15 D. Rivera-Burgos and F. E. Regnier, *J. Sep. Sci.*, 2013, **36**, 454–460.
- 16 S. M. Peterman, C. P. Dufresne and S. Horning, *J. Biomol. Tech.*, 2015, **16**, 112–124.
- 17 S. Wu, N. Tolić, Z. Tian, E. W. Robinson and L. Paša-Tolić, *Methods Mol. Biol.*, 2011, **694**, 291–304.
- 18 K. Shibata, T. Naito, J. Okamura, S. Hosokawa, H. Mineta and J. Kawakami, *J. Pharm. Biomed. Anal.*, 2017, **146**, 266–272.
- 19 H. Shida, T. Naito, K. Shibata, Y. Yamada and J. Kawakami, *Bioanalysis*, 2018, **10**, 1501–1510.
- 20 M. F. Chaplin and C. Bucke, *The preparation and kinetics of immobilized enzymes*, 1990, <http://www1.lsbu.ac.uk/water/enztech/index.html>, accessed 01/09/2018.
- 21 A. Puzkiel, G. Noé, P. Boudou-Rouquette, C. L. Cossec, J. Arrondeau, J. S. Giraud, A. Thomas-Schoemann, J. Alexandre, M. Vidal, F. Goldwasser and B. Blanchet, *J. Pharm. Biomed. Anal.*, 2017, **139**, 30–36.
- 22 Ono Pharmaceutical Co., *Summary of application data for approval of Opdivo intravenous infusion*, 2016, <http://www.pmda.go.jp/drugs/2016/P20161208002/index.html>, accessed 06/11/19.
- 23 U.S. Food and Drug Administration, *Guidance for Industry Bioanalytical Method Validation*, 2001 <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf#search=GuidanceforIndustryBioanalyticalMethodValidation>, accessed 05/01/18.

- 24 N. Yamamoto, H. Nokihara, Y. Yamada, T. Shibata, Y. Tamura, Y. Seki, K. Honda, Y. Tanabe, H. Wakui and T. Tamura, *Invest. New Drugs*, 2017, **35**, 207–216.
- 25 X. Zhao, S. Suryawanshi, M. Hruska, Y. Feng, X. Wang, J. Shen, H. E. Vezina, M. B. McHenry, I. M. Waxman, A. Achanta, A. Bello, A. Roy and S. Agrawal, *Ann. Oncol.*, 2017, **28**, 2002–2008.
- 26 S. F. Sorensen, C. Demuth, B. Weber, B. S. Sorensen and P. Meldgaard, *Lung Cancer*, 2016, **100**, 77–84.
- 27 O. Heudi, S. Barteau, D. Zimmer, J. Schmidt, K. Bill, N. Lehmann, C. Bauer and O. Kretz, *Anal. Chem.*, 2008, **80**, 4200–4207.
- 28 P. M. Ladwig, D. R. Barnidge and M. A. V. Willrich, *Clin. Vaccine Immunol.*, 2017, **24**, e00545.