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Quantification of nivolumab in human plasma by LC-MS/HRMS and LC-MS/MS, comparison with ELISA

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Abstract

Nivolumab is a fully human immunoglobulin G4 used for the treatment of several advanced solid cancers as immune checkpoint inhibitors. There are some challenges for the quantification of mAb in plasma because IgG are present intrinsically in complex biologic matrices and this determination must be based on reliable, selective, and accurate analytical methods. This study described two validated methods carried out in two separate laboratories, one developed with a triple quadrupole tandem mass spectrometry (LC-MS/MS) and the other with high resolution mass spectrometry with an orbitrap system (LC-MS/HRMS). Both methods used full-length stable isotope-labelled nivolumab-like (Arginine $^{13}\text{C}_6\text{-}^{15}\text{N}_4$ and Lysine $^{13}\text{C}_6\text{-}^{15}\text{N}_2$) as internal standard. The sample preparation was based on IgG immunocapture, then trypsin digestion was performed and one surrogate peptide was quantified in positive mode. Assays showed good linearity over the range of 5-100 $\mu\text{g/mL}$ and 5-150 $\mu\text{g/mL}$ for LC-MS/HRMS and LC-MS/MS, respectively. The limit of quantification was set at 2 and 5 $\mu\text{g/mL}$ for LC-MS/HRMS and LC-MS/MS, respectively. Acceptable accuracy (from - 13.6% to 3.0%) and precision (within 20 %) values were also obtained with both methods. The two LC-MS methods showed a very different matrix effect linked to the use of different analytical columns and elution gradients. Nivolumab plasma concentrations from 60 cancer outpatients were compared with the two mass spectrometry methods and also with a home-made ELISA method. The Bland–Altman analysis did not show any significant bias between the three methods. The Passing–Bablok linear regression analysis showed a good agreement between the three methods with a better correlation between the two mass spectrometry methods.

Key words : Nivolumab – Mass spectrometry – ELISA - Pharmacokinetics

1. Introduction

Over the last years, immune checkpoint inhibitors such as nivolumab (NIVO) have become a cornerstone of various malignancies treatment [1]. NIVO, a fully human immunoglobulin G4, can help to reinstate the antitumor immune response by targeting PD-1 receptor located on lymphocytes surface [1]. It is currently approved by the Food and Drug Administration for the treatment of advanced solid cancers such as melanoma, renal cell carcinoma, head and neck squamous cell carcinoma, urothelial cancer, colorectal cancer with microsatellite instability or hepatocellular carcinoma [2]. NIVO was firstly approved at 3 mg/kg dosing regimen every 2 weeks (Q2W). However, recent results from a quantitative clinical pharmacology approach led to the approval of new fixed dosing regimens: 240-mg flat dose Q2W and 480-mg flat dose every 4 weeks. NIVO exhibits a large interindividual variability in clinical outcomes (efficacy, safety). Besides immunologic and tumor characteristics, the moderate interindividual variability in pharmacokinetics of NIVO could also contribute to the between patient-variability to the drug response [2]. Different studies have investigated the pharmacokinetic/pharmacodynamic (PK/PD) relationship for NIVO [3-9]. All studies showed a lack of relationship between the onset of severe toxicities and plasma exposure to NIVO. In contrast, the data about the exposure-survival relationship are contradictory [3-9]. Therefore, the benefit of plasma monitoring for NIVO currently remains uncertain. The development of simple and robust analytical methods in hospital laboratory is mandatory to state on this issue.

Several ELISA methods have been published for NIVO quantification in plasma [10-12]. Two of them were used for PK/PD studies carried out in Non-Small-Cell Lung Cancer (NSCLC) patients treated with NIVO [7, 8]. However, these ELISA methods present certain limitations such as tedious/laborious assay procedure; they are time-consuming and not adaptable for multiplexing strategy such simultaneous quantification of different PD-1 antibodies (NIVO, pembrolizumab). Some assays based on liquid chromatography coupled with tandem mass spectrometry (MS) using quadripolar mass filter were previously published for NIVO [13-15]. The sample preparation proposed were based on trapping of mAb using Protein G Mag Sepharose Xtra beads or rProtein A Sepharose resin followed by trypsin digestion or nano-surface and molecular-orientation limited proteolysis [13-15]. Interestingly the critical issue of the internal standard (I.S.) for the quantification of NIVO was approached in several ways. Irie et al. did not used I.S. [13]. In another study, a synthetic peptide used as I.S, was added during the IgG elution step from the resin plate. This approach only partially

corrects variations related to sample preparation. [14]. Recently, a complex method based on the use of two I.S. was proposed: one was added at the beginning of the sample preparation to calibrate variations that arise during this step, and another one was post-column infused for the correction of matrix effect. Then both signal from I.S. were used to generate a new adjusted chromatogram for NIVO quantification in sample [15]. For an extensive investigation of PK/PD relationship for NIVO, robust MS methods with low analytical variability are mandatory. In this context, the use of a full-length stable isotope-labeled NIVO-like could contribute to minimize this analytical variability related to both the sample preparation steps and MS analysis, while being easy to handle. As far as we know, no LC/MS assay using stable isotope-labeled NIVO-like has been reported.

The objective of this work was to develop, validate and compare two LC-MS methods for the quantification of NIVO in plasma using full-length stable isotope-labeled NIVO-like as I.S. One method was developed with a triple quadrupole (QqQ) tandem mass spectrometry (MS/MS) and the other with high resolution mass spectrometry (MS/HRMS) with an orbitrap system. These two MS methods were finally compared with a previously published ELISA method [10] using 60 plasma samples from cancer outpatients treated with NIVO.

2. Materials and methods

2.1. Chemicals and reagents

NIVO (Opdivo[®], 10g/L, Bristol-Meyers Squibb, New York, NY, USA) was obtained from the hospital pharmacy. Full-length stable isotope-labelled nivolumab-like (Arginine ¹³C₆-¹⁵N₄ and Lysine ¹³C₆-¹⁵N₂) (SIL-NIVO-like), purity >95%, labeling >99%, was purchased from Promise Proteomics (Grenoble, France). The absence of a glycosylation site on the hypervariable region, where proteotypic peptides was selected, was verified by LC-MS/MS. The presence of disulfide bonds between light and heavy chains has been checked by SDS-PAGE in reduced/non reduced conditions. Based on LC-MS/MS experiments from blank and spiked samples the correct binding of SIL-NIVO-like to Protein G was verified. Stock solutions of NIVO and SIL-NIVO-like were prepared in water at 5 g/L and at 100 mg/L, respectively and stored at +4°C. Pierce[™] Protein G Spin Plate for IgG Screening from Thermo Fisher Scientific (Waltham, USA) was used as a sample cleaner. ULC/MS grade acetonitrile was obtained from Bisolve (Dieuze, France), ammonium bicarbonate from Sigma Aldrich (Darmstadt, Germany) and formic acid (FA) from Fisher Chemicals (Illkirch, France). LC-MS grade methanol was obtained from Merck-Sigma (St Louis, USA). Ultrapure

water (resistivity 18.2 mΩ.cm) was obtained using a Milli-Q Plus® system (Millipore, Molsheim, France). PBS buffer (pH 7.4, molarity 10X) was from Gibco (Thermo Fisher, Waltham, MA, USA). Trypsin Gold, Mass Spectrometry Grade and Rapid Digestion Kit-Trypsin/Lys-C were purchased from Promega (Madison, WI, USA). Drug free control human plasma was provided by the regional blood bank (EFS Rhône-Alpes, France). Low adsorption polypropylene microtubes from Dutsher (Brumath, France) were used throughout the study.

2.2. Chromatographic and mass spectrometric conditions and instrumentation

LC-MS/HRMS

The UltiMate 3000 chromatographic system including two ternary pumps and autosampler was used (Thermo Scientific, San Jose, CA). The LC system was connected to a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) via a heated electrospray ionization source (HESI) interface. Chromatography was achieved with a Luna Omega PS-C18 chromatographic column (100 x 2.1 mm, 1.6µm) maintained at 30°C and coupled with a µ-Precolumn used as on-line sample cleanup (StrataTM-X; 20 x 2,00 mm, 25 µm) both columns from Phenomenex (Torrance, CA). Pump 1 delivered at 150 µL/min through the µ-Precolumn an isocratic mobile phase composed of water and acetonitrile both with 0.1% FA (95/5; v/v). A mobile-phase gradient was used on pump 2 at flow rate 150 µL/min through the PS-C18 analytical column. The mobile phase was of 0.1% aqueous FA (A; v/v) and acetonitrile mixed with 0.1% FA (B; v/v), and the gradient elution was programmed as follows: 0–1 minutes (95% A), 1–8 minutes (from 95% to 10% A), 8–9 minutes (10% A), and 9–12 minutes (95% A). Column switching was performed using a 6-port valve to inject the sample first onto the µ-Precolumn and then to elute it in backflush mode to transfer the analytes onto the PS-C18 column. The autosampler was set at 10°C and the injection volume was 20 µL. Optimized mass spectrometer parameters were as follows: Spray Voltage at 4000 V; Vaporizer Temperature (Auxiliary Gas Heater Temperature): 300°C, Sheath Gas pressure: 30 arbitrary units (au); Ion Sweep Gas pressure: 0 au; Auxiliary Gas pressure: 10 au, Capillary Temperature: 300 °C. Nitrogen was used as collision gas in the higher-energy collisional dissociation cell and damping gas in the C-trap. Data acquisition was performed by using Parallel Reaction Monitoring (PRM) mode with mass resolution set at 70 000 FWHM. Precursor ions were selected in the quadrupole mass spectrometer with an isolation window equal to 0.7 m/z. Analysis was performed in the positive ion mode with a

normalized collision energy fixed at 20 au and a nitrogen collision pressure at 1.5 mTorr. The transitions used for NIVO peptides quantification are presented in Table 1. Data acquisition and processing were carried out using Xcalibur 2.1 software (Thermo Fisher Scientific, USA) with Qual and Quan browser.

LC-MS/MS

The chromatographic system used consisted of an Exion system with binary pumps, autosampler set at 15°C, and a column oven maintained at 40°C (Sciex, Framingham, U.S.A). Chromatographic separation of peptides was achieved on a Biozen XB-C18 chromatographic column (100 × 2.1 mm, 2.6 μm) (Phenomenex, Torrance, USA). A gradient elution program was conducted for chromatographic separation with mobile phase A (water, FA 0.1%), and mobile phase B (methanol, FA 0.1%) as follows: 0-1.5 min (100% A), 1.5-5 min (from 100 to 65 % A), 5-15 min (from 65 to 0% A) 15-19 min (0% A), 19.1-29 min (100% A). The flow rate was 100 μL/min. Analysis was performed with a 6500 QTRAP (Sciex, Framingham, U.S.A). Source conditions were optimized with the curtain gas at 20 psi, IonSpray Voltage at 5500 V, temperature at 500°C, Ion Source Gas 1 at 40 psi and Ion Source Gas 2 at 45 psi. For compound parameters, declustering potential, entrance potential and collision cell exit potential values were 60, 12 and 19 V, respectively. Analysis was performed in positive mode. The transitions used for NIVO peptides quantification were presented in Table 2. Skyline software (MacCoss,Lab, Seattle, USA) was used to process data.

2.3. Selection of peptides for quantification

The proteotypic peptides for NIVO were determined *in silico* using Skyline[®] software (<https://skyline.ms/project/home/begin.view>) and with BLAST[®] software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to verify the uniqueness of the selected peptides. The most relevant peptides, in terms of abundance, sensitivity and selectivity, were selected after analysis with LC-MS/HRMS or LC-MS/MS from samples i) after trypsin digestion of NIVO pure solution and ii) of several blank plasma prepared as described below in order to check interference.

2.4. Sample preparation for mass spectrometry methods

The preparation was based on IgG immunocapture by using the Pierce™ Protein G Spin plate. However, some steps differed between LC-MS/HRMS and LC-MS/MS method as described below. In both methods, before use, each well of the plate was washed twice with 200 µL PBS (1X) and PBS was discarded using a vacuum manifold. Twenty µL of plasma (standard, quality control, patient sample) were mixed with 80µL of PBS (1X) containing the SIL-NIVO-like (final concentration 20 µg/mL) and the mixture was distributed on the plate. The plate was then placed on an orbital shaker at room temperature. After 1 h incubation, the resin was washed three times with 200 µL PBS (1X).

Elution for LC-MS/HRMS samples

Elution was performed by using two times 150 µL elution buffer (water/acetonitrile, 50/50, v/v, with 0.1% FA), followed by a centrifugation step (1000g, 1min). The two fractions were combined and then dried at room temperature under a gentle stream of nitrogen. The residue was resuspended with 45 µL of ammonium bicarbonate (100 mM) and eluted IgG were digested for 16 h at 37°C with 2µg of trypsin Gold. After adding FA (final concentration at 1%) to stop the reaction, samples were centrifuged (5 min, 13 000 g) and 20 µL of clear supernatant were injected in chromatographic device.

Tests were also carried out using Rapid Digestion Kit-Trypsin/Lys-C with LC-MS/HRMS method. In this case the digestion was performed at 70°C for 30 min. All other steps were the same as described above.

Elution for LC-MS/MS samples

Buffer used to elute was a mix of water and acetonitrile (70/30, v/v, with 0.5% FA), and two steps of elution with 200 µL were performed before evaporation in a speed-vacuum. Resuspension was carried out with 30 µL of trypsin digestion solution. Trypsin Gold was used to digest proteins overnight at 37°C. Then, 20 µL of extracted samples were injected into LC-MS.

2.5. Method validation

The method was fully validated for linearity, sensitivity, accuracy, precision, stability, recovery, matrix effects and carry-over. The calibration curve was constructed by using six-point plasma standard (0, 5, 10, 25, 50, 75 and 100 µg/mL) with LC-MS/HRMS method and seven-point standard (0, 5, 10, 25, 50, 75 100 and 150 µg/mL) with LC-MS/MS method. Quality control (QC) for both methods including low QC (LQC), middle QC (MQC), and

high QC (HQC) at 7, 35, and 80 $\mu\text{g/mL}$ were prepared in blank human plasma. Calibration working solutions and QC samples were stored at 4°C until the end of the whole study. Evaluation of repeatability was performed with LC-MS/MS method by reanalysis of three patient samples in quadruplicate the same day. Evaluation of reproducibility was also carried out with LC-MS/HRMS method by using sample reanalysis of seven patient samples in four different days (Table 7).

The study of the linearity was based on six and four calibration curves performed in different days during the validation process with LC-MS/HRMS method and LC-MS/MS method, respectively. The calibration curves were constructed by plotting the ratio of the area of NIVO to that of SIL-NIVO-like against the concentration of calibration standards and applying weighed ($1/x$) linear least square regression.

Selectivity was demonstrated by analysis of ten different blank plasma samples obtained from healthy volunteers.

The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with an acceptable accuracy and precision ($\leq 25\%$) [16]. It was determined by analyzing three replicates of an LLOQ-standard at three different days. The upper limit of quantification (ULOQ) was defined as the highest calibration standard concentration with an acceptable precision and accuracy ($\leq 20\%$).

Carry-over effect was assessed for both MS methods by injecting triple blank samples just after the analysis of three ULOQ samples. This procedure was carried out three times consecutively. Carry-over in the triple blank sample should be $< 20\%$ of LLOQ signal.

Assessment of stability was made with the LC-MS/HRMS method. The freeze-thaw stability of NIVO was tested from patient samples following four cycles at $- 80^\circ\text{C}$. The auto-sampler stability (10 h at $+ 10^\circ\text{C}$) and a medium-term stability (5 days at $+4^\circ\text{C}$) from QC samples was tested. The long-term freezer stability at $- 80^\circ\text{C}$ was tested by re-analyzing the patient's samples three month after the first analysis.

A dilution procedure was validated with LC-MS/HRMS method if the concentration from patient sample was over the ULOQ. Plasma sample spiked with NIVO at 500 $\mu\text{g/L}$ was diluted by 1/5 in blank plasma and was extracted in sextuplicate the same day. Accuracy and precision should be below 20%.

To measure the matrix effect, pure solution of NIVO was digested and peptides were used to spike at 20 $\mu\text{g/mL}$ in one hand six different plasma samples after SPE extraction and in other hand pure distilled water. All experiments were performed in triplicate. The same

samples were then analyzed with LC-MS/HRMS and LC-MS/MS methods by comparing the NIVO signals obtained from plasma and water spiked samples.

2.6. ELISA

A home-made ELISA method previously published [10] was used for the comparison with LC-MS/HRMS and LC-MS/MS methods. All data including chemicals, reagents, sample preparation steps and validation results are available in the work of Puszkiel et al [10].

2.7. Application and method comparison

Plasma samples from 60 cancer outpatients treated with NIVO (3 mg/kg every two weeks) for NSCLC (n=38), metastatic melanoma (n=12) or metastatic renal carcinoma (n=10) were analyzed with the three methods. All patients were treated in Oncology Department of Cochin University Hospital. Blood samples were collected into 5 ml lithium heparinized Vacutainer tubes just prior to the next administration of NIVO (trough concentration). Patient samples were centrifuged ($3082 \times g$, 10 min, $4^{\circ} C$) within 2 hours after collection; then plasma was transferred to propylene tubes and stored $-20^{\circ}C$ until analysis. This study was approved by the local Review Board for Oncology.

Statistical analysis was performed using Medcalc software, version 7.2.1.0. A nonparametric regression was used to assess the relationship between two analytical methods [17]. The regression equation was expressed with the 95% confidence interval for the estimates of slope and intercept. The scatter of the result from the patient samples between methods was also shown [18]. The samples below the LLOQ of either method were excluded and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Selection of proteotypic peptides

The identification of proteotypic tryptic NIVO peptides by Skyline[®] software showed three potential surrogate peptides in heavy- or light-chain (table 1). Two peptides (AEDTAVYYCATNDDYWGQGTLVTVSSASTK (HC12 located on HCD3) and

FSGSGSGTDFTLTISSLEPEDFAVYYCQQSSNWPR (LC7 on LCDR3)) were not detected with both MS methods, probably in connection with chromatographic condition not suitable for peptides of this size and/or detection conditions (charge heterogeneity, amino-acids modifications...). Thus, only one peptide (ASGITFSNSGMHWVR - named throughout the document « ASGI ») was used as surrogate peptide for the quantification of NIVO. This peptide (MW 1649.8 g/mol) is located on the heavy chain (HC3) on HCDR1 and several amino acids (G₂₆, I₂₇, N₃₁, G₃₃) are involved in interactions with PD1 [19].

ASGI was detected with two major state of charge at +3 (ASGI³⁺: 550.5999 m/z) and at +2 (ASGI²⁺: 825.3963 m/z) and ASGI³⁺ was clearly more abundant than ASGI²⁺ with both MS methods (Fig. 1). In previous works, ASGI was also the only peptide selected for the quantification of NIVO [13-15].

3.2. Validation results

3.2.1. Selectivity with LC-MS/HRMS method

A first sample preparation method has been developed using a Rapid Trypsin/Lys C mixture for IgG digestion. However, a weak and variable signal was observed during the analysis of the peptide ASGI in PRM mode. This was related to the interference with a compound generating an ion at 550.83978 m/z in the digestion buffer solution. This compound was present in large quantities relative to the concentration of NIVO and had the same retention time as the peptide ASGI. Thus, the interfering compound essentially filled the C-Trap to the detriment of the ASGI peptide. The resolution (0.7 FWHM) set on the quadrupole before the C-Trap did not allow selecting only the ion of the ASGI peptide. The replacement of Rapid Trypsin/Lys C with Trypsin Gold, Mass Spectrometry Grade has eliminated this interference (Fig. 2). Then, the analysis of ten plasma from treatment NIVO-naive patients did not show any interference (Fig. 3).

3.2.2. Selectivity with LC-MS/MS method

The analyses of ten individual human plasma blank samples has revealed the presence of an interference that is a peptide with a close m/z and similar SRM transitions, eluting at the same retention time as the ASGI peak. The response of this interfering compound was relatively high and will contaminate the signal of ASGI peptide, and thus the quantification, if it was not separated. Thus, the gradient elution was modified in order to separate correctly

the ASGI peptide to its interference as shown in figure 4. Selectivity was then rechecked with 10 plasma from patients not treated with NIVO.

3.2.3. Calibration, accuracy, precision and LLOQ

Quantification was carried out with the most intense ions corresponding to $y_{10}^{2+} + y_{11}^{2+}$ and $y_{9}^{+} + y_{11}^{2+} + y_{13}^{2+}$, for LC-MS/HRMS and LC-MS/MS, respectively. The linearity of the LC-MS/HRMS method was evaluated by analyzing five calibration standards using the linear regression model. For all concentrations including LLOQ, inter-day precision and accuracy were within 13% for LC-MS/HRMS (Table 3). The linearity of the LC-MS/MS method was evaluated by analyzing calibration standards in at least 3 replicates using the linear regression model. For all concentrations, inter-day precision and accuracy were within 20% (Table 4).

The within-run and between-run accuracy for QC samples (LLOQ, LQC, MQC and HQC) are reported in Table 5. The LLOQ was determined to be 2 $\mu\text{g/mL}$ for LC-MS/HRMS method, and 5 $\mu\text{g/mL}$ for LC-MS/MS method. Accuracy and precision were within 15% for the 3 levels of QC with LC-MS/HRMS method. Accuracy was within 12 % and precision was within 18% for the 3 levels of QC with LC-MS/MS method. For both MS methods accuracy and precision were lower than 14% for the LLOQ. Reproducibility and repeatability were also assessed on samples from patients treated with NIVO. The results are similar to the performances obtained with QC (Tables 6 and 7).

Overall, all the results for precision and accuracy met the validation criteria, which proves both the reproducibility and reliability of the MS methods.

3.2.4. Matrix effects

Matrix effect (ME) was evaluated with both MS methods on the same samples. Interestingly, the results were very different between MS methods since ME was -90% (from -94% to -83%) and $+13\%$ (from $+6\%$ to $+23\%$) with LC-MS/HRMS and LC-MS/MS, respectively. Additional experiments were performed in order to explain the reason for this difference. Two other devices were tested with the same chromatographic conditions (column, mobile phase and gradient) used with the LC-MS/HRMS method. Both apparatus were triple quadrupole mass analyser. The first was a TSQ Quantiva (ThermoFisher, USA) with the same ion heated electrospray ionization source interface than the Q-Exactive Plus and the second was a Xevo-TQ (Waters, USA) with a z-spray source interface. The ME was from -90% to -84% with the TSQ Quantiva and was from -73% to -56% with the

Xevo-TQ. This data showed that the high ME observed using the chromatographic conditions with the LC-MS/HRMS method came from an alteration of ionization process into the source interface rather than from the filling of the C-Trap by matrix compounds. Digestion of endogenous IgG after immunocapture step generate numerous peptides and some were probably not separated chromatographically from the NIVO surrogate peptide with the LC-MS/HRMS method while the use of another analytical column associated with longer elution gradient (run time 12 min vs 29 min) made it possible to separate the compounds with the LC-MS/MS method. However, the excellent sensitivity of the LC-MS/HRMS device and the use of a labeled internal standard makes it possible to obtain a LOQ of 2 µg/mL.

3.2.5. Carryover and sample stability

When blank samples were injected just after ULOQ sample, signal of NIVO was lower than 10% of LLOQ signal and signal of SIL-NIVO-like was lower than 5% of mean signal of the lower standard). Thus, no significant carry-over was observed with both MS methods.

In all conditions tested in the present stability study, the peak area ratios (condition tested/condition initial) of NIVO concentrations were $\geq 85\%$. Thus, NIVO was stable in post-extraction conditions tested (10 h on the auto-sampler at +10°C and five days at +4°C). Furthermore, NIVO was stable in human plasma at -80°C for at least 6 months and after four freeze-thaw cycles. These data are in agreement with those previously reported [15].

3.3. Comparison of methods based on samples from treated patients

The three methods were used to assay NIVO in plasma from 60 cancer outpatients treated with NIVO. Forty-six concentrations were within the range of the calibration curve for the three methods, with values ranging from 5 to 60 µg/mL. This range of concentrations was in accordance with trough concentrations expected [7,8] for the recommended daily dose of 3 mg/Kg. The 14 remaining plasma samples were collected in NSCLC patients before treatment start (n=10) or 4 months after treatment discontinuation. For eleven samples no signal was observed with the three methods. For two additional samples, no signal was monitored with MS methods while concentrations at 5.5 and 13.5 µg/mL were obtained with ELISA method probably related to an interference with the immunologic assay. Finally, for one sample, both MS methods provided a concentration of 5 µg/mL while no signal was

observed with ELISA method. For two patients a clear discordance between both MS methods and ELISA was observed (case 1: 13 and 18 $\mu\text{g/mL}$ for MS and 35 $\mu\text{g/mL}$ with ELISA; case 2: 17 and 19 $\mu\text{g/mL}$ for MS and 58 $\mu\text{g/mL}$ with ELISA). The patients were only treated with NIVO so a cross reaction with another mAb is not an option. The most likely hypothesis was an interference from an endogenous compound with the antibody used in the home-made ELISA method. Passing–Bablok regression and Bland–Altman analysis between the three methods are presented in Fig. 6. The formula of regression equations was: LC-MS/HRMS = 0.94 (95% CI: 0.81–1.05) \times ELISA method – 1.50 (95% CI: - 4.02 – + 1.77). LC-MS/MS = 1.09 (95% CI: 0.98–1.19) \times LC-MS/HRMS method + 0.46 (95% CI: - 1.83 – + 2.78). LC-MS/MS = 0.99 (95% CI: 0.87–1.13) \times ELISA method – 1.06 (95% CI: - 5.50 – + 2.77). The Bland–Altman analysis did not show any significant bias between the three methods. The mean bias and 95% CI on the Bland-Altman analysis were: ELISA vs LC-MS/HRMS = 4.3 (-11.1 to 19.7); ELISA vs LC-MS/MS = 2.0 (-13.3 to 17.2); and LC-MS/HRMS vs LC-MS/MS = -2.2 (-11.6 to 7.2). The Passing–Bablok linear regression analysis showed also a good agreement between the three methods. These results highlight that the three methods are interchangeable. Beyond a better selectivity, MS methods also present other advantages compared to ELISA method. The use of a sample preparation protocol without capture antibody provides a lower analytical variability and less biases in the quantification. The use of a full-length stable isotope labelled NIVO-like standard is otherwise renowned as optimal for reaching an accurate quantification. Indeed, when added to a sample, it allows to compensate for precision in the extraction protocol and LC retention times since it behaves exactly the same as the native analyte [20]. MS methods are less time-consuming because of a rapid and easy pretreatment step. Furthermore, LC-MS/HRMS methods exhibit a lower LLOQ (2 $\mu\text{g/mL}$) compared to ELISA (5 $\mu\text{g/mL}$) in the present work [10]. Recently, the quantification of NIVO by ELISA has been published in serum and cerebrospinal fluid with a very low LOQ at 1 ng/mL [12]. A threshold value of 10 $\mu\text{g/mL}$ of NIVO would allow for >90% of maximum achievable receptor occupancy of circulating T lymphocytes T [21]. A recent study proposes a target concentration of 4.5 $\mu\text{g/mL}$ [22], which suggests that the use of MS methods would be more relevant for the investigation of exposure-survival relationship. Finally, these MS methods could be adaptable to the development of a multiplexing quantification of two anti-PD1 antibodies, NIVO and pembrolizumab. Taken together, these different elements argue for the implementation of MS methods in hospital laboratories for NIVO monitoring in plasma.

4. Conclusion

We have developed and validated new LC- MS/MS and LC-MS/HRMS bioanalytical methods for the quantification of NIVO in human plasma. Both methods were based on a sample preparation using an IgG immunocapture. Completing all of the steps of sample preparation (except digestion time) was relatively rapid since requiring approximately 4 hours. Additionally, these methods are the first that used a full-length stable isotope-labeled NIVO-like as internal standard. For both mass spectrometry methods this internal standard containing proteotypic peptides derived from NIVO's originator sequence was used. The addition to the plasma sample before extraction allowed to correct the loss of analyte because of sample preparation (extraction from sample, variability of the digestion,...) and MS response (matrix effect). The use of the SIL-NIVO-like conferred a robustness of both MS assays. The results demonstrated that these MS methods are simple and sensitive in analysing NIVO in plasma from patient treated by this immunotherapy. Finally, an excellent correlation was observed between these two methods and an ELISA method. Taken together, these different elements support the use of LC-MS/MS and LC-MS/HRMS methods to investigate pharmacokinetics of NIVO in PK/PD studies or to perform plasma drug monitoring in daily clinical practice.

Declaration of competing interest

P. Bros, D. Lebert and G. Picard work for the company Promise Proteomics.

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Figure 1. Chromatogram of nivolumab with LC-MS/HRMS method (surrogate peptide ASGITFSNSGMHWVR) using full scan mode according to the state of charge: ASGI³⁺: 550.5999 m/z and ASGI²⁺: 825.3963 m/z.

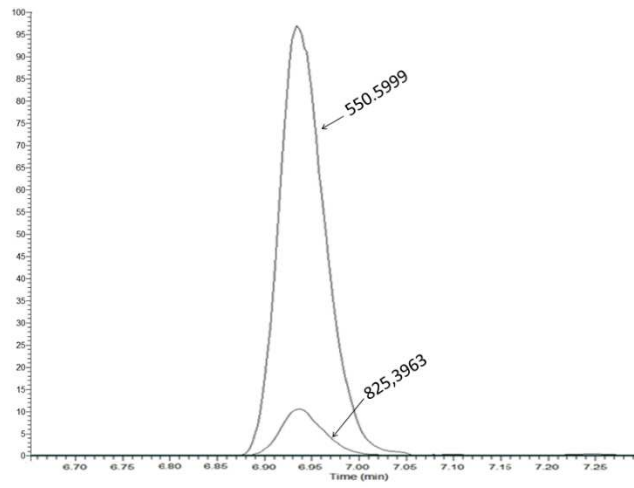
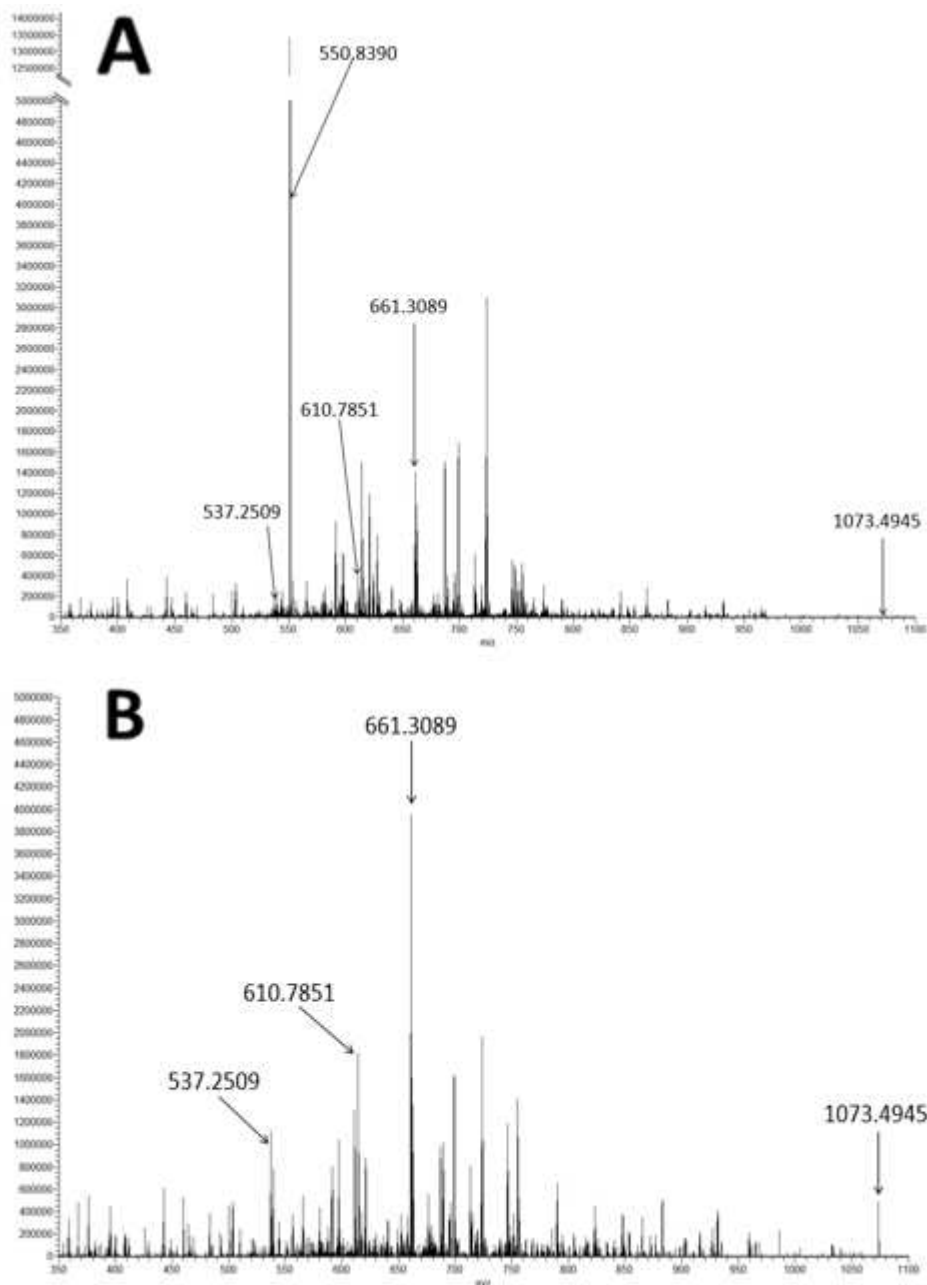
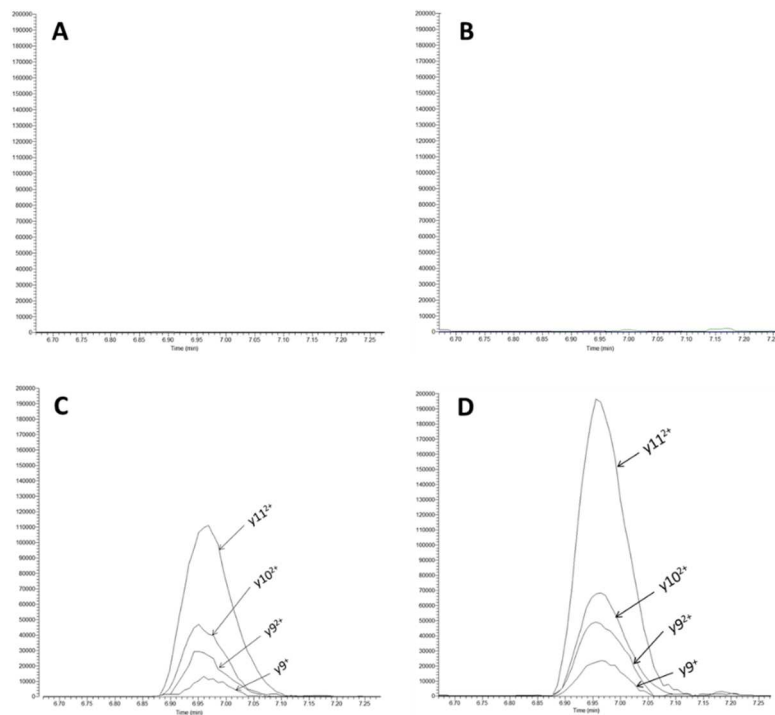


Figure 2. Mass spectrum acquired by full MS/data dependent mode from plasma spiked with 50 $\mu\text{g/mL}$ of nivolumab using (A) Rapid Trypsin/Lys C and (B) Trypsin Gold for sample preparation.



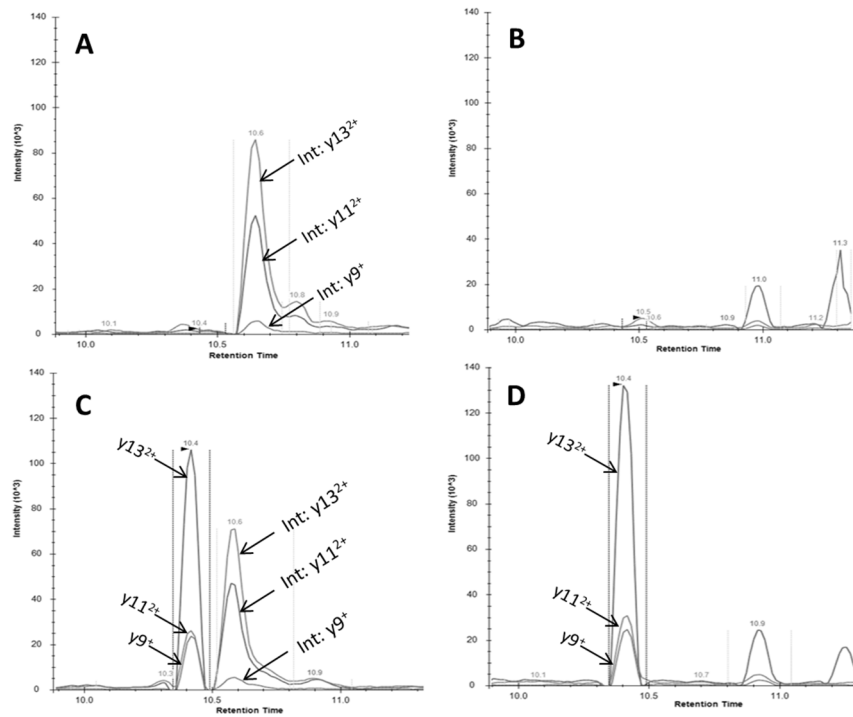
Precursor ion selected at m/z 550.5999 (corresponding to ASGI^{3+} surrogate peptide) with a isolation window set at 0.7 m/z for quadrupolar mass analyzer, HCD collision cell fixed at 15 eV and orbitrap resolution set at 70 000.

Figure 3. Chromatograms of nivolumab (NIVO) (left) and internal standard (full-length stable isotope-labeled nivolumab-like; SIL-NIVO-like) (right), obtained with LC-MS/HRMS method. A and B: double blank sample; C: NIVO at 5 $\mu\text{g/mL}$ (lower standard) and D: SIL-NIVO-like at 20 $\mu\text{g/mL}$.



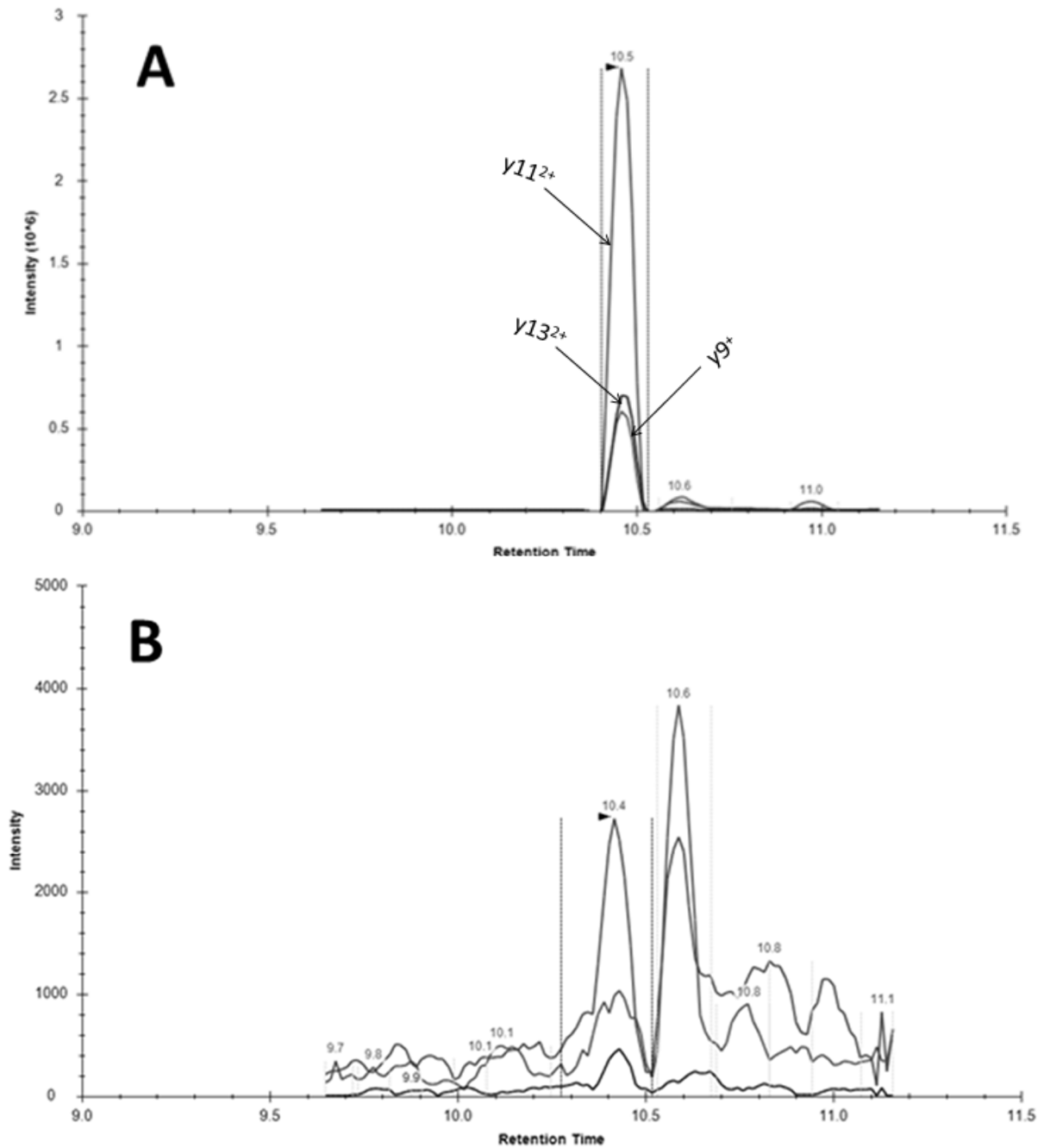
Surrogate peptides followed for NIVO and SIL-NIVO-like: ASGITFSNSGMHWVR. Product ions for NIVO: y_{11}^{2+} : 661.3089, y_{10}^{2+} : 610.7851, y_9^{2+} : 537.2509, y_9^+ : 1073.4945 and for SIL-NIVO-like: y_{11}^{2+} : 666.3131, y_{10}^{2+} : 615.7892, y_9^{2+} : 542.2550, y_9^+ : 1083.5028.

Figure 4. Chromatograms of nivolumab (NIVO) (left) and internal standard (full-length stable isotope-labeled nivolumab-like; SIL-NIVO-like) (right), obtained with LC-MS/MS method. A and B: double blank sample; C: NIVO at 5 $\mu\text{g/mL}$ (lower standard) and D: SIL-NIVO-like at 20 $\mu\text{g/mL}$. Retention time of ASGI peptide and plasma interference were 10.4 and 10.6 min, respectively.



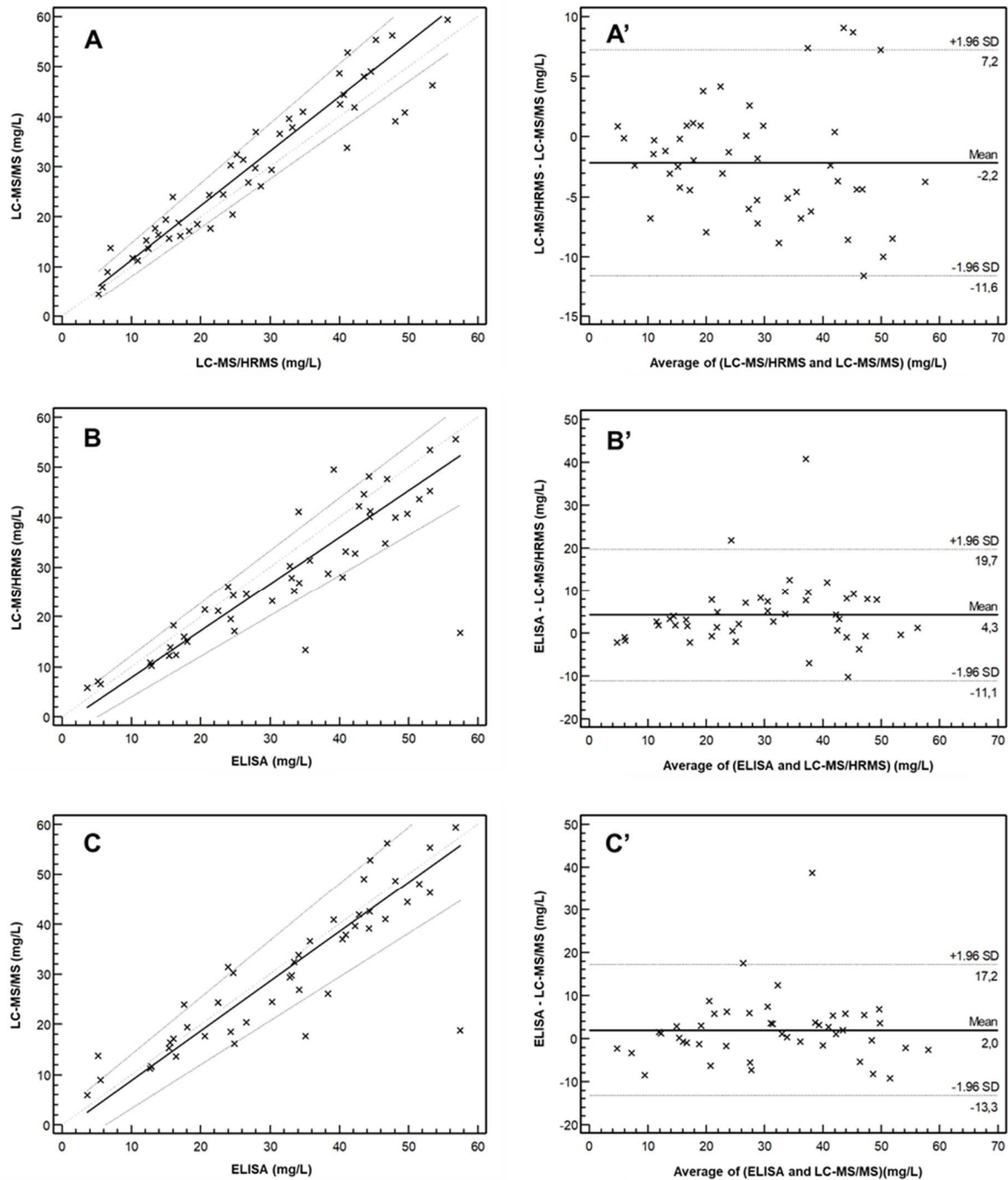
Transition monitored (m/z): 550.6 \rightarrow 1073.5 (y9⁺), 550.6 \rightarrow 746.4 (y13²⁺) and 550.6 \rightarrow 661.3 (y11²⁺). Int.: interfering ion.

Figure 5. Study of carryover using the LC-MS/MS method. Chromatograms of upper standard at 150 $\mu\text{g/mL}$ after three injections (A) and blank (B) analyzed just after.



Retention of surrogate peptide: 10.4 min; Transition monitored (m/z): 550.6 \rightarrow 1073.5 (y_9^+), 550.6 \rightarrow 746.4 (y_{13}^{2+}) and 550.6 \rightarrow 661.3 (y_{11}^{2+}). Int.: interfering ion.

Figure 6. Analysis of 46 patient sample with the three methods. Passing–Bablok regression (left) and Bland–Altman (right) analysis between the LC-MS/HRMS method and the LC-MS/MS method (A, A'), the LC-MS/HRMS method and the ELISA method (B, B') and the LC-MS/MS method and the ELISA method (C, C').



For Passing–Bablok analysis, the solid and dashed lines indicate the regression line and confidence interval for the regression line, respectively. For Bland–Altman analysis, the solid line indicates the mean difference between the methods.

Table 1. Surrogate peptides used for Nivolumab (NIVO) quantification by LC-MS/HRMS method using the internal standard (SIL-NIVO-like).

Compound	Selected peptide	Precursor ion		Product ion		
		(m/z)	charge	Ion	(m/z)	charge
NIVO	ASGITFSNSGMHWVR	550.5999	+3	y9	1073.4945	+1
				y11	661.3089	+2
				y10	610.7851	+2
				y9	537.2509	+2
SIL-NIVO-like (I.S.)	ASGITFSNSGMHWVR	553.9360	+3	y9	1083.5028	+1
				y11	666.3131	+2
				y10	615.7892	+2
				y9	542.2550	+2

I.S.: Internal Standard (full-length stable isotope-labeled nivolumab-like); Amino acid in bold was stable isotope-labeled

Table 2. Surrogate peptides used for Nivolumab (NIVO) quantification by LC-MS/MS method using the internal standard (SIL-NIVO-like).

Compound	Selected peptide	Precursor ion		Product ion		
		(m/z)	charge	Ion	(m/z)	charge
NIVO	ASGITFSNSGMHWVR	550.6	+3	y9	1073.5	+1
				y13	746.4	+2
				y11	661.3	+2
SIL-NIVO (I.S.)	ASGITFSNSGMHWVR	553.9	+3	y9	1083.5	+1
				y13	751.7	+2
				y11	666.3	+2

I.S.: Internal Standard; Amino acid in bold was stable isotope-labeled

Table 3. Inter-day validation of the determination of nivolumab in plasma by LC-MS/HRMS method (n = 5). Result obtained for the six standards.

Spiked ($\mu\text{g/mL}$)	Quantifying ions ($y10^{2+} + y11^{2+}$)		
	Found ($\mu\text{g/mL}$) (mean \pm s.d.)	Precision (%)	Accuracy (%)
5	4 +/- 1	12.7	86.4
10	9 +/- 1	12.5	93.1
25	25 +/- 3	10.0	100.0
50	52 +/- 4	5.3	103.0
75	76 +/- 5	6.1	101.6
100	99 +/- 3	3.1	98.5

Table 4. Inter-day validation of the determination of nivolumab in plasma by LC-MS/MS method (n = 3). Result obtained for the seven standards.

Spiked ($\mu\text{g/mL}$)	Quantifying ions ($y9^+ + y11^{2+} + y13^{2+}$)		
	Found ($\mu\text{g/mL}$) (mean \pm s.d.)	Precision (%)	Accuracy (%)
5	4 +/- 1	19.3	86.8
10	10 +/- 1	3.2	94.9
25	24 +/- 2	8.9	97.5
50	51 +/- 4	8.3	102.8
75	70 +/- 2	2.3	92.7
100	90 +/- 15	17.1	89.5
150	146 +/- 14	9.5	97.4

Table 5. Assessment of accuracy and precision for nivolumab in plasma using either the LC-HRMS or LC-MS/MS method. Result obtained for the three level of quality control and lower limit of quantification (LLOQ).

	Concentration ($\mu\text{g/mL}$)		Precision (%)		Accuracy (%)
	Spiked	Found (mean \pm s.d.)	Within-day	Between-day	
LC-HRMS	2 (LLOQ)	2.2 +/- 0.3	11.4	13.8	107.8
	7	6 +/- 1	5.7	11.7	89.4
	35	33 +/- 4	5.5	11.9	94.5
	80	78 +/- 9	2.6	13.1	96.8
LC-MS/MS	5 (LLOQ)	4.6 +/- 0.4	3.3	8.5	91.1
	7	6 +/- 1	13.1	15.9	88.0
	35	39 +/- 6	12.4	16.3	111.5
	80	76 +/- 9	9.0	17.8	95.0

Table 6. Measure of repeatability using sample reanalysis of three patient samples prepared in quadruplates with the LC-MS/MS method.

Patient	Found ($\mu\text{g/mL}$) (mean \pm s.d.)	Repeatability (%)
P26	24 +/- 2	9.0
P27	12 +/- 1	11.9
P28	15 +/- 2	9.6

Table 7. Measure of reproducibility using sample reanalysis of seven patient samples in four different days with the LC-MS/HRMS method.

Patient	Found ($\mu\text{g/mL}$) (mean \pm s.d.)	Reproducibility (%)
P1	42 +/- 5	12.0
P4	35 +/- 4	10.4
P8	55 +/- 4	6.5
P14	19 +/- 2	12.2
P19	37 +/- 4	11.5
P20	< 2	-
P24	19 +/- 1	3.5

Graphical abstract

