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Combined use of UV and MS data for ICH Stability-Indication Method: Quantification and isoforms identification of intact nivolumab

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ABSTRACT

Nivolumab (Opdivo®) is a fully human immunoglobulin G4 isotype approved for the treatment of many cancers. It acts as an immune checkpoint inhibitor by blocking the interaction between PD-1 (Programmed Cell Death Protein 1) – an inhibitory receptor expressed on activated T cells- and its ligands, PD-L1 and PD-L2. The quantification of therapeutic proteins in their medicines and pharmaceutical preparations remains challenging because the protein content, a critical quality attribute, must be rigorously calculated using a validated stability-indicating method, such as that indicated by the International Conference on Harmonization (ICH) quality guidelines, and this requires the analysis of the drug in the presence of its degraded products. In this work, we present an strategy based on the combined use of the UV and MS data to full file the requirement of the ICH-Q2 (R1) to develop and validated as stability indicated a (RP)UHPLC/UV-(HESI/Orbitrap™)MS method for the quantification of nivolumab in medicinal products. A comparative study of all figures of merit of the method using UV or MS data are shown and discussed. The results show that linearity was similar for the two detectors and was established over a range of 4–45 μg/mL and 1–45 μg/mL for the UV and (HESI/Orbitrap™)MS signals, respectively. The sensitivity of the method was higher when using the (HESI/Orbitrap™)MS signal (0.2 μg/mL) than with the UV(2.0 μg/mL). However, the UV signal provided better accuracy and precision than the (HESI/Orbitrap™)MS signal, which did not meet the criteria for method robustness and system suitability. In spite of this, the MS signal plays a crucial role in this methodology by obtaining the molecular weight profile of the nivolumab isoforms, so enabling us to propose the glycans profile and detect structural modification due to degradation. The specificity of the method was evaluated by conducting forced degradation tests on samples of nivolumab in medicine form. The aim was to find out whether nivolumab suffers structural modifications when subject to stress. Structural modifications were detected by analysing the MS isoform profile, as changes of this kind promote new isoforms that are not chromatographically separated or detected by the UV signal. In this way, we demonstrated that the (RP)UHPLC/UV-(HESI/Orbitrap™)MS method was capable of detecting nivolumab degradation, and was suitable for use in nivolumab stability studies. Thus, the protein content in the daily surplus of the Opdivo® medicine, stored either at room temperature (20 °C) or refrigerated at 4 °C, could be tracked for 15 days.

1. Introduction

Nivolumab (Opdivo®) is a humanized monoclonal antibody (mAb) IgG4 isotype approved for the treatment of a wide range of cancers, including malignant melanomas, non-small cell lung cancer, renal cell cancer, squamous cell cancer of the head and neck, urothelial cancer and classical renal lymphoma [1]. The FDA approved nivolumab for the treatment of melanoma in December 2014 [2], non-small cell lung cancer in March 2015 [3], renal cell carcinoma in November 2015 [4], and Hodgkin lymphoma in May 2016 [5]. Nivolumab is produced by Bristol-Myers Squibb and works by blocking the interaction between PD-1 receptor and its ligands, PD-L1 and PD-L2. Nivolumab binds to the PD-1 molecule and generates steric hindrance that prevents PD-1 from binding to its ligands. It also activates the PD-1 pathway-mediated

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immune response, including the anti-tumor immune response [6].

Since mAbs are used as therapeutic substances in medicines, they must comply with strict quality requirements to guarantee patient safety. Their efficacy is also carefully controlled. In the hospital context, quality control of mAbs plays a pivotal role in ensuring that they can be safely and effectively administered to patients [7]. Without such control, mAbs could induce immunogenic responses related to antidrug antibodies generated by patients, which could potentially lead to life-threatening situations [8]. The recommendations for ensuring the quality of biotechnological products are set out in the ICH Q5E guidelines issued by the International Conference on the Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use [9]. Particular specifications with regard to the test procedures and the acceptance criteria for biotechnological/biological products are set out in the ICH Q6B guidelines [10]. It is therefore important to develop validated analytical methods that can characterize the mAb structure and any modifications it may undergo. All analytical methods used to characterize mAbs should comply, as far as possible, with the requirements of international drug regulatory organizations. These include, for example, those specified in the ICH Q2(R1) guidelines [11], which include validation principles covering analytical use.

Due to the complex nature of therapeutic mAbs, a variety of orthogonal analytical methods must be used to achieve appropriate characterization. Different experimental strategies could be applied in order to carry out structural characterization: (i) a "top-down" approach, based on intact mAb analysis; (ii) a "bottom-up" approach, based on a prior enzymatic digestion of the mAb and a subsequent analysis of the digested peptides; and (iii) a "middle-down" approach, in which the mAb is partially digested. The "top-down" approach, also called intact protein analysis, does not require the samples to be treated in any way, and they can therefore be analysed more quickly. The disadvantage of intact analysis is that it does not offer as much information as the other approaches. In spite of that, it is widely used in the detection of PTMs, including the glycan isoforms in mAbs [12,13].

Ultra-high performance liquid chromatography hyphenated with mass spectra detection (UHPLC-MS) is one of the most commonly used techniques for characterizing mAbs. This technique can be applied in two different modes, i.e. denaturing mode and native mode, depending on the mobile phase solvents used. In denaturing mode the protein is denatured, while in native mode the protein structure is maintained [14,15]. Native mode can be performed in size exclusion chromatography (*SEC*), ion exchange chromatography (IXC) and hydrophobic interaction chromatography (HIC). Recent advances have allowed this mode of chromatography to be coupled to mass detectors using volatile salts in the mobile phases. *SEC* is the standard method for analysing mAbs aggregates [16–19], IXC is used to separate the mAb charge variants [20,21] and HIC allows the mAbs to be separated on the basis of their hydrophobicity under native conditions [22,23].

The denaturing mode includes reverse-phase (RP) chromatography, which is generally considered to be more efficient and more sensitive for the analysis of intact biotherapeutic proteins than other modes of liquid chromatography [24–26]. UHPLC in reverse-phase (RP) needs higher pressures in the system (around 1000 bar) than HPLC. This significantly enhances the efficiency of the separation of intact proteins using common organic solvent composition in the mobile phase, such as mixtures of water and acetonitrile and common ion pairing agents, such as trifluoroacetic acid (TFA) or formic acid (FA) [27]. Column temperatures of up to 80 °C are also required [28,29], and a gradient must be applied during chromatographic analysis. All these conditions can also be applied in intact protein analysis when coupled to a mass spectrometer [27], allowing for the separation, quantification and identification of the intact mAbs and its fragments [27,30,31].

In our previous research into the quantification of intact mAbs, we focused on the development and validation of (RP)HPLC methods for analysing single therapeutic mAbs using a wide porous particle size packed column [24–26]. We also assessed the development and validation of (RP)UHPLC-UV-(HESI/Orbitrap™)MS methods for the simultaneous separation, quantification, and identification of mAbs mixtures with a monolithic column [27]. In the research presented here, we used the latter strategy to develop and validate a quantification and identification (RP)UHPLC-UV-(HESI/Orbitrap™)MS method for the analysis of intact nivolumab in a top-down analysis strategy. We also present a comparative analysis of the validation results obtained using either the UV signal or the MS signal for quantification purposes.

Several ELISA and (RP)LC-MS methods for the quantification of nivolumab are proposed in the bibliography we consulted. They are all intended for use in plasma samples analysis [32–36]. One of these also included an (RP)LC-MS/HRMS method [37]. However, these methods using human plasma cannot easily be adapted for quality control analyses of nivolumab and they would also need to be validated for this purpose. In this research, we propose an strategy based on the combined use of the UV and MS data to full file the requirement of the ICH-Q2(R1) to develop and validated as stability indicated a (RP)UHPLC/UV-(HESI/Orbitrap™)MS method, which allows for the rigorous quantification of nivolumab for quality control purposes and for the identification of isoforms. Validation was performed using both the UV and MS signals, in both cases according to different well-known international guidelines. This involved the study of different performance parameters such as linearity, accuracy, precision, detection limits, quantification limits, specificity, system suitability and robustness. A comparative discussion of the results obtained for nivolumab quantification using the two analytical signals is presented here in order to weigh up their respective benefits. The method was used to analyse nivolumab (protein content -quantification- and isoforms identification) in opened vials of the medicine Opdivo® (daily surplus). These vials were stored in darkness and kept at 4 °C and 20 °C for two weeks in order to provide new data about the stability of this expensive biotechnological medicine.

2. Materials and methods

2.1. Chemicals and reagents

Reverse-osmosis-quality water (purified with a Milli-RO plus Milli-Q station from Merk Millipore, Darmstadt, Germany) was used throughout the study. The reagents used were LC-MS purity grade. Acetonitrile (ACN) was purchased from VWR International S.A.S. (Fontenay-sous-Bois, France), while formic acid (FA) was supplied by Thermo Fisher Scientific (Geel, Belgium).

2.2. Nivolumab standard and solutions

Opdivo® (Bristol-Myers Squibb Pharma EEIG, Dublin, Ireland) is the tradename of the medicine format of nivolumab. The Pharmacy Unit of the University Hospital "San Cecilio" (Granada, Spain) kindly agreed to supply us with their daily surplus of this drug. Nivolumab is presented as an aqueous solution for injection containing 10 mg/mL. The medicine indicates that each mL of concentrate contains 10 mg of nivolumab. There are 40, 100 and 240 mg of nivolumab in each single-use vial of 4, 10 and 24 mL, respectively. Each vial also contains sodium citrate dihydrate, sodium chloride, mannitol (E421), pentetic acid (diethylenetriaminepentaacetic acid), polysorbate 80, sodium hydroxide, hydrochloric acid and water for injections, so producing the final concentration of 10 mg/ml.

All standard solutions of nivolumab were prepared from the medicine Opdivo®, as a suitable reference material for nivolumab was impossible to obtain. To this end, appropriate dilutions in reverse-osmosis-quality water were prepared using the medicine Opdivo®. This was done within the expiry date indicated in its technical reports [1], so as to avoid any degradation of the nivolumab and ensure full integrity of the medicine. All nivolumab solutions were freshly prepared daily as required for the experiments. The following batches were used during this study: ABK3907, ABL3361 and ABL3906.

2.3. (DAD)UV absorption spectrum of nivolumab

Fresh samples of Opdivo® (10 mg/mL nivolumab) were used to determine the UV absorption spectrum of nivolumab. This was measured using an Agilent 8453 UV–Visible Spectrophotometer G1103A (Agilent Technologies, U.S.) equipped with a photodiode array (DAD) for simultaneous measurement of the complete ultraviolet-to-visible light spectrum. The UV spectrum was recorded from 190 to 300 nm.

2.4. (RP)UHPLC/UV-(HESI/Orbitrap™)MS analysis of intact nivolumab

For the purposes of this analysis, a proper analytical platform was used (Thermo Scientific, Waltham, MA, USA). Chromatographic separation was performed using a Dionex Ultimate 3000 chromatograph equipped with two ternary bombs, a degasser, an auto-sampler, a thermostatted column compartment and a multiple-wavelength detector (MWD-3000 Vis-UV detector). The UHPLC system was coupled in-line to a Q-Exactive hybrid quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific, Waltham, MA, USA). The chromatographic instrument was operated by Chromeleon™ 7 and Xcalibur™ 4.0 software and the mass spectrometer by Tune™ Software. The BioPharma Finder™ software version 3.1 with ReSpect algorithms was used to obtain the isoform profile of nivolumab by mass spectra deconvolution. All these software programmes were provided by Thermo Fisher Scientific.

The chromatographic analysis of nivolumab was conducted using a MAbPac™ RP column, 4 μm, 2.1 mm × 50 mm (Thermo Fisher Scientific, P/N 088648). The mobile phase flow rate was 0.3 mL/min and the column temperature was set at 70 °C. Nivolumab samples were analysed in a gradient mode using an injection volume of 10 μL. The eluent system was composed of 0.1 % FA in deionised water (eluent A) and 0.1 % FA in ACN (eluent B). Gradient conditions were as follows: 25 % B increased to 45 % B in 2.5 min, with a further increase to 80 % B in 0.5 min with a 2 min isocratic hold. Initial conditions were then restored in 0.2 min and maintained for a further 5.8 min to achieve column re-equilibration. Total run time for the analysis was 11 min. The wavelength selected to register chromatograms was 214 nm, using $\lambda = 350 \pm 10$ nm as the reference wavelength, and it was used to obtain all the figures of merit of the method and throughout the work. Chromatograms at 220 nm and 280 nm were also used to register chromatogram, using $\lambda = 350 \pm 10$ nm as the reference wavelength, to corroborate (or not) the protein nature of the chromatographic peaks.

The mass spectrometric parameters used here consisted of full positive polarity MS scans at a resolution setting of 17,500 (defined at *m/z* 200) with the mass range set to 1500–4500 *m/z* and an AGC target value of 3.0×10^6 with a maximum injection time of 100 ms and 10 microscans. In-source CID was set to 100 eV. Protein mode was selected to perform the analysis. MS instrumental tune parameters were set as follows: spray voltage − 3.8 kV, sheath gas flow rate − 35 AU, auxiliary gas flow rate − 10 AU, capillary temperature − 275 °C, probe heater temperature − 175 °C and S-lens RF voltage − 80 V.

2.5. Validation of the nivolumab quantification method (protein content)

The (RP)UHPLC/UV-(HESI/Orbitrap™)MS method we developed was validated in terms of linearity, sensitivity, precision, accuracy, specificity, robustness and system suitability according to the guidelines established by the International Conference on Harmonization (ICH Q2 (R1)) [11]. The Food and Drug Administration (FDA) recommendations [38] and Hsu and Chien criteria [39] were also used. The method was validated using the analytical signals from the UV and the MS detectors. The UV (registered at 214 nm with $\lambda = 350 \pm 10$ nm as reference wavelength) and MS (registered at total ion) chromatograms were employed to obtain the figures of merit for this method. A comparative discussion of the results was conducted in order to enable us to select the most appropriate strategy (UV or MS) for quantifying nivolumab.

2.5.1. Linearity and detection and quantification limits

Linearity was assessed by means of a calibration curve established using five standard solutions at 5, 15, 25, 35 and 45 μg/mL of nivolumab. Each standard solution was prepared in quadruplicate and injected into the chromatograph. The least-squares regression method was used to fit a linear calibration function, by relating chromatographic peak areas with concentrations. All the characteristic parameters of linear function, such as slope or the coefficient of determination (R^2), were calculated with the Statgraphics centurion 18® program (Statgraphics Technologies Inc., U.S.). A $R^2 > 0.995$ was established as the criterion for the linearity of the method.

The detection limit (DL) and quantification limit (QL) were estimated from the calibration function. DL was calculated as $3(SD(a)/b)$ whereas QL was calculated as $10(SD(a)/b)$, where $SD(a)$ is the standard deviation of the intercept and b is the slope of the calibration function.

2.5.2. Precision

Precision was evaluated as the repeatability (intraday precision) and the intermediate precision (interday precision). Both were given as relative standard deviations (RSDs).

The repeatability of our experiment was determined from the results of our analysis of standard solutions of nivolumab prepared at the same concentration on the same day. Nine samples with low concentration levels (3 samples of 5 μg/mL), medium concentration levels (3 samples of 25 μg/mL) and high concentration levels (3 samples of 45 μg/mL) were used for this purpose. The intermediate precision was determined from the analysis of standard solutions at the same concentration levels as were used to assess repeatability (5, 25 and 45 μg/mL), but over six consecutive days. Three separate samples of each concentration mentioned were freshly prepared and analysed daily.

Given that the ICH Q2(R1) does not establish any precision criteria for acceptance of the method, we decided to follow the Hsu and Chien precision criteria for HPLC methods for pharmaceutical analysis of biologics [39]. According to these criteria, the method can be considered acceptable in terms of precision when the RSD value is < 5 %.

2.5.3. Accuracy

Accuracy over the established linear range was assessed via the average recovery (%) value, which was calculated by analysing three standard solution replicates at low, medium and high concentrations of nivolumab (5, 25 and 45 μg/mL respectively). The FDA [38] accuracy acceptance criteria for HPLC methods for pharmaceutical analysis were used once again due to the lack of such criteria in the ICH Q2(R1). A recovery percentage value of 100 ± 3 % was established as the criterion for acceptance.

2.5.4. Specificity

Specificity was assessed by studying the forced degradation of nivolumab in its medicine form Opdivo® 10 mg/mL. The chromatograms of the sample solutions submitted to degradation were recorded not only to assess the specificity of the method, but also to detect degradation of nivolumab due to mishandling during manufacturing or in a clinical situation. With this in mind, three different stresses were selected for testing. The mild temperature and light exposure stress tests evaluate the behaviour of the medicine when exposed to heat and daylight respectively, while the high ionic strength test assesses how the medicine would behave if it were mistakenly prepared with a higher concentration of NaCl aqueous solution than indicated in its technical report [1].

The effect of high temperature was assessed by placing aliquots (opened vials) of nivolumab original medicine (Opdivo®) in an Eppendorf ThermoMixer® C (Hamburg, Germany) at a temperature of 60 °C for 1 h.

When studying the effect of high ionic strength, strong conditions were applied. We used a solution of 1.5 M of NaCl. The stressing agent concentration was 40 % (v/v) (160 μL of stressing agent was added to 40

μL of nivolumab medicine). The sample was analysed 24 h after preparation.

In order to investigate the effects of light exposure, aliquots of the medicine (Opdivo®) were placed in an accelerated stress test chamber to simulate sunlight (Solarbox 3000e RH, Cofomegra, Milan, Italy). The samples were light irradiated with a xenon lamp using an S208/S408 UV filter made of soda lime glass to simulate indoor exposure conditions with infrared rejection coating to reduce the temperature of the samples. The temperature was maintained at 20 °C, irradiation was set at 250 W/m^2, between 320 and 800 nm and the samples were exposed to the light for 24 h.

Once the chromatograms for the stressed samples had been registered, they were then compared with the chromatogram for the control sample of nivolumab. We also evaluated the isoform profiles for the stressed samples and compared them with those for the control sample. The control sample was not subjected to any stresses.

2.5.5. Robustness test

The robustness of our method was evaluated by making small changes to the flow rate, the temperature of the column and the mobile phase composition. Several aliquots of nivolumab standard solution of 25 μg/mL were prepared and analysed at each modified condition. In this way, we evaluated the impact of these changes on the analysis results. The robustness of our method was then estimated by means of the retention time, the number of theoretical plates (N) and the capacity factor (*k'*) in the corresponding chromatogram.

2.5.6. System suitability test

The system suitability test was carried out using standard samples of 25 μg/mL of nivolumab. ICH Q2(R1) does not indicate acceptance criteria for system suitability. We therefore decided to use the FDA criteria for HPLC methods of pharmaceutical analysis [38]. As a result, N and *k'* were evaluated in the chromatograms. The Hsu and Chien criteria for biologics were used to assess injection repeatability [39]. The acceptance criteria were $N > 2000$, $k' > 2.0$ and injection repeatability RSD (%) of the chromatographic area of the nivolumab chromatographic peak < 5 %.

2.6. Nivolumab isoform profile identification from MS data

The nivolumab sequence was obtained from the DrugBank database [40]. Mass spectra were used to obtain the *N*-glycan-based mass isoform profiles of nivolumab using freshly prepared standard solution samples and stressed samples (both at 25 μg/mL nivolumab) by deconvolution of the mass spectra using BioPharma Finder™ 3.1 software (Thermo Fisher Scientific). To perform the deconvolution, some post-translational modifications (PTMs) commonly found in mAbs produced in CHO cell line -such as nivolumab- were taken into account. According to the information extracted from the nivolumab sequence, these PTMs were 2C-term lysine clipping and 2 *N*-term pyroglutamate formation. The fact that nivolumab has 16 disulphide bonds was also taken into account.

2.7. Application of the method in a long-term stability study of the medicine

Within the framework of a long-term (15 days) stability study, the protein content of the medicine Opdivo® in opened vials (daily surplus) was tracked by analysing two independent aliquots (10 mg/mL, batch ABL3906), each stored at two conditions, i.e., in a refrigerator at 4 °C and on the bench-top at 20 °C. In both cases, the aliquots were protected from daylight. The stability of the solution was assessed using the validated stability-indicating method. This involved measuring its concentration over time for 15 days at intervals of 0 (D0), 1 (D1), 3 (D3), 6 (D6), 7 (D7), 10 (D10) and 15 (D15) days. To this end, we analysed 10 μL of the aliquots previously diluted at 25 μg/mL (the target concentration) with NaCl 0.9 %. The recovery of nivolumab (expressed as the percentage of recovery with regard to the day of preparation, D0) was calculated at each time point and plotted against time.

3. Results and discussion

3.1. Chromatographic analytical procedure

Quantification is an important step in the manufacturing and quality control process of any biopharmaceutical drug. It is a critical quality attribute, given the obligation to specify the protein content (the active product ingredient) of any pharmaceutical product or medicine. One of the best ways of quantifying the protein content of mAbs is via chromatographic analysis. As a starting point for this analysis, we decided to use the same experimental conditions applied in a chromatographic analytical procedure previously developed for the analysis of a group of different therapeutic mAbs [31]. We then optimized these conditions for the quantification of nivolumab. In brief, we used a MAbPac™ RP column because of its specificity for mAbs quantification analysis, and because it has demonstrated higher resolution capacity for mAbs analysis, as shown in our previous work with the chromatographic separation of at least four similar mAbs. The mobile phase was highly MS compatible as it consisted of a combination of water, acetonitrile (ACN) and formic acid (FA) as the additive. Another advantage is that it did not require the use of stronger eluotropic solvent systems, which are widely used in mAbs analysis to reduce column interaction due to the high hydrophobicity of mAbs [41].

The first experiments were carried out using the chromatographic conditions optimized in our previous research [31]. However, nivolumab, whose structure corresponds to IgG4, proved to be a more non-polar protein than the IgG1 analysed in our earlier research. This is because it was more strongly retained in the column. To solve this problem, the isocratic hold had to be increased to 2 min, to ensure the elution of nivolumab. In addition, the re-equilibration time had to be extended from 3.8 to 5.8 min, as more time was required to restore the column to its initial conditions. The rest of the chromatographic experimental conditions were as described in the previous study [31] and are set out here in the experimental section. With the optimized chromatographic conditions, the elution of intact nivolumab took place at a retention time of 4.25 ± 0.25 min, with a total analysis time of 11 min. This procedure produced rapid results, which is important for routine quality control analysis.

Moving onto the UV spectrum recorded by the DAD detector, three different wavelengths were selected i.e. λ = 214 nm, 220 nm and 280 nm for recording the chromatograms. The reference wavelength was λ = 350 ± 10 nm. The figures of merit for the proposed method were calculated using signals from λ = 214 nm, as this enabled higher sensitivity due to the higher absorbance of nivolumab at this λ. Another advantage was that the analytical signal recorded at this λ was less affected by the inherent heterogeneity of nivolumab. With all these chromatographic and UV conditions, suitable peaks of nivolumab were obtained (Fig. 1a) to enable us to carry out the validation of the quantification procedure. Signals from 220 nm and 280 nm were also used to corroborate the protein nature of the chromatographic peaks, since the absorption at these two wavelengths is characteristic of proteinaceous compounds, improving the specificity of the method.

3.2. LC-MS nivolumab N-glycan-based isoform profile identification

The MS conditions applied in our previous work [31] proved to be useful here for the intact analysis of nivolumab and did not require any further adjustment. Fig. 1b shows a representative total ion chromatogram (TIC) of nivolumab standard solution (25 μg/mL), while Fig. 2 presents the MS results after deconvolution. *N*-terminal glutamate to pyroglutamate conversion was identified. Five *N*-glycoforms were identified, whose masses coincided with those previously published in nivolumab samples [42,43]. A2G0F/A2G0F was identified as the main

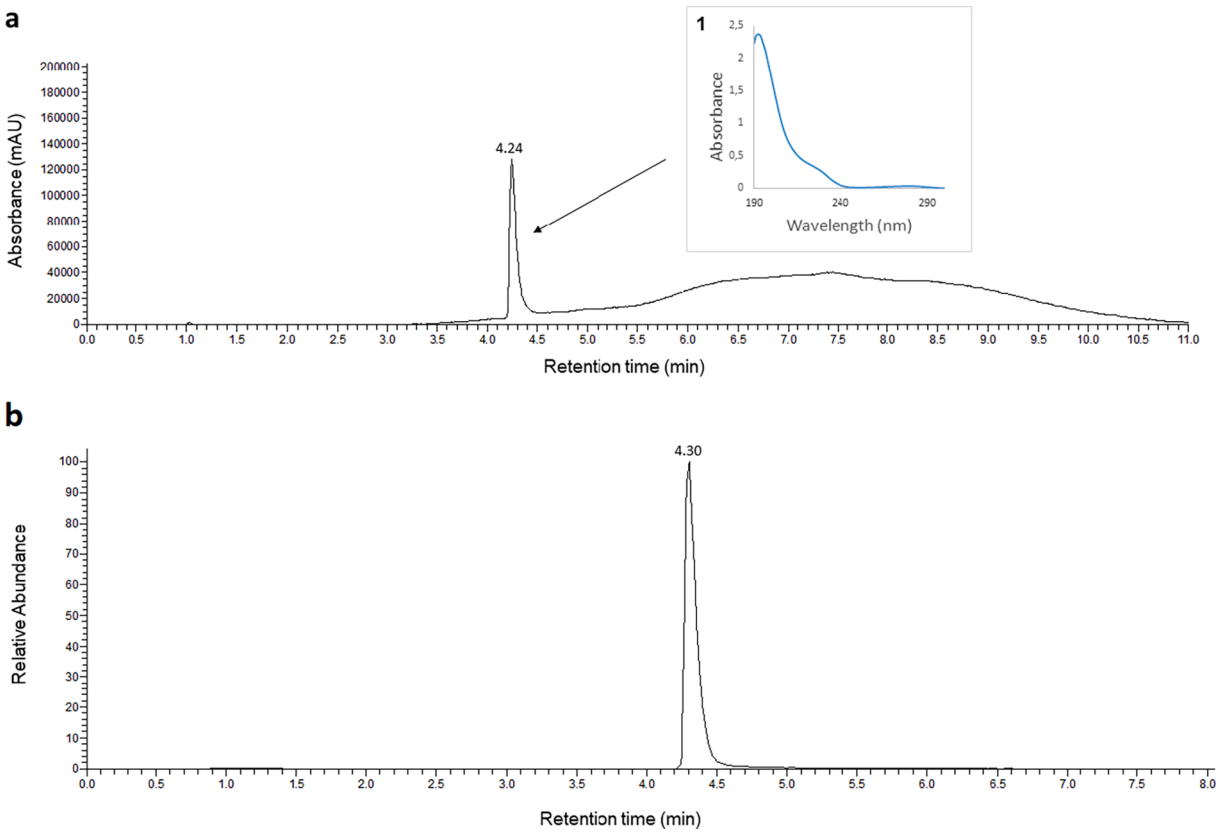

Fig. 1. UV chromatogram (a) and TIC (b) of standard solution of nivolumab at 25 μg/mL. UV absorption spectra of standard solution of nivolumab at 25 μg/mL D0 (a)1.

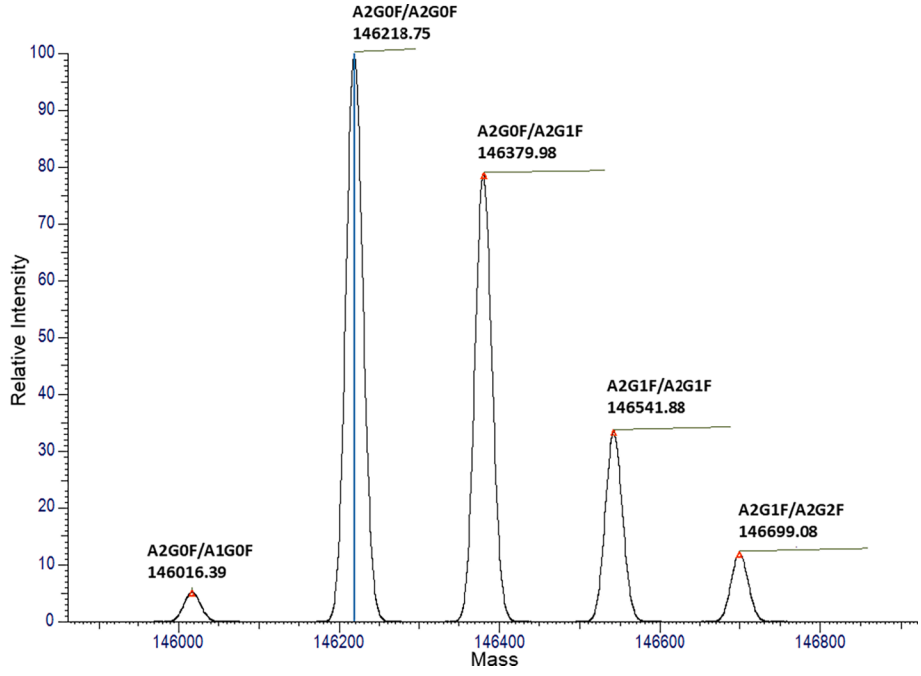

Fig. 2. Deconvoluted mass spectrum for nivolumab at 25 μg/mL showing the mass isoform profile and the *N*-glycoforms association obtained with the proposed method.

glycoform, followed by A2G0F/A2G1F, A2G1F/A2G1F, A2G1F/A2G2F and A2G0F/A1G0F. This glycosylation pattern was identical in the different batches that were studied in this research and the same 5 *N*-glycoforms were detected. Table 1 shows the experimental masses obtained for each isoform and the glycoforms associated with their relative abundance (%) and error (ppm) for the control sample of nivolumab (25 μg/mL). Theoretical masses were calculated considering 2C-terminal lysine clipping, 2 *N*-terminal pyroglutamate formation and

Table 1
Experimental mass for the intact nivolumab in control and stressed samples. The relative abundances of *N*-glycoforms are also reported.

	Associated glycoforms	Experimental mass (Da)	Theoretical average mass (Da)	Mass difference (ppm)	Relative abundance (%)
Control	A2G0F/A1G0F	146016.4	146017.0	3.8	2.2
	A2G0F/A2G0F	146218.7	146220.2	10.0	43.6
	A2G0F/A2G1F	146380.0	146382.3	15.7	34.3
	A2G1F/A2G1F	146541.9	146544.4	17.3	14.6
	A2G1F/A2G2F	146699.1	146706.6	51.0	5.2
Temp 60 °C	A2G0F/A1G0F	146012.5	146017.0	30.3	2.0
	A2G0F/A2G0F	146217.3	146220.2	19.4	44.1
	A2G0F/A2G1F	146380.3	146382.3	13.7	34.0
	A2G1F/A2G1F	146540.7	146544.4	25.7	15.8
	A2G1F/A2G2F	146701.9	146706.6	31.8	4.0
NaCl	A2G0F/A1G0F	146013.6	146017.0	23.1	1.6
	A2G0F/A2G0F	146218.3	146220.2	12.9	44.0
	A2G0F/A2G1F	146379.9	146382.3	16.1	33.5
	A2G1F/A2G1F	146540.7	146544.4	25.4	15.8
	A2G1F/A2G2F	146697.0	146706.6	65.6	5.1
Light	Not identified	146239.4	–	–	46.5
	Not identified	146587.2	–	–	53.5

16 disulphide bonds.

3.3. Validation of the analytical procedure for nivolumab quantification

The optimized procedure for RP chromatographic analysis described above was validated for nivolumab quantification purposes, using UV and MS signals to compare the figures of merit for the method when using one detector or the other. A review of the ICH Q2(R1) guideline is currently ongoing [44], which will define the common characteristic for the validation of procedures involving hyphenated techniques like LC-MS. In the meantime, we followed above all the current ICH Q2(R1) [11]. However, given that none of the ICH guidelines provide acceptance criteria, we also followed the FDA recommendations for chromatographic methods and procedures [38] and the Hsu and Chien criteria [39].

3.3.1. Linearity and detection and quantification limits

The results of the linearity study are shown in Table 2 for signals from UV and MS detectors. The coefficient of determination (R^2) was used to check the goodness of fit of the linear regression model.

If we consider the signals from the UV detector, a linear relationship can be observed over the 5 – 45 μg/mL nivolumab concentration range. This relationship was confirmed by the regression line established by the least squares method, with an R^2 value of 0.998, which indicates that the estimated regression line is a good fit for the experimental data. In addition, the intercept was not significant since the corresponding P-value was over 5 %. Linearity was also checked using the signals from the mass detector (total ions signals) for the same concentration range, i.e. 5 – 45 μg/mL (Table 2). In this case, results showed a slight decrease in the R^2 (0.944). However, a matrix effect was detected due to the significant intercept value (p-value < 5 %). This indicates that the UV signal produced better results than the MS signal.

UV and MS detectors obtained similar results for DL and QL with slightly lower values for MS (Table 2).

Table 2
Analytical parameters of the UHPLC method using UV and (HESI/Orbitrap™) MS signals.

Parameter	Values from UV	Values from (HESI/Orbitrap™)MS
Intercept (*a*)	-15,948	332,242,879
Slope (*b*)	24,198	83,190,184
Standard deviation of the intercept *s (a)*	8,161	137,211,598
Probability of significance of the intercept *P (%)*	6.64	2.62
Standard deviation of the slope *s(b)*	284.1	4,777,095
Coefficient of determination R^2	0.998	0.944
Linear range (μg/mL)	4.0–45.0	1.0–45.0
Quantification limit (μg/mL, estimated from SD(*a*))	4.0	1.0
Detection limit (μg/mL, estimated from SD(*a*))	2.0	0.2

3.3.2. Accuracy and precision

As stated in the ICH Q2(R1) guidelines [11], accuracy and precision were assessed across the linear range of the analytical procedure. The accuracy and precision results were expressed as recovery (%) and RSD (%), respectively. Both parameters were calculated by considering UV and MS signals separately. The results are shown in Table 3. Satisfactory values were obtained for the UV signals, with recovery values close to 100 % for each concentration checked, so fulfilling the acceptance criteria (value within the 100 ± 3 % range). As regards precision, the criteria were not fulfilled for the lower concentration, with a RSD (%) > 5 %, but precision –both inter and intraday- increases in line with concentration, as expected. This means that for the target concentration (25 mg/mL), the analytical procedure fulfilled the criteria.

By contrast, the accuracy and precision results for the (HESI/Orbitrap™)MS signals were significantly worse across the linear range than those obtained for the UV signals (described above) and the criteria were not fulfilled. The recovery values were<100 %, and did not meet the criteria for any of the concentration levels checked. Intra- and interday precision results did not meet the selected criteria either, and in all cases the RSD (%) was over 5 %.

3.3.3. Specificity

To evaluate the specificity of the proposed analytical procedure, forced degradation studies were performed according to the ICH Q2(R1) guidelines [11]. Another aim of these studies was to find out more about

Table 3
Accuracy and precision from UHPLC using the-UV and (HESI/Orbitrap™)MS signals.

Analytical signal	**Concentration (μg/mL)**	**Recovery (%)**[a]	**RSD (%)**[b]	
			Intraday	Interday (6 days)
UV	5	99.18	8.07	10.60
	25	103.0	0.71	2.54
	45	100.18	1.35	2.53
(HESI/Orbitrap™) MS	5	52.34	50.0	41.03
	25	93.33	8.55	12.19
	45	93.25	5.70	6.75

[a] Recovery value based on three measurements.
[b] Relative standard deviation based on three measurements. Intraday means on the same day, whereas interday means on six different days.

the possible degradation of nivolumab samples during real use in hospitals prior to administration to patients. With this in mind, in this study, we selected three stress factors that could potentially affect the stability of nivolumab when handled in hospitals. These are discussed below.

It is important to remember that slight physicochemical modifications, such as the presence of oligomers, will not be reflected in the chromatograms, unless UV-spectra were recorded to perform similarity analysis, as described in the literature [24–26]. The UV chromatograms recorded at three λ (214, 220 and 280 nm) in this study will not reflect these modifications. Nevertheless, a comparison between control and stressed samples was carried out in order to detect significant modifications, such as the breaking of the protein chains. As previously mentioned, three stresses were performed using Opdivo® samples to evaluate the specificity: thermal stress at 60 °C; high ionic strength, as the medical samples are diluted in 0.9 % NaCl for clinical administration; and light exposure due to the fact that therapeutic proteins are exposed to light during preparation for administration to the patient. In all cases, the UV chromatograms for the stressed and the control (fresh) samples of nivolumab were similar, in terms of both the profile and the area under the peaks. New chromatographic peaks were not detected in any of them and the main chromatographic peak appears at the same retention time in stressed and fresh samples (Fig. 3a), therefore indicating that none of the samples suffered protein breakdown.

Consequently, specificity was assessed using the mass detector (MS results), which allows small physicochemical changes to be detected by evaluating the isoform profile. As regards the thermal stress at 60 °C, TIC chromatograms did not show any variation in the TIC chromatogram profiles for the stressed sample as compared to the control sample (Fig. 3b). Moreover, the MS isoform profile showed the same *N*-glycoforms in both the stressed and the control (fresh) samples (Fig. 4), indicating that there was no chemical degradation of nivolumab at this temperature. In the high ionic strength stress test, there was a decrease in the relative abundance of the TIC, which was attributed to the suppression of the mass signal due to the higher concentration of salt in the medium (Fig. 3b). We believe that the new peak detected at the front of this chromatogram (RT = 0.68 min) is an artefact, as its mass information was not related to nivolumab and it was not detected in the UV chromatogram. In this case, the MS isoform profile was similar in both stressed and control samples, and the same *N*-glycoforms were detected (Fig. 4). Finally, as regards light exposure stress, important modifications were detected by the mass analysis of the isoforms. The results showed that the molecular weight of the isoforms was different from that of the control samples, clearly indicating that chemical modifications had taken place (Fig. 4). An increase in the number of isoforms was also observed in the stressed sample, and the main isoform was different from that of the control (fresh) nivolumab sample. This shows that exposure to light caused important chemical modifications in the isoform profile of nivolumab, so indicating that it had suffered degradation. This highlights the need to use a high-resolution mass detector to detect such modifications, in that the isoforms promoted by light degradation were not chromatographically separated in RP mode, and they were not detected by changes in any of the three UV chromatograms registered at three different λ. Moreover, Fig. 4b shows that two original nivolumab isoforms remain after exposure to light as they are in the same mass range as the isoforms of the control sample. However, these two isoforms have different masses than the isoforms of fresh nivolumab, which means that no *N*-glycoforms can be attributed to these two masses. According to previous publications [16,45], when exposed to light, proteins can undergo photo-oxidation in residues such as tryptophan, tyrosine and phenylalanine. This suggests that the variation in the mass of the isoforms produced by light-stressed samples may be due to photo-oxidation of nivolumab. This is currently being investigated in our group using a bottom-up strategy (peptide mapping by LC-MS/MS).

After gathering together all the results of the degradation study to

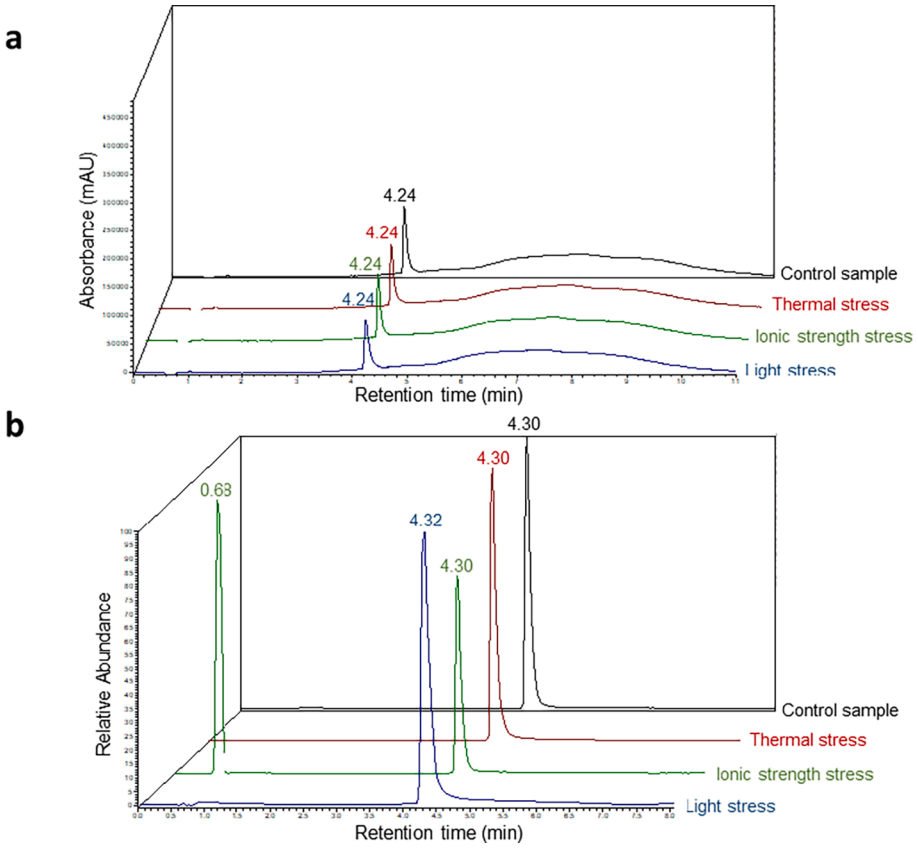

Fig. 3. Controlled degradation study of nivolumab 25 μg/mL using the (RP)UHPLC-UV-(HESI/Orbitrap™)MS method. Representative UV chromatogram (a) and TIC (b) of: control (fresh), thermal stress, ionic strength stress, and light stress samples. The chromatograms are displayed normalized from the main peak.

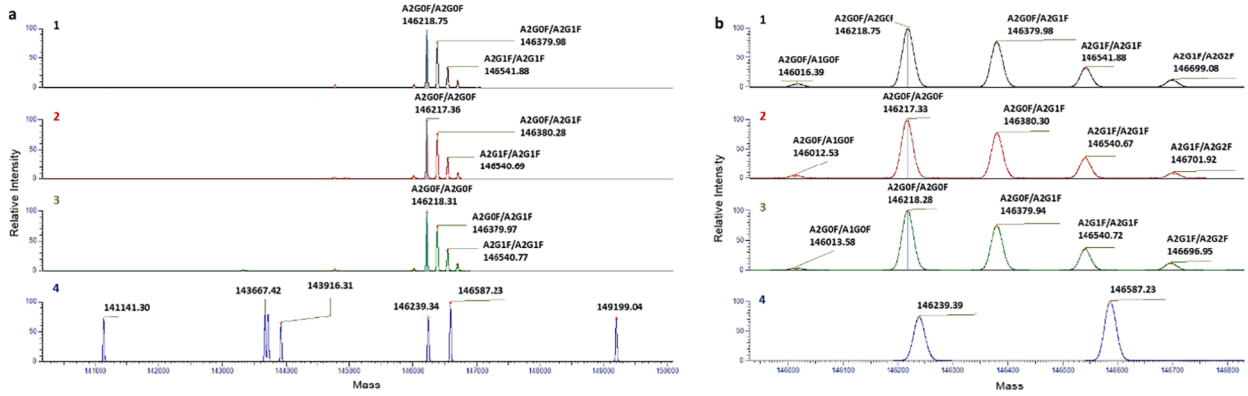

Fig. 4. Deconvoluted mass spectra for nivolumab 25 μg/mL showing the mass isoform profile with the associated *N*-glycoforms (a) and the corresponding expansion (b). Control (fresh) sample (1), thermal stress sample (2) ionic strength stress sample (3) and light stress sample (4).

assess the specificity of the proposed (RP)UHPLC-UV-(HESI/Orbitrap™) MS method, these indicated that the degraded nivolumab could not be chromatographically separated. This means that degraded nivolumab cannot be differentiated from control nivolumab, as the elution time is similar for both degraded and non-degraded forms of this mAb (Fig. 3). Nevertheless, modifications in the isoforms were clearly detected by HRMS (Fig. 4). If we look at the results for each stress test, the isoform profile for the thermal and high ionic strength tests remained unchanged compared to the deconvoluted mass spectrum for the control samples. However, the light exposure stress produced a different isoform profile, with a different total number of isoforms. Fig. 4a shows an increase in the number of isoforms with regard to control, thermal stressed and high ionic strength stressed samples, which means that light stress had a more noticeable effect on nivolumab than other stresses, which did not alter the chemical structure. Table 1 shows the masses obtained for the isoforms of the stressed and the control (fresh) samples of nivolumab and the associated *N*-glycoforms. It also includes the masses of the *N*-glycoforms and their relative abundance (%) and error (ppm). As occurred in previous research [24–26], although the proposed method was unable to chromatographically separate degraded nivolumab, it was capable of detecting and identifying, via HRMS, slight physicochemical modifications in the product. The method can therefore be considered as stability-indicating in nivolumab medicine samples, in which it can rigorously detect degradation.

3.3.4. *Robustness test*

The results of the study performed to assess the robustness of the method are summarized in Table 4. The retention time was most affected by small variations in the flow rate, and was only slightly altered by the changes in mobile phase composition and temperature. The same results were observed for both the UV and the HRMS detection techniques. This indicates that the flow rate was the most important factor influencing the robustness of the method.

The results set out in Table 4 indicate with regard to the UV signals that in all cases the N and k' values were within the FDA acceptance criteria (≥2000 and > 2.0, respectively). However, when the MS signals were used, k' met the acceptance criteria, but N did not. This shows that the method is more robust when using signals from the UV detector than when using MS signals.

3.3.5. *System suitability test*

The results of the system suitability test are summarized in Table 5. This test was conducted using standard nivolumab samples at a

Table 5
Results for the system suitability of the method using UV and (HESI/Orbitrap™) MS signals.

Technique	Nivolumab standard sample	Retention time	N[a]	k'[b]
UV	25 μg /mL	4.24	2348.10	4.3
(HESI/Orbitrap™) MS	25 μg /mL	4.30	98.85	6.68

[a] Theoretical plates.
[b] Capacity factor.

Table 4
Results for the robustness of the method using UV and (HESI/Orbitrap™)MS signals. Control optimized parameters of the proposed method are shown in bold type.

Technique	Gradient variation (%)B	T (°C)	Flow (mL/min)	Retention time (min)	N[a]	k'[b]
UV	**80**[c]	**70**[c]	**0.3**[c]	**4.24**[c]	**2488.25**[c]	**5.42**[c]
	80	68	0.3	4.25	2229.94	5.44
	80	72	0.3	4.23	2979.05	5.41
	80	70	0.28	4.48	2621.44	5.40
	80	70	0.32	4.03	3089.83	5.30
	78	70	0.3	4.26	2653.81	5.44
	82	70	0.3	4.26	3007.28	5.44
(HESI/Orbitrap™)MS	**80**[c]	**70**[c]	**0.3**[c]	**4.30**[c]	**144.67**[c]	**6.68**[c]
	80	68	0.3	4.31	59.49	7.11
	80	72	0.3	4.28	28.14	9.17
	80	70	0.28	4.54	71.38	6.53
	80	70	0.32	4.07	60.10	15.28
	78	70	0.3	4.31	64.30	22.94
	82	70	0.3	4.31	48.72	29.79

[a] Theoretical plates.
[b] Capacity factor.
[c] Selected optimized conditions.

concentration of 25 μg/mL. The mean values of N and k' were found to be within FDA acceptance criteria (≥2000 and > 2.0, respectively), with RSD values < 5 % when the UV signals were used [38,39]. However, the N values did not meet the acceptance criteria when the MS signals were used. Once again, from the chromatographic experimental point of view, the system suitability is greater when tested using the signal from the UV detector than when using the MS signal from the HRMS.

3.4. Long-term stability of the medicine (Opdivo®) using UV detection

The results of the evaluation of the protein content in the daily surplus of the medicine Opdivo® stored for 15 days either at room temperature (20 °C) or refrigerated at 4 °C, both daylight protected, are shown in Fig. 5. After storage for 24 h at the two different storage conditions, nivolumab recovery values were > 90 % in both cases. After 15 days, nivolumab recovery values were > 80 % in both storage conditions (Table 1, supplementary material). In all the UV chromatograms registered, the nivolumab peak was detected at 4.24 ± 0.01 min and the shape remained unchanged for the two storage conditions (Fig. 1, supplementary material). On the basis of these UV detection results for the protein content in the medicine samples, the refrigerated samples could be considered stable for 15 days (recovery close to 90 %), while those stored at room temperature (20 °C) could be considered stable for 7 days. To evaluate possible modifications in the chemical structure of nivolumab, the MS isoform profile was analysed every day during the experimental period. Fig. 6 shows the variations in the isoform profile. In both storage conditions (4 °C and 20 °C), the three most abundant isoforms remain unchanged, while the other two (less abundant isoforms) were not always detected. This was attributed to the low intensity of these isoforms which were not always detectable by the mass spectrometer. Tables 2 and 3 of the supplementary material show the relative abundance (%) and error (ppm) of the associated glycoforms over the 15 days of the stability study for storage at 4 °C and 20 °C, respectively.

This long-term stability study was carried out, above all, for the purpose of applying and testing the stability-indicating method we had developed. Assessing the stability of nivolumab was only a secondary objective. This will be further assessed in greater depth in future stability studies using different techniques focused on different quality attributes of the medicine.

4. Conclusions

For the first time, a (RP)UHPLC/UV-(HESI/Orbitrap™)MS method for nivolumab quantification (protein content) was successfully developed and validated as stability-indicating according to the ICH quality guideline Q2(R1), in that it was able to detect structural modifications in the degraded protein.

A comparative study of the figures of merit for the validated method using the UV and the (HESI/Orbitrap™)MS signals was conducted. Apart from the specificity and sensitivity parameters, all others worked better when using data from the UV signal. In the robustness and system suitability tests using the (HESI/Orbitrap™)MS signals, not all the acceptance criteria were met. Nevertheless, these signals played a crucial role in assuring method specificity in order to validate it as stability-indicating. This was achieved by identifying the molecular weight isoform profile of nivolumab. This provided information about structural characteristics (such as the *N*-glycosilation pattern or pyroglutamation identification). It also identified the main isoforms and detected any modifications due to protein degradation when they occurred. The method can therefore be classified as stability-indicating, according to the ICH Q2(R1).

The method was successfully applied to track the protein content in the daily surplus of the medicine Opdivo® (nivolumab 10 mg/mL) over the course of 15 days. This method complied with all the requirements to enable it be used for the analysis of protein content, a critical quality factor in any quality control program in the pharmaceutical field.

CRediT authorship contribution statement

Anabel Torrente-López: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Jesús Hermosilla:** Conceptualization, Validation, Formal analysis, Investigation. **Raquel Pérez-Robles:** Writing – original draft, Supervision. **Antonio Salmerón-García:** Conceptualization, Formal analysis, Resources, Funding acquisition. **José Cabeza:** Formal analysis, Resources, Funding acquisition. **Natalia Navas:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Funding acquisition, Project administration.

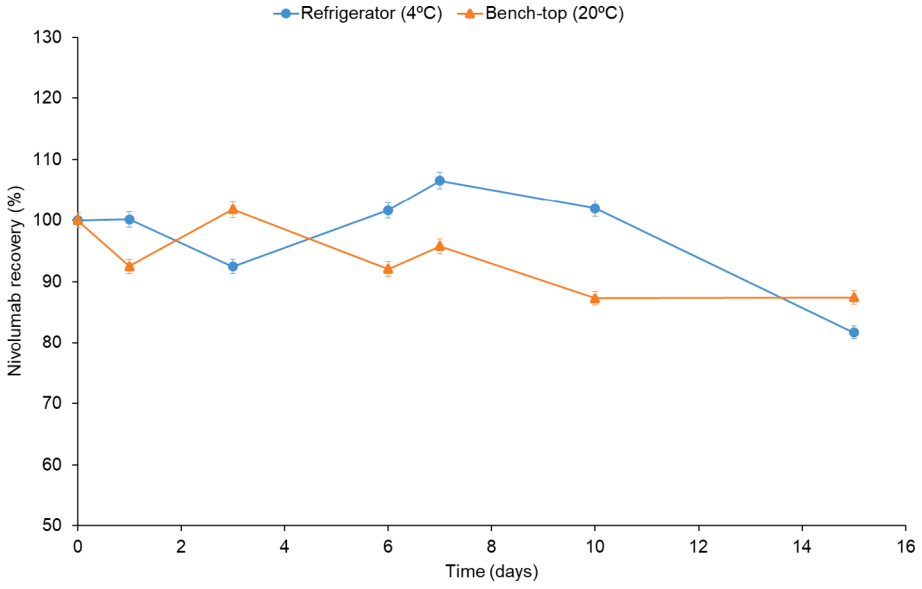

Fig. 5. Results of the stability study over time: protein content of the daily surplus of Opdivo® (nivolumab 10 mg/ml) solution during storage for 15 days under two different conditions: in a refrigerator at 4 °C (blue) and on the bench-top at 20 °C (orange). Nivolumab 25 μg/mL was the target concentration for (RP)UHPLC-UV-(HESI/Orbitrap™)MS method analysis.

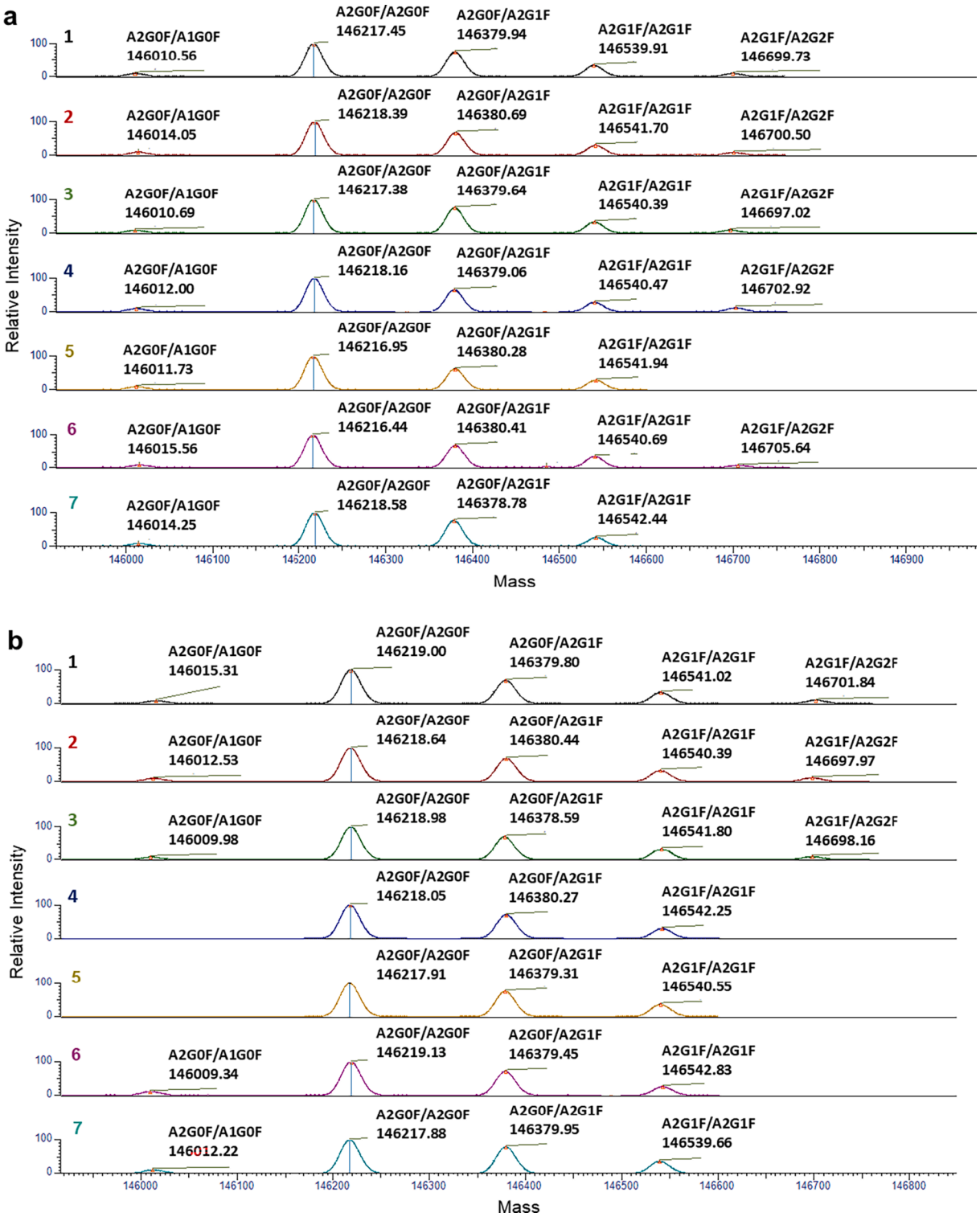

Fig. 6. Results of the stability study along time: Deconvoluted mass spectra (isoform profile including the associated *N*-glycoforms) resulted from the analysis of nivolumab in the daily surplus of the medicine Opdivo®. Two storage conditions: in a refrigerator at 4 °C (a) and on the bench-top at 20 °C (b). Checked days: Day 0 (1), day 1 (2), day 3 (3), day 6 (4), day 7 (5), day 10 (6), and day 15 (7).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.107896.

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