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Enzyme linked immunosorbent assay for the quantification of nivolumab and pembrolizumab in human serum and cerebrospinal fluid

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ABSTRACT

Immunotherapy with monoclonal antibodies targeting the programmed-death-1 (PD-1) receptor has become standard of care for an increasing number of tumor types. Pharmacokinetic studies may help to optimize anti-PD-1 therapy. Therefore, accurate and sensitive determination of antibody concentrations is essential. Here we report an enzyme linked immunosorbent assay (ELISA) capable of measuring nivolumab and pembrolizumab concentrations in serum and cerebrospinal fluid (CSF) with high sensitivity and specificity. The assay was developed and validated based on the specific capture of nivolumab and pembrolizumab by immobilized PD-1, with subsequent enzymatic chemiluminescent detection by anti-IgG4 coupled with horse radish peroxidase (HRP). The lower limit of quantification for serum and CSF was 2 ng/mL for both anti-PD-1 agents. The ELISA method was validated and showed long term sample stability of >1 year. This method is reliable, relatively inexpensive and can be used in serum and CSF from pembrolizumab and nivolumab treated patients.

1. Introduction

Nivolumab and pembrolizumab are both monoclonal antibodies against Programmed-Death-1 (PD-1), which received FDA and EMA approval for immunotherapeutical treatment of a wide range of tumors including non-small cell lung cancer (NSCLC), melanoma, renal cell, urothelial, and microsatellite instability (MSI) high colorectal cancer. In the phase III trials both compounds showed better response rates with increased overall and progression free survival compared to standard chemotherapy [1,2]. Furthermore, nivolumab and pembrolizumab were associated with fewer high-grade treatment-related adverse events than other second-line therapy [3]. Little is known, however, about the impact of immunotherapy in patients with metastatic disease to the central nervous system. Clinical trials of immunotherapy excluded patients with active brain metastases due to a poor prognosis and uncertainty about the ability of the drugs to cross the blood brain barrier (BBB). However, current studies suggest that systemically administered immunotherapeutic antibodies demonstrate a similar durable response in the brain as in extra-cerebral sites [4]. Studies with other monoclonal antibodies indicate that median concentrations of monoclonal antibodies may be up to 400-fold lower in the central nervous system (CNS) than in serum [5], due to the BBB limiting penetration of molecules with molecular weights up to 200 kDa (nivolumab 144 kDa, pembrolizumab 146 kDa) [4–6]. To the best of our knowledge no data has been published of nivolumab and pembrolizumab levels in cerebrospinal fluid (CSF). CSF is relatively easily accessible, and clinical studies suggest that drug concentrations in CSF are reasonably accurate in predicting CNS exposure [9]. Therefore, CSF may be used as a surrogate for the interstitial fluid (ISF) in the CNS and may be used for assessing CNS exposure because tumor biopsies are considered unethical to collect for pharmacokinetic purposes.

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Monitoring of nivolumab and pembrolizumab concentrations in serum and CSF may enable individualized treatment strategies and lead to a better understanding of pharmacokinetic (PK) –pharmacodynamic (PD) effect relationships of these agents. Puszkiel et al. recently reported the development and validation of an ELISA for the quantification of nivolumab in plasma from NSCLC patients [10]. This assay has a lower limit of quantification (LLQ) of 5 μg/mL. Although this is sensitive enough for the quantification of trough plasma levels, a more sensitive assay is needed for the quantification in CSF. A five-fold more sensitive Liquid Chromatography–Mass Spectrometry (LC/MS) method has been developed that shows a LLQ of 0.977 μg/mL [11]. Although this method is more sensitive, it may still be not possible to accurately determine trough concentrations in CSF. In addition, LC/MS is unable to show if the measured antibodies are functionally active. Furthermore, this assay relies on costly lab equipment that is not readily available at standard clinical laboratories. When properly optimized, chemiluminescent ELISA is one of the most sensitive immunoassays available with typical detection ranges of 0.01–0.04 fmole per mL [12]. Here, we report the successful development and validation of an ELISA with a lower limit of quantification of 2 ng/mL, which enables the accurate quantification of both nivolumab and pembrolizumab in serum and CSF.

The applicability of the presented assay is demonstrated with the analysis of serum and CSF samples from cancer patients treated with these drugs.

2. Materials and methods

2.1. Reagents and chemicals

BD Vacutainer® SST II 5 mL tubes were obtained from Becton Dickinson (Franklin lakes, NJ, USA). Ficoll-paque™PLUS was obtained from General Electric Healthcare (Little Chalfont, UK). Nunc MaxiSorp™ white 96-well plates were purchased from VWR (Amsterdam, the Netherlands). Phosphate buffered saline (PBS) was purchased from GIBCO BRL (Gaithersburg, MD, USA). Protifar Plus low fat milk powder (ELK) was from Danone (Amsterdam, the Netherlands). Eppendorf® LoBind micro-centrifuge 2.0 mL tubes, bovine serum albumin (BSA), fetal calf serum (FCS), glycerol, thimerosal, and Tween-20 were purchased from Sigma (St. Louis, MO, USA). PBSTF consisted of PBS with 0.1% (v/v) Tween-20 and 1% (v/v) Ficoll. Ipilimumab, nivolumab and pembrolizumab were a kind gift from the Antoni van Leeuwenhoek hospital pharmacy. Mouse anti-human IgG4 Fc antibody-HRP conjugate originated from Thermo Fisher (Landsmeer, the Netherlands) as 200 μg lyophilized powder per vial, which was stored at −20 °C after reconstitution with 200 μl of 50% (v/v) glycerol, 0.05% (w/v) thimerosal, and 1% (w/v) BSA. Recombinant human PD-1 (His Tag) protein was purchased from Sino Biological Inc. (Beijing, China) as 100 μg of lyophilized powder, which was stored at −80 °C in small aliquots after reconstitution with 5.0 mL PBS. Pierce™ standard Electro Chemical Luminescence (ECL) western blotting substrate was from Pierce (Waltham, MA, USA). The ECL reagent PeroxyGlow™ was from Trevigen (Gaithersburg, MD, US). Biorad Clarity ECL was from Biorad (Veenendaal, the Netherlands). Unless stated otherwise, serum used was pooled from 6 healthy human volunteers.

2.2. Nivolumab and pembrolizumab concentrations in the clinical stocks

The concentrations of nivolumab and pembrolizumab in the clinical stock vials were determined spectrophotometrically at 280 nm with a DS-11 (DeNovix, Wilmington, DE, USA) using the following formula:

$$c_{Ab} = 10 \cdot A_{280\,nm}/(\varepsilon_{Ab*L})$$

A_{280} = measured absorbance of nivolumab and pembrolizumab solution at 280 nm

c_{Ab} = concentration of nivolumab and pembrolizumab (mg/mL)

ε_{Ab} = extinction coefficient of human IgG_4 ($13.6\ A^{280\,nm} \cdot 1\%^{-1} \cdot cm^{-1}$) [13]

L = optical path length DS-11 (1 cm)

2.3. Serum preparation

Blood was collected in 5 mL BD Vacutainer® SST II tubes. Tubes were immediately inverted 5 times. After 30 min of coagulation at room temperature (RT), tubes were centrifuged at 1200 *g* for 10 min in a swing-out rotor. Next, serum was snap-frozen in liquid nitrogen in 2.0 mL vials before storage at −80 °C.

2.4. ELISA

Nunc MaxiSorp™ white 96-well flat-bottom plates were coated overnight at 4 °C with 50 μl of 2 μg/mL PD-1. The next day, wells were emptied and washed 4 times with 300 μl of PBSTF.

Standard curves were prepared in 2 mL Eppendorf® LoBind vials on the day of analysis by serial dilution of a 11.0 mg/mL nivolumab clinical stock solution to 100, 50, 20, 10, 5, 2, and 0 ng/mL in ice-cold 10% (v/v) serum in PBSTF. Quality controls (QCs) were prepared from different nivolumab and pembrolizumab stock solutions, independently from the standard curves, at 5, 20, and 160 μg/mL in serum, and stored at −80 °C. On the day of analysis, patient serum and QCs were diluted 10-fold with PBSTF, and CSF was diluted 2-fold with 20% serum (v/v) in PBSTF, in order to have the same 10% serum (v/v) in PBSTF final matrix. If necessary, CSF and serum were additionally diluted 2- and 100-fold, respectively, with 10% (v/v) serum in PBSTF. The 10-fold diluted QCs were additionally diluted 100-fold to 5, 20, and 160 ng/mL with 10% (v/v) serum in PBSTF. Next, QC160 was further diluted 2-fold to 80 ng/mL with 10% serum (v/v) in PBSTF. Patient serum, CSF, and QCs were analyzed as 50 μl duplicates per plate. Samples were added as 50 μl triplicates per plate, which was subsequently sealed and incubated for 2 h at RT. Then, the plate was emptied and washed 4 times with 300 μl of PBSTF. After addition of 50 μl of 1 μg/mL anti-human IgG4-HRP in PBSTF, plates were sealed and incubated for 1 h at RT. Next, plates were emptied and washed 4 times with 300 μl of PBSTF. Subsequently, 100 μl of Pierce standard ECL was added and luminescence was measured within 15 min using a Tecan Infinite 200 Pro plate reader at 1 s per well of read time.

2.5. Optimization of anti-human IgG4-HRP concentration

Nivolumab standard curves were prepared at concentrations of 100, 50, 20, 10, 5, 2, and 0 ng/mL in ice-cold 10% (v/v) serum in PBSTF. In triplicate 50 μl of each standard was incubated for 2 h at RT on plate. After 3 washes with 300 μl of PBSTF, 50 μl of 1:500, 1:1000, and 1:2000 in PBSTF diluted anti-human IgG4-HRP was added and incubated for 1 h at RT. Subsequently, the plate was washed and luminescence was measured after addition of ECL, as described in the ELISA section.

2.6. Serum matrix effect

The effect of different concentrations of serum on the quantification of nivolumab was determined in triplicate in standard curves prepared in 2 mL Eppendorf® LoBind vials on the day of analysis by

serial dilution of 11.0 mg/mL nivolumab clinical stock solution to 100, 50, 20, 10, 5, and 2 ng/ml in ice-cold PBSTF containing 0, 10%, and 20% (v/v) serum.

To assess the dilution integrity, nivolumab was spiked in triplicate at 1000 μg/mL in serum and 2 μg/mL in CSF. Next, serum and CSF were diluted 1000 and 2-fold to 1 μg/mL, respectively, as described in ELISA. Further 2-fold serial dilutions with 10% serum (v/v) in PBSTF were then applied to serum and CSF to a final nominal nivolumab concentration of 62.5 ng/mL. The accuracies of the back-calculated nivolumab concentrations relative to the nominal spike concentrations at each serial dilution level were determined.

2.7. Specificity and limit of detection

Wells coated with and without PD-1 were incubated in triplicate with 100 μl of 0 and 100 ng/mL nivolumab in PBSTF. Next, plates were washed and incubated with secondary antibody as described under ELISA. After 4 washes, 100 μl of Pierce standard, Biorad Clarity, and Trevigen Peroxyglow™ ECL were added and luminescence was measured.

The effect of three of the most commonly used blocking agents was tested. Wells coated with PD-1 were incubated for 3 h at RT with 300 μl of 2% and 5% (w/v) BSA in PBS, 2% and 5% (w/v) ELK in PBS, 40% and 100% (v/v) FCS in PBS, and PBS as negative control. Next, wells were emptied and incubated for 2 h with 50 μl of 10% (v/v) serum in PBSTF.

Treatment of nivolumab is sometimes combined with ipilimumab, which is a fully human monoclonal antibody against cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). Although its target is different, a possible analytical interference can not be ruled out. Therefore, we spiked 0, 20, and 80 ng/mL of nivolumab in 10% (v/v) serum in PBSTF and 50% (v/v) CSF containing 10% (v/v) serum in PBSTF. After addition of 0, 100, 200, and 500 ng/mL of ipilimumab, these samples were analyzed by ELISA, as described.

The limit of detection (LOD) was defined as the average background level plus 5 times the standard deviation and was determined in serum from 10 healthy volunteers and in CSF from 10 immunotherapy naïve cancer patients.

2.8. Standard curve fitting

Calibration curves are commonly fit using polynomial or logistic models [14]. We compared the goodness of fit of a quadratic and 4-parameter logistic model on 21 standard curves using Graphpad Prism 6. Net luminescence was calculated as the luminescence of samples minus the average luminescence of the duplicate blank samples. Net luminescence of standards 2–100 ng/mL was plotted against the nominal nivolumab concentration. Curve fits were not forced through 0, and back-calculated concentrations had to be within 15% of the nominal concentrations for all 7 calibration standards.

2.9. Lower limit of quantification

The lower limit of quantification (LLQ) was determined in triplicate in ice-cold PBSTF, containing 10% (v/v) serum from 7 different volunteers, spiked with 1, 2, 3, 4, 5 ng/mL of nivolumab or pembrolizumab. CSF from 6 immunotherapy naïve patients was diluted 2-fold with ice-cold PBSTF containing 20% (v/v) serum, which was spiked with 1, 2, 3, 4, and 5 ng/mL of nivolumab or pembrolizumab. The LLQ was defined as the nominal input level at which the nivolumab and pembrolizumab concentrations could be determined with a precision ≤20% and an accuracy of 80–120%. Furthermore, the analyte response at the LLQ should be at least five times the response compared to the blank response.

2.10. Between- and within-day precision and accuracy

Samples containing 5, 20 or 80 ng/mL nivolumab in 10% (v/v) serum in PBSTF were measured in triplicate on six consecutive days. The between-day (BDP) and within-day precision (WDP) were calculated by one-way analysis of variance (ANOVA) for each spike level using the run day as classification variable using the software package SPSS v15.0 for windows (SPSS, Chicago, USA). The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentrations across run days were used. The WDP% and BDP% for each spike level were calculated using the formulas:

$$WDP\% = (ErrMS)^{0.5}/GM \times 100\%$$

$$BDP\% = [(DayMS - ErrMS)/n]^{0.5}/GM \times 100\%$$

With n being the number of replicates within each run.

Accuracy was determined as the relative difference between the nominal input concentration and measured concentration. Imprecisions ≤15% and accuracy between 85–115% were considered acceptable.

2.11. Pembrolizumab quantification

Standard curves were prepared in 2 mL Eppendorf® LoBind vials on the day of analysis by serial dilution of 11.0 mg/mL nivolumab and 27.8 mg/mL pembrolizumab clinical stock solutions to 100, 50, 20, 10, 5, and 2 ng/mL in ice-cold 10% (v/v) serum in PBSTF. These standards were analyzed in triplicate on three consecutive days. The concentrations of the pembrolizumab standards (Mw = 146,286 Da) were back-calculated from the nivolumab (Mw = 143,597 Da) standard curves. After correction for the 1.87% difference in molecular weight, the back-calculated pembrolizumab concentrations had to be within 15% of the nominal pembrolizumab concentrations.

2.12. Stability

To assess the long-term storage stability, nivolumab and pembrolizumab were spiked at 0.1, 1, 10, and 100 μg/mL in serum. This largely covers the whole range of concentrations found in patient serum along the PK curve. Aliquots of 50 μl of spiked serum were snap-frozen in liquid nitrogen and stored for 0, 7, 120, 360, and 480 days at −80 °C. At these time points nivolumab and pembrolizumab concentrations were determined in triplicate, after dilution to 50 ng/mL in ice-cold 10% (v/v) serum in PBSTF.

Stability of nivolumab and pembrolizumab at 10 and 50 ng/mL, diluted in ice-cold 10% (v/v) serum in PBSTF, was tested after 0, 6, and 24 h on ice, using freshly prepared nivolumab standard curves.

Freeze-thaw stability was tested for nivolumab and pembrolizumab spiked at 10 and 100 μg/mL in serum. Nivolumab and pembrolizumab concentrations were determined after 0, 1, 2, and 3 snap-freeze/thaw cycles, after dilution to 100 ng/mL with 10% (v/v) serum in PBSTF.

Nivolumab and pembrolizumab concentrations were considered stable if the determined concentrations were within 15% of the nominal concentrations.

2.13. Clinical applicability

The clinical application of the ELISA method was demonstrated in serum from seven patients treated once every 2 weeks with nivolumab (n = 4) or once every 3 weeks with pembrolizumab (n = 3). Patient 1 received concomitantly ipilimumab at 3.3 mg/kg

(Table 3). Blood was drawn from these patients at day 0 (predose + end of infusion), and predose at cycle 2.

To demonstrate clinical applicability of the ELISA for determination of nivolumab in CSF, CSF was collected from 15 patients with a solid tumor and a clinical suspicion of leptomeningeal metastases but a normal or equivocal MRI who underwent a diagnostic lumbar puncture (LP). All patients have been included in a diagnostic CSF study at the NKI comparing the sensitivity and specificity of immunoflowcytometry assays for circulating tumor cells (CTC) detection with CSF cytology. Five patients were treated with nivolumab. Three out of these five patients had melanoma and concomitantly received ipilimumab at 3 mg/kg (Table 4). The other 10 patients had not received any immunotherapy prior to sampling and served as a negative control group. An aliquot of 1–2 mL of CSF was collected in 2.0 mL vials and stored at −80 °C.

Both clinical studies have been approved by the Ethics Committee of the Netherlands Cancer Institute and subjects provided whole blood and CSF samples after written informed consent.

2.14. Statistical analysis

Statistical evaluation was performed using the unpaired two-tailed student *t*-test in Excel, unless indicated otherwise. Matrix effects were analyzed using the paired two-tailed *t*-test in Excel. The slopes and intercept of nivolumab and pembrolizumab standard curves were compared using linear regression analysis in Graphpad Prism 6. *P*-values of ≤0.05 were considered to be significant.

2.15. Method validation

Validation of the ELISA method was performed based on the guidelines for bioanalytical assays provided by the FDA [15].

3. Results

3.1. Optimization of anti-human IgG4-HRP concentration

We tested anti-human IgG4-HRP at dilutions of 1:500, 1:1000, and 1:2000 in PBSTF. The 1:1000 dilution resulted in a significantly higher ($P < 0.001$) signal to noise ratio, as compared to the other dilutions, over the whole range of spiked nivolumab concentrations from 2 to 100 ng/ml (Supplementary Table 1).

3.2. Serum matrix effect

We found that addition of 10% and 20% (v/v) serum to PBSTF had a significant effect on the accuracy of nivolumab quantification over the whole standard curve concentration range with an average of decrease in nivolumab concentration of 14.1% at 10% (v/v) serum to PBSTF ($P < 0.001$) and 21.4% at 20% serum to PBSTF ($P < 0.001$) (Supplementary Table 2).

Therefore, we used 10% (v/v) serum in PBSTF, for both serum and CSF samples, as well as for the standard curves and quality controls (QCs), to assure accurate quantification of nivolumab.

Next, dilution integrity was assessed in triplicate in quality controls, spiked with nivolumab at 160 μg/mL, after a standard 1000-fold dilution followed by an additional 2-fold dilution. The back-calculated nivolumab concentration did not deviate more than 15% from the nominal spike concentration, which indicates good dilution integrity. Furthermore, samples spiked with nivolumab at 1000 and 2 μg/mL in serum and CSF, respectively, which required an additional 16-fold dilution after the standard 1000-fold dilution, also showed adequate dilution integrity (Table 1).

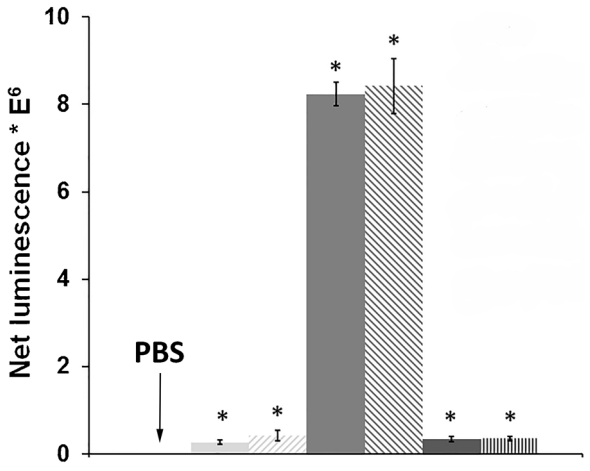

Fig. 1. Background signal after 3 h of incubation with 300 μl of the following blocking solutions in phosphate buffered saline (PBS): PBS as control ■; 2% and 5% bovine serum albumin (BSA); 2% and 5% low fat milk powder; 40% and 100% fetal calf serum (FCS). Results ± SD of 3 different samples are shown. * Indicates significant $P < 0.05$ higher background relative to PBS.

3.3. Specificity

The signal to noise ratios of nivolumab using Pierce standard ECL, Biorad Clarity, and Trevigen Peroxyglow were 363, 100, and 2000, respectively. Although, Peroxyglow showed superior signal to noise ratio, we chose to develop the ELISA with about 10-fold less expensive Pierce standard ECL.

The detection of nivolumab was very specific: wells coated with PD-1 showed luminescence of 5762 ± 182, which was not significantly higher than the luminescence of 5439 ± 454 for wells not coated with PD-1. This ensures the absence of any meaningful interaction between the secondary antibody and PD-1, and indicates that net luminescence, defined as measured luminescence minus background signal from ECL, originates only from the reaction of the secondary antibody with nivolumab.

There was a large difference in background signal after blocking with different agents (PBS only, BSA, FCS, ELK). The lowest background of $10.2 \times 10^3 \pm 552$ arbitrary luminescent units (ALU) was obtained without blocking, which are the wells incubated with PBS only. In sequence of increasing background signal, 2% BSA, 40 and 100% FCS, 5% BSA, and 2 and 5% ELK, resulted in significant ($P < 0.001$) higher backgrounds of 270×10^3, 338×10^3, 363×10^3, 423×10^3, 823×10^4, and 842×10^4 ALU, respectively (Fig. 1). To put this in perspective, 100 ng/mL nivolumab resulted on average in net luminescence of 240×10^4 ALU. Based on these results, we concluded, that blocking should be omitted in this ELISA.

Addition of ipilimumab had no significant effect on the quantification of nivolumab in both serum and CSF (Supplementary Table 3). Furthermore, the background level was not significantly increased by 500 ng/mL of ipilimumab (data not shown).

The mean background level of 10% (v/v) serum from 10 different volunteers in PBSTF was 0.22 ± 0.039 (range 0.089–0.37) ng/mL. The mean background of 50% (v/v) CSF in PBSTF containing 10% (v/v) serum from 10 patients was 0.31 ± 0.011 (range 0.21–0.45) ng/mL. From these backgrounds, limits of detection (LOD) for nivolumab in serum and CSF of 0.65 ng/mL, and 0.75 ng/mL, respectively, were calculated.

3.4. Lower limit of quantification (LLQ)

The LLQ of nivolumab and pembrolizumab in serum and CSF was 2 ng/mL. In serum, nivolumab was determined at the LLQ with a mean accuracy of 101% (range 97.4%–110%, n = 7), and mean

Table 1
Dilution integrity was assessed, after indicated number of serial 2-fold dilutions with 10% (v/v) serum in PBSTF, for serum spiked at 160 (Quality Control) and 1000 μg/mL, and CSF spiked at 2 μg/mL nivolumab. Results are the average of three replicate measurements. PBSTF = phosphate buffered saline supplemented with 0.1% Tween-20 and 1% Ficoll.

nivolumab spiked	total dilution factor	number of 2-fold serial dilutions	nominal conc. ng/ml	determined conc. ± SD μg/mL	accuracy ± SD (%)
QC at 160 μg/ml	2000	1	80	153 ± 9.6	95.6 ± 6.0
	1000	1	1000	129 ± 5.7	13.4 ± 1.7
	2000	2	500	251 ± 14.9	25.5 ± 5.3
serum at 1000 μg/mL	4000	4	250	483 ± 21.7	48.2 ± 3.2
	8000	8	125	880 ± 21.4	87.9 ± 3.4
	16,000	16	62.5	921 ± 44.4	94.9 ± 9.0
	2	1	1000	0.26 ± 0.011	12.9 ± 4.4
	4	2	500	0.50 ± 0.030	25.1 ± 5.9
CSF at 2 μg/mL	8	4	250	0.97 ± 0.043	48.3 ± 4.5
	16	8	125	1.75 ± 0.085	87.2 ± 4.9
	32	16	62.5	1.84 ± 0.089	96.3 ± 3.2

Table 2
Imprecisions and accuracy at indicated nivolumab and pembrolizuamb nominal input levels after dilution of quality control samples prepared in 100% serum to a final matrix composition of 10% (v/v) serum in PBSTF. Imprecisions were calculated from triplicate measurements on three consecutive days by one-way analysis of variance (ANOVA) for each spike level using the run day as classification variable. Accuracy is determined as the ratio between the measured and nominal concentration. WDP = within-day precision; BDP = between-day precision.

nominal input ng/mL	nivolumab			pembrolizumab		
	WDP %	BDP %	accuracy %	WDP %	BDP %	accuracy %
5	3.3	4.1	102.5	6.1	5.3	98.1
20	3.4	4.1	99.5	6.5	6.6	101.9
80	4.2	4.6	100.8	5.1	0.6	105.7

Table 3
Patients and treatment characteristics used to demonstrate applicability of the ELISA in serum. Patients received indicated dose of nivolumab at day 1 of every course. In addition, melanoma patients received 3 mg/kg ipilimumab; NSCLC = non-small cell lung cancer; q2w and q3w = administration every 2 and 3 weeks, respectively.

patient #	tumor type	therapeutic antibody	dosing regime	dose mg/kg	dose mg
1	melanoma	nivolumab	q2w	1.3	100
2	melanoma	pembrolizumab	q3w	2.1	200
3	NSCLC	nivolumab	q2w	2.8	140
4	NSCLC	nivolumab	q2w	5.7	240
5	NSCLC	pembrolizumab	q3w	3.0	200
6	melanoma	pembrolizumab	q3w	2.5	150
7	NSCLC	nivolumab	q2w	2.6	240

precision of 3% (range 0%–9.5%). Pembrolizumab was determined at LLQ with a mean accuracy of 100% (range 91.4%–105%, n = 7), and mean precision of 3.9% (range 1.6%–5.8%). In CSF, nivolumab was determined at LLQ with a mean accuracy of 103% (range 101%–106%, n = 6) and mean precision of 2.2% (range 0.4%–4.2%). Pembrolizumab was determined at LLQ with mean accuracy of 102% (range 98.9%–105%, n = 6), and mean precision of 3.4% (range 0.4%–4.2%).

3.5. Between- and within-day precision

Nivolumab was measured at 6 consecutive days in triplicate at 5, 20, 80 ng/mL spiked in 10% (v/v) serum in PBSTF. The mean within- and between day imprecisions, and the nivolumab quantification accuracy at these nominal input levels were within 15%, and 85–115%, respectively (Table 2).

3.6. Pembrolizumab quantification

Concentrations of nivolumab and pembrolizumab, back-calculated from nivolumab standard curves, were compared by linear regression analysis. No significant differences in slope and intercept were found, which indicates that assay response over the investigated standard curve concentration range is the same for both antibodies (Supplementary Table 4). In addition, the Pearson correlation coefficient (r) of 1.00 indicates good correlation between the quantification of both antibodies. Therefore, we conclude that pembrolizumab can be accurately quantified against standard curves prepared from nivolumab if the 1.87% molecular weight difference is taken into account.

3.7. Stability

Nivolumab and pembrolizumab were stable at 0.1, 1, 10, and 100 μg/mL spiked in PBSTF containing serum, in storage at −80 °C for at least 480 days. Furthermore, samples containing nivolumab and pembrolizumab, at 10 and 50 ng/mL in 10% (v/v) serum in PBSTF, could be stored on ice for 6 h without significant decrease in concentration of both antibodies. However, after 24 h of storage on ice nivolumab and pembrolizumab concentrations decreased significantly by 13% (P = 0.026) and 19% (P = 0.005), respectively.

Samples containing 10 and 100 μg/mL of nivolumab and pembrolizumab spiked in 10% (v/v) serum in PBSTF were subjected to 3 freeze-thaw cycles. The measured drug concentrations, after 1000-fold dilution of samples in 10% (v/v) serum in PBSTF, did not differ significantly from the spiked concentrations (Supplementary Table 5).

3.8. Clinical applicability

Nivolumab (n = 4 patients) and pembrolizumab (n = 3 patients) serum concentrations were determined in seven patients treated with different doses of nivolumab and pembrolizumab (Fig. 2). Predose nivolumab and pembrolizumab serum concentrations for all seven patients were below the limit of detection. At end of infusion, we found nivolumab C_{max} concentrations of 43.9–65.1 μg/mL for two patients treated with nivolumab at 2.6 and 2.8 mg/kg, which is within the concentration range reported by EMA of 61.3 ± 16.2 μg/mL for patients treated with nivolumab at 3 mg/kg (n = 13 patients) [16]. Patients 1 and 4 were treated with nivolumab doses that were about a factor 2 below and above this 3 mg/kg level, which resulted in nivolumab serum concentrations of 19.6 and 107 μg/mL, respectively. Trough nivolumab serum concentrations ranged from 3.1 for patient 1 (1.3 mg/kg) to 56.2 μg/mL for patient 4 (5.7 mg/kg). Pembrolizumab serum concentrations at end of infusion were 43.9, 46.5, and 65.1 μg/mL for the three patients treated with a 200 mg dose of pembrolizumab, which is within the range reported by EMA of 67.5 ± 23 μg/mL (n = 150) for patients treated at this dose [17]. Trough pembrolizumab concentrations ranged from 8.01 to 22.8 μg/mL.

Table 4
Measured nivolumab concentrations in serum and CSF from 5 patients receiving the indicated dose of nivolumab at day 1 of every course. In addition, melanoma patients received 3 mg/kg ipilimumab. Results are the average of three replicate measurements ± SD. CSF = cerebrospinal fluid; NSCLC = non-small cell lung cancer; PK = pharmacokinetics; C = course; D = day; q2w and q3w = administration of nivolumab every 2 and 3 weeks, respectively.

patient #	tumor type	nivolumab dosing regime	PK sample	dose mg/kg	dose mg	measured nivolumab concentration ± SD in ng/mL		ratio
						serum	CSF	serum/CSF
137	breast cancer	q3w	C1D16	1	61	4481 ± 287	15 ± 0.9	299
123	melanoma	q3w	C1D21	1	80	1831 ± 138	35 ± 0.9	52
113	melanoma	q3w	C1D21	1	77	4410 ± 324	39 ± 1.9	113
135	melanoma	q2w	C1D12	3	245	13,759 ± 311	150 ± 2.5	92
114	NSCLC	q3w	C3D14	3	240	33,454 ± 705	304 ± 11	110

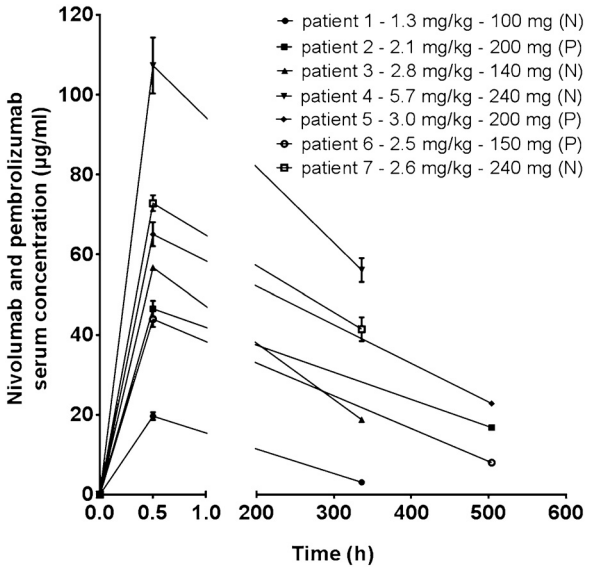

Fig. 2. Pharmacokinetics of nivolumab (N; n = 4) and pembrolizumab (P; n = 3) in serum from 7 patients treated with indicated doses (mg/kg). Blood was drawn at baseline (0 min), end of infusion (30 min), and predose course 2 (336 and 504 h). Results are expressed as the means ± SD of 3 different samples.

The concentrations of nivolumab in CSF of five patients treated with 1 or 3 mg/kg nivolumab ranged from 14.5 to 304 ng/mL and levels of nivolumab in concomitantly drawn serum ranged from 1.8 to 33.5 µg/mL (Table 4). The serum/CSF ratios of nivolumab ranged from 52–299. Although, the sample size is small and inter-patient variability in nivolumab levels in CSF is substantial, these data indicate that there is a low penetration of nivolumab in the brain.

4. Discussion

Pembrolizumab and nivolumab are both anti-PD-1 monoclonal IgG4 antibodies, which have been approved for various advanced cancers, showing improved overall and progression free survival compared to standard-of-care in phase III trials [18–22]. Intracranial activity of these agents has been observed in progressing brain metastases in patients with melanoma and NSCLC [23,24]. Studies show a rapid and durable brain metastasis response rate of 22% in 18 melanoma patients and 33% in 18 NSCLC patients. Despite these encouraging data, many patients fail to respond to anti-PD-1 treatment in the brain or on extra-cerebral sites. Additional combination therapies and biomarker development will be important, particularly in patients with brain metastases who may have a different disease biology than patients with extra-cerebral disease. It is unclear whether the effect of anti-PD-1 agents in brain metastases is due to systematically activated T-cells that cross the blood-brain barrier or whether the anti-PD-1 agent actually has its action mechanism in the brain itself and therefore has to cross the BBB [23]. Our data now show that only minimal nivolumab concentrations reach the brain/CSF with serum to CSF ratios of 52–299.

Recently, Puszkiel et al. reported the first ELISA for the determination of nivolumab in plasma [10]. Puszkiel et al. have demonstrated that their ELISA is sensitive enough to measure trough nivolumab levels in patients receiving nivolumab at 3 mg/kg. However, our results indicate that treatment of patients with nivolumab at 1–1.3 mg/kg can result in trough levels below the 5000 ng/mL lower quantification limit of their ELISA (Fig. 2 & Table 4).

Here, we report the development and validation of a sensitive, quick and inexpensive ELISA which can be used to measure both nivolumab and pembrolizumab concentrations in biological fluids. Most ELISAs describe the use of time consuming blocking steps with BSA, FCS, and ELK-based protein solutions to prevent nonspecific binding of antibodies [10]. These blocking agents, however, prevented the sensitive detection of nivolumab in our ELISA due to an increase of background signal that originates from nonspecific binding of the secondary anti-IgG4-HRP antibody. Therefore, we tried the highly branched hydrophilic polysaccharide Ficoll as an alternative blocking agent, as suggested by Huber et al. [25]. Furthermore, the original developers of the ELISA [26] described that addition of Tween-20 in the antibody and washing solutions is sufficient to reduce nonspecific binding. Based on these findings, we omitted a separate blocking step and combined both the Ficoll and Tween-20 in the antibody and washing solutions. Further enhancement in sensitivity was obtained through chemiluminescent detection of the anti-IgG4-HRP. An advantage of this assay is a 100-fold reduction in the amount of recombinant PD-1 used for coating the ELISA plates, which significantly reduces the cost of the assay. The method has a LLQ of 2 ng/mL for both nivolumab an pembrolizumab, which will most likely be sensitive enough to allow quantification of both peak and trough levels of nivolumab and pembrolizumab in serum and CSF from most patients.

Clinical trials are showing promising results from the combination of nivolumab and pembrolizumab with ipilimumab [27,28]. We showed that quantification of nivolumab with our ELISA was not affected by analytical interference from an 25-fold excess of ipilimumab. Moreover, the background of the assay was not significantly increased by 500 ng/mL of ipilimumab. Therefore, our ELISA can be used to accurately quantify nivolumab and pembrolizumab in plasma and CSF from patients receiving combination therapy.

5. Conclusions

We developed and validated a sensitive ELISA for the quantitative determination of nivolumab and pembrolizumab in serum and CSF. The ELISA has a LLQ of 2 ng/mL, which enables accurate quantification of the low levels of these anti-PD-1 antibodies found in CSF.

To our knowledge, this is the first evaluation of nivolumab concentration levels in CSF. The concentrations of nivolumab in CSF

ranged from 14.5 to 304 ng/mL, at trough nivolumab serum levels in 5 patients receiving nivolumab at 1 and 3 mg/kg, respectively. The method is accurate, precise, and shows good long-term sample storage stability using standard laboratory equipment and techniques. This quantitative ELISA for nivolumab and pembrolizumab can be used in future clinical trials.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.10.025.

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