Research Article **IJHP, 2023, 7:42**

International Journal of Hospital Pharmacy (ISSN:2574-0318)

Development and Validation of Analytical Methods to Determine the Prolonged in-use Stability of Anticancer Monoclonal Antibodies in Clinical Practice

A. Hedvig Arnamo[1], Dick Pluim[1], Alwin D.R. Huitema[1,2,3], Bastiaan Nuijen[1], Jos H. Beijnen[1,4]

[1.]Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek hospital - Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; [2.] Department of Clinical Pharmacy, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands; [3.] Department of Pharmacology, Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands; [4.] Division of Pharmacoepidemiology and Clinical Pharmacology, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, David de Wied building, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands

ABSTRACT

Background/purpose: Personalized dosing of monoclonal antibodies (mAbs) based on body weight and surface area may result in leftovers that need to be discarded due to the absence of stability data. After manufacturing, these mAbs undergo extensive quality control with a panel of methods. With some of these similar methods, these mAbs could be tested to see if stability is maintained after the opening of the pharmaceutical product vial and dilution for administration in a daily clinical setting. Prolonged in-use stability may reduce unnecessary waste and minimize the financial loss of these expensive drugs.

Methods: Previously, based on extensive literature research, a complementary panel of methods was selected including visual inspection colour, apparency and, clarity (CAC), size exclusion chromatography (SEC), enzyme-linked immunosorbent assay (ELISA), dynamic light scattering (DLS), thermal denaturation (Tm), and aggregation (Tagg) determination, pH and dye ingress method. We evaluated, developed, and validated these methods with respect to the accuracy, precision, reproducibility, stability-indicating capability, carry-over, limit of quantification (LOQ) and limit of detection (LOD) for nivolumab and pembrolizumab as prototype anticancer mAbs.

Results: Analytical results show that stability can be determined

***Correspondence to Author:**
Hedvig Arnamo, MSc
Department of Pharmacy & Pharmacology Antoni van Leeuwenhoek – The Netherlands Cancer Institute
Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands

How to cite this article:
A. Hedvig Arnamo, Dick Pluim, Alwin D.R. Huitema, Bastiaan Nuijen, Jos H. Beijnen.Development and Validation of Analytical Methods to Determine the Prolonged in-use Stability of Anticancer Monoclonal Antibodies in Clinical Practice. International Journal of Hospital Pharmacy, 2023, 7:42.

eSciPub LLC, Houston, TX USA.
Website: https://escipub.com/

from a physiochemical, biological, and microbiological point of view with the selected methods. Forced degradation studies showed that even small instabilities can be detected using the panel of methods together, therefore the stability-indicating capability has been established. CAC shows mainly aggregation and discolouration. SEC shows aggregation and fragmentation/degradation, DLS gives information about turbidity and soluble aggregates, T_m and T_{agg} give information changes intra- and inter conformational changes, respectively, in the mAb molecule. ELISA shows potential loss in bioactivity and the pH of the sample is essential to the chemical composition of the mAb molecules in the solution.

Conclusion: A panel of analytical methods was selected, enabling in-use stability determination of anticancer mAbs. This panel will be used in future studies to determine the prolonged use of leftover anticancer mAb products and infusion solutions, starting with nivolumab and pembrolizumab.

Keywords: Stability, mAbs, Nivolumab, Pembrolizumab, SEC, DLS, ELISA, T_m

Introduction

In clinical practice today personalized dosing of monoclonal antibodies (mAbs), based on body weight and surface area, may result in leftovers of mAb products. Due to the absence of stability data, these leftovers sometimes need to be discarded. After manufacturing, these mAbs undergo extensive quality control with a panel of methods. To examine the prolonged stability in a clinical setting, some of these methods, or similar methods, could be used to test if stability is maintained after the opening of the pharmaceutical product vial and, dilution for administration in a daily clinical setting. Prolonged in-use stability may reduce unnecessary waste and minimize the financial loss of these expensive drugs. In this manuscript, we describe a panel of assays that can be used to examine the in-use stability of nivolumab (Opdivo®, Bristol-Myers Squibb (BMS)) and pembrolizumab (Keytruda® Merck & co. (MSD)). Both are immunoglobulin (Ig) G4 programmed cell death protein 1 (PD-1) targeted monoclonal antibodies (mAbs) frequently used in cancer immunotherapy. Both checkpoint inhibitors have a shared indication for melanoma and non-small cell lung cancer (NSCLC). Before administration to the patient, Opdivo® and Keytruda®, which both come as a concentrate, have to be diluted in normal saline (NS) or glucose (D5W) infusion solution. In daily practice, due to the nivolumab and pembrolizumab patient dose, the limited number of product formats, and overage of mAb concentrate per vial, leftovers of nivolumab and pembrolizumab concentrates are accumulated. The current expiration time for both pharmaceutical products, however, is limited from a microbiological as well as a physicochemical point of view (Table 1)[1,2].

As these products carry a high economic value, it is warranted to examine if in-house stability data can be generated to use concentrate leftovers or infusion solutions over a prolonged period of time. To this purpose, based on extensive literature research, we have assessed a stability-indicating panel of assays that may substantiate the use of nivolumab and/or pembrolizumab concentrate and infusion solution beyond the expiration timeframe indicated by the manufacturer (see Table 2).

Assays

Colour, apparency, and clarity (CAC)

Colour, apparency, and clarity (CAC) examination is performed by visual inspection of the sample. Mainly the inter-molecular stability is regarded here, regarding aggregation formation but also other instabilities that might cause colour change or other changes in appearance. Both physiochemical and microbiological changes can be seen as insoluble particles and discolouration[3–5].

Size exclusion Chromatography (SEC)

Due to its precision, robustness, and simplicity, Size Exclusion Chromatography (SEC) is often used for protein analysis. This method separates particles according to size due to the pore structure of the SEC column. The amount of time a solute remains within a pore is dependent on the size of the pore. Larger solutes will have access to a smaller volume and vice versa. Therefore, a smaller solute will elute from the column later because it remains within the pore for a longer period compared to a larger solute [6–9]. Coupled to a high performance liquid chromatography (HPLC) system with a diode array detector (DAD), we can detect and quantify the different eluting particles[9,10]. SEC allows detection of mAb fragmentation and small size aggregations and therefore gives us information about the quaternary structure[11]. Another benefit of SEC is that desalting is not necessary for samples diluted in saline.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is performed to examine the hydrodynamic diameter, the absolute diameter of the molecular sphere, and compare it to theoretical and fresh sample values. This will indicate if there is any turbidity or soluble aggregates in the sample solution. This method can determine particle sizes between 0.1 nm – 10 µm[12] and therefore give us an evaluation of the tertiary and quaternary structure of the mAb[7,8,13]. The method also provides information about the polydispersity index (PDI) of the sample, giving us information about the distribution of different sized particles in the sample.

Thermal denaturation (T_m) and Thermal aggregation curves (T_{agg})

Thermal denaturation curves give intramolecular conformational information about the denaturation/(melting) point (T_m) and intermolecular information about the thermal aggregation temperature (T_{agg}). T_m is defined as the temperature at which half of the protein molecules are denatured, while T_{agg} indicates at what temperature aggregation starts. The T_m curves for proteins such as mAbs are usually measured with ultraviolet (UV) detection. T_m and T_{agg} can also provide important information about the purity of the sample, by looking at any shift in the melting curve[2,17,18].

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is one of the most used immunoassays for determining the biological activity of mAbs. Different kits are commercially available In this study, ELISA is used to determine the biological activity of mAbs by quantification of the amount of mAbs bound to the programmed death-1 (PD-1) coating on the plates. [16,17].

pH

The pH of a sample is very important for the stability of mAbs. The pH should be lower than the isoelectric point (pI) of the mAb, otherwise, the tertiary, quaternary, secondary, and also primary structures could change due to interactions within the mAb[18].

Microbiological stability (Dye ingress method)

The microbiological stability is crucial and must be examined to ensure that there is no contamination while preparing the infusion solution for patients or when storing the samples. A study to ensure safe handling, from a microbiological safety point of view, has been conducted previously at our Institute[19]. The integrity of already opened vials was examined by the dye ingress method developing a method

of safe handling of the mAb vials so that no contamination occurs, and that the integrity was assured after opening the vials.

In this manuscript, we examine and describe the selected methods and their validation in detail to study mAb stability from a pharmaceutical point of view. The aim is to see if these methods have stability-indicating capability for nivolumab and pembrolizumab. After evaluating this we can then set up a panel of the best methods to later be used to examine the prolonged stability of mAbs in a clinical setting. An overview of the methods is given in Table 2.

Material & Methods

Chemicals

Nivolumab (Opdivo®) 10 mg/ml pharmaceutical product was purchased from Bristol Myers Squibb, Rueil-Malmaison, France. Pembrolizumab (Keytruda®) 25 mg/ml pharmaceutical product Merck & Co., Darmstadt, Germany. Saline (0.9% NaCl) was from Braun (Melsungen, Germany). Potassium dihydrogen phosphate (KH_2PO_4), di-potassium phosphate (K_2HPO_4), potassium chloride (KCl), hydrochloric acid 25% (w/v) (HCl), sodium hydroxide 50% (w/v) (NaOH), hydrogen peroxide 30% (w/v) (H_2O_2), and saccharose ($C_{12}H_{22}O_{11}$) originated from Merck (Darmstadt, Germany). Bovine Serum Albumin (BSA), Tween-80 (Polysorbate 80), dithiothreitol (DTT), L-histidine, and D-mannitol were bought from Sigma Aldrich (Steinheim, Germany). Water (H_2O), with an analytical grade for Ultra Performance LC (UPLC) grade, were purchased from Biosolve Ltd (Valkenswaard, The Netherlands). Gold nanobeads 10 nm ± 2 nm with hydrodynamic diameter 19 nm were purchased from Nanocomposix (Prague, Czech Republic), 60 & 200 nm Nanosphere standard was purchased from Thermo Fisher Scientific (Landsmeer, The Netherlands). Nunc MaxiSorp™ white 96-well plates come from VWR (Amsterdam, The Netherlands). Phosphate buffered saline (PBS) was purchased from GIBCO BRL (Gaithersburg, MD, USA). Ficoll-paque™PLUS was from General Electric Healthcare (Little Chalfont, UK). Tween-20 was from Sigma (St. Louis, MO, USA). Mouse anti-human IgG_4 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 PBS, and 0.05% (w/v) thimerosal. 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).

Preparation of solutions

Nivolumab 10 mg/ml was diluted in 0.9% NaCl (saline) to a final concentration of 1 mg/ml according to the Summary of Product Characteristics (SmPC).[1] Pembrolizumab 25 mg/ml was diluted in saline to a final concentration of 1 mg/ml.[2] In the development phase of our study, different concentrations were tested. Later, in all experiments, solutions of 1 mg/ml were used for the analysis except for the ELISA where the samples were further diluted using PBS supplemented with 0.1% (v/v) Tween and 1% Ficoll (v/v) (PBSTF). A freshly opened vial (<24h at 2-8°C) of each mAb was used to prepare the reference standards in all experiments.

Forced degradation samples

The forced degradation samples were prepared for both nivolumab and pembrolizumab. The samples were made in duplicates with the initial concentration of 1 mg/ml[1,2]. The matrices for the forced degradation samples were first prepared. The oxidizing condition was prepared by adding 30 % H_2O_2 to water to obtain a concentration of 1M. The basic condition was made by adding 50% NaOH to water to a concentration of 1M. The acidic condition by using 25% HCl added to water to a final concentration of 1M. Samples were treated with both 1 M and 0.1 M of the acidic, basic, and oxidative solutions. Samples that underwent UV exposure, heat 80°C and

shaking were prepared as the standard samples under *2.2 Preparation of solutions*. UV samples were exposed to 344 nm (and also 254 nm for nivolumab) by a CAMAG Tamson UV lamp (Zoetermeer, The Netherlands). The heat samples were put in a Julabo TW8 water bath of 80°C (Julabo Labortechnik GmbH, Seelbach, Germany). The samples that underwent shaking (only nivolumab) were put on a shaking table set to 450 rpm (Vibramax 100, Heidolph Instruments, Schwabach, Germany). All forced degradation samples were centrifuged in a Centrifuge 5427R (Eppendorf, VWR, Amsterdam, The Netherlands) at 5000 rpm for 30 seconds. The acidic, basic, and heated samples were stored at room temperature or 80°C for 6 h, the UV, shaken, and oxidation samples were kept at room temperature for 24 h (and in dark for the oxidation sample) and thereafter put at 2-8°C until analysis.

Visual inspection – Colour, Appearance, and Clarity (CAC)

Before every run, the physical stability was examined by visual inspection against a white surface to determine if there was any discolouration or visible particles in the sample. A fresh sample was compared with a stored sample or a sample that had gone through forced degradation.

Size Exclusion Chromatography (SEC)

Preparation of calibration (CAL and quality control (QC) samples

Calibration standards (CAL1-6) were prepared by diluting nivolumab 10 mg/ml and pembrolizumab 25 mg/ml in saline to concentrations of 500, 700, 900, 1200, 1300, and 1500 µg/ml, while CAL0 contained no mAb product. QC samples had a concentration of 600, 1000, and 1400 µg/ml for QC-Low, QC-Mid, and QC-High, respectively. The calibration standards and quality control solutions were prepared from different batches of nivolumab and pembrolizumab.

For the forced degradation analysis and LOD/LOQ determinations, the calibration curve was extended from 0.25-1500 µg/ml with duplicates of 0.25, 5, 10, 30, 50, 100, 250, 500, 750, 1000, 1500 µg/ml.

HPLC-SEC-DAD/PDA method

The chromatographic system consisted of a solvent delivery pump (Ultimate LPG-3400M), autosampler (Ultimate WPS-3000TSL), diode array detector (DAD) (Ultimate DAD-3000), and a column oven (TCC-3000SD) from Thermo Fisher Scientific (Waltham, MA, USA). A Yarra™ SEC-3000 (300 x 7.8 mm 3 µm particle size) analytical column with SecurityGuard Guard Cartridge Kit and SecurityGuard Cartridges GFC 3000 (4 x 3.0mm ID, 10/Pk) were used (Phenomenex, Utrecht, The Netherlands). The eluent consisted of 50 mM potassium phosphate buffer in water (KH_2PO_4/K_2HPO_4 in H_2O) with 250 mM potassium chloride (KCl) with a pH of 6.7. Isocratic elution was applied at a flow rate of 1 ml/min. The tray was held at 4°C and the column oven was held at 25°C. The injection volume was set to 10 µl. Photodiode array (PDA) spectra between 190 nm and 800 nm were obtained to determine the purity of the peaks and quantification and the main analysis were performed at 280 nm. The peak purity was assessed by an algorithm based on the UV spectra comparison over the peak. Before the start of an analytical run, a system suitability test (SST) (6-fold injection followed by a 2-fold injection of a concentration of 1000 and 500 µg/ml respectively) was executed. The analytical batch analysis was started only when the RSD of the area of the mAb (nivolumab or pembrolizumab) was ≤5%. All collected data were analysed with the acquisition software Chromeleon (version 7.2 SR5, Waltham, MA, USA).

Method validation SEC

Validation of the SEC method was based on ICH guidelines [20]. The validation of the method was performed on three separate occasions. Furthermore, before the start of each analytical batch, a system suitability test was performed (see section 2.4.2).

Linearity

Duplicates of calibration standards at each concentration level were freshly prepared from a nivolumab (10 mg/ml) and pembrolizumab (25 mg/ml) vial respectively before each validation batch. The peak areas were plotted against the corresponding nominal concentration. Linear regression was applied using a weighting factor of 1/x, where x is the mAb concentration. The calibration model was found to be linear when the deviation from the back-calculated concentration for CAL1 was within ±10% and ±5% for the other CAL2-6 levels.

Accuracy & Precision

Quality control samples at every concentration level were freshly prepared five-fold before each validation batch. The nivolumab (10 mg/ml) and pembrolizumab (25 mg/ml) used were from another vial than for the calibration standards. The intra- and inter-assay accuracy were calculated per QC level (QC-Low, QC-Mid, and QC-High). The accuracy was expressed as the bias from the measured concentration of nivolumab or pembrolizumab from the nominal concentration. The accepted criteria for the bias were within ±10% for QC-Low, ±5% for QC-Mid, and QC-High. The intra- and inter-assay precisions were determined by calculating the relative standard deviation (RSD) and these values should be ≤10% for QC-Low and ≤5% for QC-Low and QC-High.

Carry-over

Carry-over was examined by injecting two blanks after the highest calibration concentration level (CAL6) in every validation run and the chromatograms were examined. A level of ≤0.1% carry-over (peak area in the first blank versus the mAb area in CAL6) was considered to be acceptable.

Lowest limit of detection (LOD) and quantification (LOQ)

LOD and LOQ were calculated with the signal-to-noise approach using samples with low concentration samples of nivolumab and pembrolizumab ranging from 0.25-250 µg/ml. The ratio between signal and noise for LOD was determined as 3:1 and 10:1 for LOQ.

Stability-indicating capability (Forced degradation study)

The nivolumab and pembrolizumab samples that had been treated in the different forced degradation conditions were tested 2-fold for each duplicate on two different days, and results were compared to a fresh reference sample. The results from these samples were compared with a fresh sample to see if the method had a stability-indicating capability.

Dynamic Light Scattering (DLS)

A Zetasizer Nano SD (Malvern Panalytical, Malvern, United Kingdom) was used to determine the hydrodynamic diameter and the PDI of the samples. The samples with a concentration of 1 mg/ml were transferred to 10 x 10 x 45 mm polystyrol/polystyrene cuvettes (Sarstedt AG & Co, Nümbrecht, Germany). The method was developed at 25°C to take 3 runs and provide the average value result from the particle peak. The number of measurements per run was automized by the instrument and differed between the runs, all runs had >60 measurements (except the acidic, basic and 80°C samples that had fewer measurements), however, most runs were around 100 measurements. The PDI was calculated for each peak as follows: (standard deviation/mean)^2. Normally a monodisperse sample has a PDI between 0 and 0.1, whereas moderately polydisperse samples have values between 0.1-0.4[21].

Method development and validation DLS

Reproducibility, accuracy, and precision

For the validation, 3 x >60 DLS measurements of the beads were made on two separate days. The nivolumab and pembrolizumab samples were measured in three runs of >60 measurements each on 7 separate days. The RSD of the measurements of the beads and mAbs was ≤5%, which is acceptable. Calibration of the instrument was performed using 60 and 200 nm beads.

Stability-indicating capability (Forced degradation study)

The forced degraded nivolumab and pembrolizumab samples were measured in threefold, and results were compared to a fresh reference sample measured in threefold. The concentrate vial was shaken for 24h as well as the diluted samples as an addition to this experiment.

Thermal denaturation (T_m) and thermal aggregation (T_{agg}) analysis

Method settings

The experiments were conducted with a Prometheus NT.48, with PR.TermControl software (Nanotemper, Munich, Germany). Temperature was increased by 1°C/min ranging from 25 to 90°C with UV detection at 330 and 350 nm combined. T_m and T_{agg} were calculated by the Prometheus software from the denaturation and scattering curves.

Method validation T_m/T_{agg}

Reproducibility, accuracy, and precision

The validation included BSA (reference), nivolumab, and pembrolizumab. BSA was used at 1 mg/ml in saline solution (0.9% NaCl). The test was carried out 48-fold for BSA on 3 different days and nivolumab and pembrolizumab was tested in 3-fold on 7 separate days to investigate whether the T_m (and T_{agg}) values were reproducible. accurate and precise.

Stability-indicating capability (Forced degradation study)

The forced degraded nivolumab and pembrolizumab samples were tested two-fold on two different days, and results were compared to a fresh reference sample.

ELISA – Stability-indicating capability (Forced degradation study)

The ELISA method was previously developed and validated[22]. The ELISA was developed for the quantification of nivolumab and pembrolizumab in PBSTF, and human plasma and cerebrospinal fluid samples. Saline was shown to give no interference. Here, we used this method to assess the stability of the biological activity of nivolumab and pembrolizumab in the formulated product before and after forced degradation.

pH measurements

A pH meter (Metrohm, Barendrecht, The Netherlands) was used to test the acidity or alkalinity of the solution. Before every pH run, the instrument was calibrated with Centripur® buffer solutions pH = 4.00 and pH = 7.00 (Merck, Darmstadt, Germany).

Results

CAC

Visual inspection showed that all untreated solutions remained clear without any change of colour or appearance. Visual inspection of the samples from the forced degradation study showed that nivolumab and pembrolizumab were not stable under heat, acidic and basic conditions, which resulted in the appearance of white precipitation most prominent in the 80°C heated samples, second most prominent in the acidic samples, and least prominent in the basic samples. The other samples did not show any visible changes.

SEC

To develop the SEC, different columns and eluent, flow, and column temperatures were tested. A BioSep SEC (300 x 78 mm, 5 µm particle size) from Phenomenex (Utrecht, The Netherlands) was initially tested with different concentrations of NaH_2PO_4, diluted in water, as eluent[2]. However, after a few initial runs and literature research[23,24] a new column; Yarra™ SEC-3000 (300 x 7.8 mm, 3 µm particle size) analytical column with SecurityGuard Guard Cartridge Kit and SecurityGuard Cartridges GFC 3000 (4 x 3.0mm ID, 10/Pk) were applied (Phenomenex, Utrecht, The Netherlands). The eluent was mixed to 50 mM KH_2PO_4/K_2HPO_4 buffer with 250 mM KCl in H_2O with a pH of 6.7. The method was then further examined with a reduction performed with DTT of the mAb to separate the heavy chain (HC) and the light

chain (LC) from each other, an aggregated sample (treated at 60° C for 2 h) was also included to make sure the SEC method could separate aggregates, intact mAb, HC and LC from each other in separated peaks. Fronting of the pembrolizumab peak in the SEC results was first suspected to appear from overloading the column. We tried to overcome this by using half the injection volume (5 µl), and with concentrations starting from 5 µg/ml[25]. However, the fronting of the peak remained. The peak shape was nevertheless considered acceptable combined with the purity data of the main peak. The flow was set to 1 ml/min, and the injection volume was set to 10 µl. Different concentrations and injection volumes were tested, and a sample concentration of 1 mg/ml and the injection volume of 10 µl was decided to be used to be able to see small degradation peaks and prevent dilution errors. The column oven was held at 25°C and the tray at 4°C, the run time was 15 min.

Method validation SEC

The calibration model was linear for the tested range of 500-1500 µg/ml with correlation coefficients (R^2) > 0.995 in all validation runs for nivolumab and R^2 > 0.997 for pembrolizumab. The purity of the mAb peak was over 99.5% in all samples; full mAb peak purity can be seen in Table 3. All calibration standards were within criteria established for accuracy and precision, respectively, ≤10% for QC-Low and ≤5% QC-Mid and QC-High. Accuracy and precision values are summarized in Table 4. Carry-over was tested and no peaks were observed in the first blank that was injected after the highest calibration standard (1500 µg/ml) in any of the runs for both mAbs. LOD was determined to be 5 µg/ml and LOQ to 17 µg/ml.

Stability-indicating capability

The forced degradation study revealed that nivolumab and pembrolizumab were not stable under most of the tested conditions. In the nivolumab and pembrolizumab samples, the chromatograms showed distorted peaks and an additional peak appearing under all conditions except for the shaken solutions. Most of the conditions show a clear alteration in the chromatograms, however, for the samples treated with UV at 344 nm, the chromatogram needs to be examined in a zoomed-in version to see any new arising peaks. In the heated nivolumab samples, only the excipient peaks remained untouched however in the heated pembrolizumab samples there were no peaks detected at all. Chromatograms can be seen in Figures 1 & 2. Since there were additional peaks with different elution times from the original mAbs, with concomitantly decreased mAb concentrations in most of the treated samples, the method was considered to be stability indicating.

DLS development and validation

Development and testing of the performance of the DLS included leftovers from several different mAbs diluted to 1 mg/ml (trastuzumab, rituximab, bevacizumab, cetuximab, ipilimumab, olaratumab, panitumumab, pembrolizumab, atezolizumab, tocilizumab, pertuzumab, infliximab). The method showed that dynamic diameter can be correctly measured for all named mAb, except for infliximab which detected bigger and several particles in almost all samples. The average hydrodynamic diameter for the mAbs (excluding infliximab) was 11.46 with an RSD of 2.99%.

The validation of the method included nivolumab, pembrolizumab and 10 ± 2 nm gold beads (hydrodynamic diameter 19 ± 2 nm). The results are presented as the average of the 3 mean values from each of more than 60 measurements. The hydrodynamic diameter of nivolumab and pembrolizumab were 9.94 nm and 9.98 nm with an average day precision of 3.41% and 2.74%, respectively as measured from runs performed on 6 different days. The average PDI for the measurements of nivolumab and pembrolizumab were both 0.11. The 10 nm (±2nm) beads, that had a nominal hydrodynamic diameter of 19, were shown to have a hydrodynamic diameter of 19.02 nm ± 1.03%, using our DLS method.

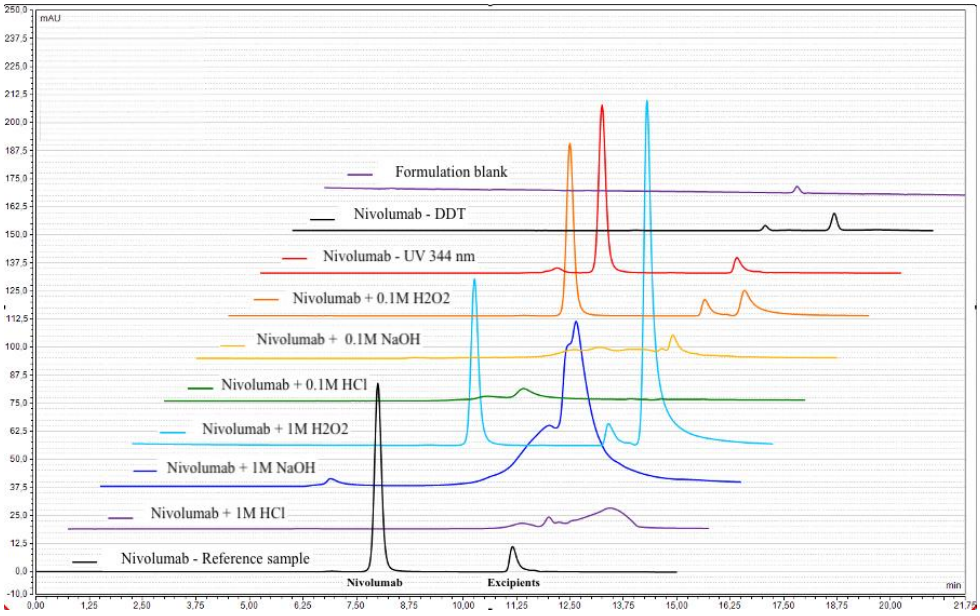

Figure 1 *HPLC-SEC-UV* chromatograms obtained at 280 nm of the nivolumab forced degradation samples of 1 mg/ml. From bottom up; Fresh/untreated nivolumab 1 mg/ml (black trace). Nivolumab elutes at 7.44 min. 6 h at room temperature in 1 M HCl (purple trace), 6 h at room temperature in 1 M NaOH (blue trace), 24 h at room temperature and protected from light in 1 M H_2O_2 (turquoise trace), 6 h at room temperature in 0.1 M HCl (green trace), 6 h at room temperature in 0.1 M NaOH (yellow trace), 24 h at room temperature and protected from light in 0.1 M H_2O_2 (orange trace), 24 h at room temperature under UV light of 344 (red trace), treated with DTT (black trace), formulation blank (purple trace). Heat sample not shown (only excipient peak detected).

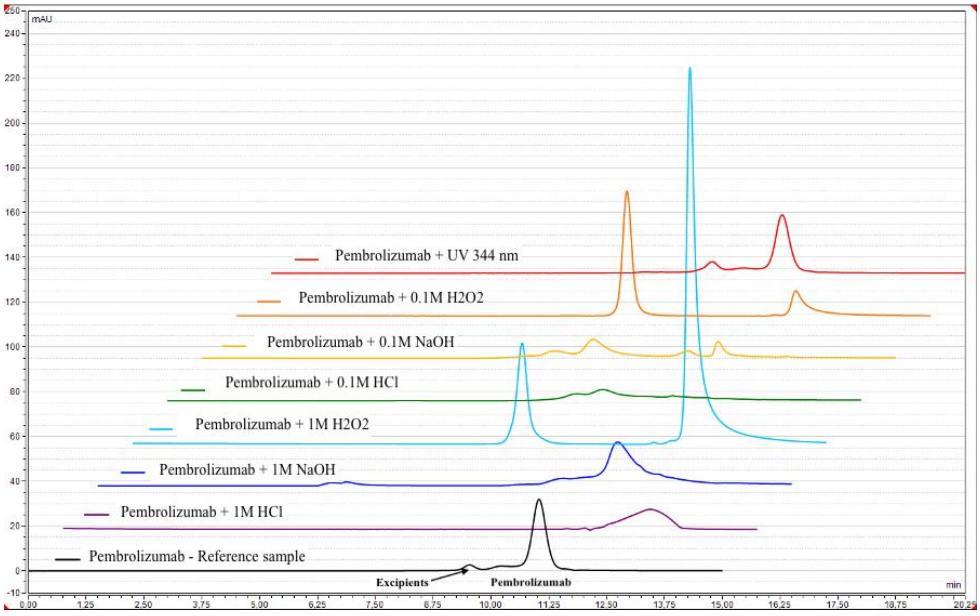

Figure 2 Chromatograms obtained at 280 nm of the pembrolizumab forced degradation samples of 1 mg/ml. From bottom up; Fresh/untreated pembrolizumab 1 mg/ml (black trace). Pembrolizumab elutes at 10.29 min. 6 h at room temperature in 1 M HCl (purple trace), 6 h at room temperature in 1 M NaOH (blue trace), 24 h at room temperature and protected from light in 1 M H_2O_2 (turquoise trace), 6 h at room temperature in 0.1 M HCl (green trace), 6 h at room temperature in 0.1 M NaOH (yellow trace), 24 h at room temperature and protected from light in 0.1 M H_2O_2 (orange trace), 24 h at room temperature under UV light of 344 (red trace). Heat sample not shown (only excipient peak detected).

Table 1 Composition and stability data retrieved from SmPC from EMA[1,2]. *When this study started the stability of Opdivo® was stated to be 24 h (incl. 8 h at 25°C)

mAb	Commercial name	Manufacturer	Form	Shelf life	Stability (Undiluted and diluted in saline)	Excipients
Nivolumab	Opdivo®	BMS	10mg/ml (4, 10 or 24 ml)	3 y	30 days (incl. 24h at 25°C)*	2.5mg/ml Na, Sodium citrate dihydrate, NaCl, Mannitol (E421), Pentetic acid, Polysorbate 80, Sodium hydroxide (for pH adjustment), Hydrochloric acid (for pH adjustment), Water for injections
Pembrolizumab	Keytruda®	MSD	25mg/ml (4ml)	3 y	96 h (incl. 6 h at 25°C)	L-histidine, L-histidine hydrochloride monohydrate Sucrose, Polysorbate 80, Water for injections

Bristol-Myers Squibb (BMS), Merck & Co (MSD)

Table 2 Overview of the methods described, developed, and validated.

Stability Category	**Method**	**Analytical procedure**	**Purpose**	**Ref**
Biological ***Immunoassay affinity***	**ELISA**	In indirect ELISA, the wells of the plate are coated with the target antigen, the examined mAb can bind to the target and an anti-Ig mAb conjugated to a detectable compound (e.g. HRP) can be used for the detection. Well-established and specific method.	Biological activity. The quantification of active mAb is then determined. Detects loss of affinity.	28–35
Physicochemical ***Size related, Visible particle detection***	**Visual inspection**	A shaken sample is visually analysed in front of a white background and compared to a reference to detect aggregation, crystallisation precipitation, and discolouring.	Easy to implement and severe physical stability issues can be detected.	10,30,36–38
Subvisible particles and aggregates	**DLS**	Samples are transferred to a cuvette and inserted in the instrument. The instrument uses fluctuating scattered light to measure the hydrodynamic diameter and PDI of the mAb sample. The results can be compared to a reference.	Sensitive and non-destructive method to detect physical instabilities. The quaternary structure is examined, and non-visible and soluble aggregates can be detected.	6–9,21,39,40
Aggregates, fragments, degradation products and concentration	**SEC**	Sample particles are separated according to hydrodynamic size and interaction with the column. High molecular weight particles normally elute first. Robust, precise, easy, well-established method.	A separative method, detection of both low and high molecular weight particles. Degradation products and concentration differences can be detected.	6–9,39,41–44
Unfolding	**Thermal denaturation with UV detection**	Samples are inserted in instrument and the temperature is increased while measuring the scattered light with UV.	T_m and T_{agg} curves can be determined. Difference in these values indicates instabilities due to changes in intermolecular interactions.	39,45–47
Chemical environment	**pH measurements**	Measures the potential difference between two electrodes and converts it to pH units. Simple to perform and non-destructive analysis.	mAbs are normally stable at pH 6.5-8.4. Outside this range, affinity is inhibited. pH also needs to be stable for administration.	8,36,48,49
Microbiological ***Risk of preparation contamination***	**Bioburden**	Broth simulation of the preparation method. Total viable count testing.	Gives a number of how many aerobic organisms, yeasts, and moulds are in a sample. Measures the contamination risk of the preparation continuously.	50–54
Risk of storage contamination	**Dye ingress method**	Punctured vials are inserted in a vacuum leak tester and integrity is examined.	Risk of contamination when punctured vial is stored outside of a sterile environment is examined.	54

Enzyme-Linked Immunosorbent Assay (ELISA), Horseradish peroxidase-labelled streptavidin (HRP), Dynamic Light Scattering (DLS), Polydispersity Index (PDI), Size Exclusion Chromatography (SEC), Ultra-Violet (UV), Thermal denaturation (T_m), Thermal Aggregation (T_{agg})

Table 3 Purity of mAb main peak for the HPLC-SEC-UV method.

Purity of main peak	Nivolumab %	RSD %	Pembrolizumab %	RSD %
Run 1	99.8	0.26	99.8	0.25
Run 2	99.9	0.09	99.6	0.56
Run 3	99.7	0.53	99.5	0.69

Table 4 Accuracy, and within and between run precision for the determination of nivolumab with HPLC-SEC-UV method.

QC sample	Run	Nominal concentration (µg/ml)	Accuracy (%) nivolumab	Bias (%) pembrolizumab	Precision (%) nivolumab	Precision (%) pembrolizumab	Number of replicates (nivolumab/ pembrolizumab)
QC low	1	600	0.91	0.02	0.90	0.70	5
	2	600	0.96	1.07	0.77	0.79	5
	3	600	0.73	1.30	1.39	4.00	5/4
	Between run	600	0.87	0.76	0.98	2.10	15/14
QC mid	1	1000	1.61	2.79	0.47	1.12	5
	2	1000	0.66	0.56	0.51	4.60	5
	3	1000	1.19	2.29	0.38	0.96	5
	Between run	1000	1.15	1.50	0.58	2.95	15
QC high	1	1500	1.44	-2.69	0.97	0.40	5/3
	2	1500	0.11	2.69	0.94	0.27	5
	3	1500	-0.27	1.22	1.71	0.61	5
	Between run	1500	0.42	0.41	1.39	0.84	15/12

Table 5 Hydrodynamic diameter (nm) and area% of each particle peak measured with DLS and the overall PDI of the 3 measurements per sample. *Only one measurement detected particle. **Only two measurements detected particles.

Nivolumab	Peak 1 (nm)	Peak 1 (%)	Peak 2 (nm)	Peak 2 (%)	Peak 3 (nm)	Peak 3 (%)	PDI
Reference	9.94	100	ND	ND	ND	ND	0.11
HCl (1 M)	*171.4	100	ND	ND	ND	ND	0.59
HCl (0.1 M)	15.53	99.6	*5074	0.40	ND	ND	0.14
NaOH (1 M)	7.16	58.33	657.27	28.03	19.90	13.63	0.30
NaOH (0.1 M)	9.68	86.43	311.96	10.63	*121.33	2.47	0.31
H_2O_2 (1 M)	9.66	93.60	479.43	6.40	ND	ND	0.23
H_2O_2 (0.1 M)	9.66	100	ND	ND	ND	ND	0.05
UV 344 nm	10.14	100	ND	ND	ND	ND	0.09
80°C**	304.15	43.55	0.16	29.55	5.13	18.15	0.77
Shake	9.42	100	ND	ND	ND	ND	0.10
Shake (concentrate)	9.68	100	ND	ND	ND	ND	0.06
Pembrolizumab	Peak 1 (nm)	Peak 1 (%)	Peak 2 (nm)	Peak 2 (%)	Peak 3 (nm)	Peak 3 (%)	PDI
Reference	9.98	100	ND	ND	ND	ND	0.11
HCl (1 M)	53.24	100	ND	ND	ND	ND	0.81
HCl (0.1 M)	17.29	100	ND	ND	ND	ND	0.16
NaOH (1 M)	603.53	25.7	3.97	40.03	0.33	34.3	0.67
NaOH (0.1 M)	7.02	85.87	667.90	8.87	*0.17	5.27	0.22
H_2O_2 (1 M)	6.49	100	ND	ND	ND	ND	0.16

H_2O_2 (0.1 M)	9.87	100	ND	ND	ND	ND	0.07
UV 344 nm	8.86	99.53	*5104	0.47	ND	ND	0.17
80°C	ND	ND	ND	ND	ND	ND	ND
Shake	9.49	100	ND	ND	ND	ND	0.12
Shake (concentrate)	9.44	100	ND	ND	ND	ND	0.15

Table 6 Within and between run precision for the determination of denaturation/melting (T_m) and aggregation (T_{agg}) temperatures for bovine serum albumin, nivolumab and pembrolizumab measured with the Prometheus NT.48.

Sample	**Run**	**T_m (°C)**	**RSD (±%)**	**T_{agg} (°C)**	**RSD (±%)**	**Number of replicates (T_m/T_{agg})**
BSA	1	66.3	0.26	82.9	0.92	48/48
	2	66.5	0.22	82.0	0.72	48/48
	3	66.7	0.37	N/A	N/A	48/0
	Between run	66.5	0.36	82.4	1.15	144/96
Nivolumab		67.3	0.10	66.1	0.11	21/21
Pembrolizumab		69.3	0.08	67.9	0.59	21/21

Aggregation temperature (T_{agg}) was only recorded in the first two runs for bovine serum albumin (BSA). T_m = melting temperature; RSD = relative standard deviation; N/A – not applicable.

Table 7 T_m and T_{agg} results from forced degraded samples measured with the Prometheus NT.48. The difference is the temperature shift from the reference sample. N/A – not applicable. ND – not detected. *Only detected in one of the two measurements.

Nivolumab	T_m (°C)	Difference T_m (°C)	T_{agg} (°C)	Difference T_{agg} (°C)
Reference	67.4	N/A	66.2	N/A
HCl (1 M)	ND	N/A	ND	N/A
HCl (0.1 M)	ND	ND	ND	N/A
NaOH (1 M)	51.9	- 15.5	21.2	45
NaOH (0.1 M)	51.4*	- 16	47.4*	- 18.8
H_2O_2 (1 M)	57.6	9.8	54.8	- 11.4
H_2O_2 (0.1 M)	60.7	- 6.7	79.1	12.9
UV 254 nm	66.8	- 0.5	65.3	- 0.8
UV 344 nm	67.3	0.1	66.1	0.1
80°C	ND	N/A	ND	ND
Shake	67.3	0	66.0	0.1
Pembrolizumab	T_m (°C)	Difference T_m (°C)	T_{agg} (°C)	Difference T_{agg} (°C)
Reference	69.3	N/A	67.9	N/A
HCl (1 M)	ND	N/A	86.8*	18.9
HCl (0.1 M)	79.0*	9.7	ND	N/A
NaOH (1 M)	ND	N/A	ND	N/A
NaOH (0.1 M)	45.9	-23.4	42.1*	-25.8
H_2O_2 (1 M)	67.7	-1.6	65.8*	-2.1
H_2O_2 (0.1 M)	69.5	0.2	68.0	0.1
UV 344 nm	64.4	-4.9	63.1	-4.8
80°C	ND	N/A	ND	N/A

Table 8 Fraction of nivolumab and pembrolizumab detected by ELISA compared to a fresh sample.

Sample	% detected Nivolumab	% detected Pembrolizumab
HCl (1 M) HCl (0.1 M)	0% 3.6%	0% 1.5%
NaOH (1 M) NaOH (0.1 M)	0% 0%	0% 0%
H_2O_2 (1 M) H_2O_2 (0.1 M)	96.9% 97.8%	96.1% 96.8%
UV 254 nm UV 344 nm	93.4% 95.6%	N/A 96.3%
80°C	0.0%	0%
Shake	96.5%	N/A

Table 9 Overview of the stability indicating capability of the different methods based on the forced degradation samples, x marks detected instability.

	Tested condition					
Method	Acidic	Basic	Oxidative	UV	Heat	Shake
CAC	x	x	-	-	x	-
SEC	x	x	x	x	x	-
T_m	x	x	x	x	x	-
T_{agg}	x	x	-	-	x	-
DLS	x	x	x	-	x	-
ELISA	x	x	-	-	x	-

Stability-indicating capability

DLS measurements showed that there was aggregation in most samples. The oxidation samples and both concentrates and diluted shaken samples did not show any aggregation. All measurements from the heat-treated samples showed no particles except for one measurement that showed aggregation. In the other treated samples, the size of the particle in the main peak was sometimes changed and, in several samples, both aggregation and degradation could be detected in form of more peaks with smaller or bigger particle size. Full results can be seen in Table 5.

T_m and T_{agg}

The T_m and T_{agg} data for BSA, nivolumab and pembrolizumab are presented in Table 6. The standard deviation in temperature for the instrument is 0.24°C for T_m and 0.95° for T_{agg}.

Stability-indicating capability

The forced degradation samples showed signs of stability-indicating capability when looking at T_m and T_{agg}. Several samples showed that both T_m and T_{agg} had shifted significantly relative to baseline results (reference), see Table 7.

ELISA

A fresh vial of formulated nivolumab and pembrolizumab pharmaceutical products was used for calibration and QC samples. The concentration of both mAbs in the shaken, UV, and oxidated samples was decreased. However, we could not conclude that there was a significant loss of bioactivity as the decrease did not exceed the 15% method precision. On the other hand, the acidic, basic, and heat-treated samples showed a significant decrease in bioactivity.

Stability-indicating capability

In conclusion, the combination of the methods shows that we can detect physiochemical and biological instabilities of mAbs with the complementing methods, (see Table 9).

Discussion

In this study, we investigated which methods are most suitable to study stability of monoclonal antibodies in pharmaceutical products in daily pharmacy practice environment. The methods were chosen to cover major aspects of protein stability and based on extensive literature research (see Table 2). The methods were then developed and tested with forced degradation samples and the stability-indicating capability was established for each method.

For the SEC method, the LOD and LOQ showed that the sensitivity is sufficient for determining nivolumab and pembrolizumab fragmentation and aggregation stability. Strong cation exchange (SCX) is sometimes used to complement the results from SEC. In this study, we complemented the SEC results with a panel of other methods to do a more extensive evaluation of the stability. Due to the similar structure of nivolumab and pembrolizumab, the retention time was expected to be almost the same. However, it seems that besides size, other interactions may also play a role in the chromatography of these compounds, given them different retention times. The added excipients are likely the cause of these secondary interactions or change the hydrodynamic volume and are causing interference in the elution of the mAbs. As seen in Table 1, nivolumab and pembrolizumab have different excipients in their formulations. The pH of the nivolumab concentrate solution is around 6 and for pembrolizumab 5.2-5.8. When pembrolizumab is slightly oxidized, it does not seem to degrade (at least not initially) or gives rise to more peaks in the SEC, but instead, the hydrodynamic diameter changes causing the main peak to elute earlier. This may confirm the impact of the excipient. By running the method with a formulation blank, we can see that the excipient compounds are detected with the method, therefore the purity of the peak is confirming full separation. Purity is defined as the similarity of the spectrum in all wavelengths in the PDA spectra (190 nm to 800 nm) in the leading edges and the maximum of the peak. The purity is presented as a percentage provided by the algorithm in the acquisition software. The purity data of the mAbs peaks was over 99% in all validation runs with a fresh sample so this will be the future limit for stability studies. We could conclude that the method was stability indicating looking at the purity and the forced degradation data. Further analysis of these samples with SEC, reveals instability issues in the oxidation sample of nivolumab with the formation of a new peak that could be a light chain breaking off the main Ab. For pembrolizumab, it seems that the mAb remains intact but that the hydrodynamic diameter or secondary interactions with the excipients have changes as mentioned above. The UV (254 nm) treated sample showed several new peaks in SEC. The UV (344 nm) samples also showed several small additional peaks. Therefore, we believe the SEC method is very sensitive as it gives a lot of information about the degradation in the form of new peaks arising. The heat-treated sample indicated that the excipients remain intact and are still present in the nivolumab samples, while the mAb has completely aggregated as its peak was completely gone after centrifugation. Complete mAb aggregation also occurred in the pembrolizumab heat treated samples, but in addition also all excipients in these samples were affected and undetectable by SEC-UV. Both the acidic and the basic conditions resulted in precipitation of mAbs as well. However, visual inspection revealed that these precipitations were much less than for the heat-treated samples.

The DLS method showed that it was possible to measure a broad spectrum of tested mAbs, with the exception of infliximab. Infliximab showed soluble particles in all the tests. This could be an

aggregation or part of the initial conformation in its pharmaceutical formulation. Therefore, the method was not reliable enough to show if there were any aggregates formed in a prolonged clinical setting for infliximab. Other investigators have drawn the same conclusion in previous studies[9]. The hydrodynamic diameter of a wide range of mAbs measured was in correspondence with a value of 10.58 nm for IgG reported in literature[26]. Due to slight size differences, secondary interactions with the excipients and the non-globular shape of the mAb, the hydrodynamic diameter can differ slightly between the different mAbs, as seen in our results where pembrolizumab and nivolumab have a smaller hydrodynamic diameter than the overall average of the other mAbs. The DLS method confirmed instabilities in all conditions except the UV samples. The method was also considered stability-indicating since soluble aggregates could be detected in several samples after forced degradation and centrifugation as seen in Table 9. In the SmPC for both mAbs, it is mentioned that the vials and the infusion solutions should not be shaken. However, the shaken samples showed no significant changes in any of the measured parameters, indicating that shorter shaking impact (≤24h, 450 rpm) at room temperature on the mAb may not inflect any stability issues. Nevertheless, we cannot exclude that some soluble aggregates could still be formed while shaking, but that the DLS measurements were not sensitive enough to detect these. However, when the concentrate stands on the shake table for 24 h for DLS analysis, this not resulted in any aggregation formation. The statement in the SmPC might be to avoid foaming from the Polysorbate 80 in the solution since this was noticed to happen when shaking infusion solution samples by hand.

Thermal denaturation gives information about the conformational integrity of the mAbs. If the T_m and/or T_{agg} had changed, there have been alterations in the intramolecular structure and intermolecular interactions. If the interactions within and between the mAb molecules change, the thermal energy that is needed to denature the mAb or aggregate them also changes. Other studies have shown a T_m for BSA to be 66.7°C[14], suggesting that the accuracy of our instrument is high, as our method measured 66.5°C. Since the BSA was tested by diluting it from its pure form without any excipients, a theoretical comparison can be made. However, the nivolumab and pembrolizumab samples were composed of both mAb and excipients in a saline dilution, therefore it would not be correct to compare the T_m is to the theoretical values, instead we used pure mAb references standards solubilized in the same excipient solution as used for the clinical vials. The forced degradation studies showed that both T_m and the T_{agg} shifts significantly for most conditions (heat, acidic, basic, oxidative, and UV for pembrolizumab), as seen in Table 7, and is therefore stability indicating.

The ELISA method is discussed in previous research[22]. In addition, the method showed that the forced degradation study significantly affected the biological activity of nivolumab and pembrolizumab in the acidic, and basic exceeding the 15% variation limit from the nominal concentration that was set in the validation of the method[22]. No readable results were obtained with ELISA after heating at 80°C as the mAb was completely aggregated and/or degraded. This indicates that the method can be used to indicate the stability of stored mAbs.

In our consideration, the pH method did not need to be assessed nor validated, the instrument can instead be calibrated with known calibration standards before every run.

The concentration of 1 mg/ml was chosen since it falls in the clinical administration range of both mAbs (1-10 mg/ml[1,2]) and this concentration was also suitable for all the experiments except ELISA, where smaller concentrations were required, to obtain readable results.

Our mAb analysis with different assays and using forced degraded samples, it is evident that not one or our selected assays is fully conclusive for determining the overall physiochemical and

biological stability as seen in Table 9. It may seem that SEC gives the most information, however, stability issues that affect the biological activity and subtle protein changes cannot be seen in this method if the mAb is still largely intact. Since the goal is to use these experiments to examine the use of mAbs beyond the timeframe that is indicated by the manufacturer in our hospital pharmacy, we cannot assume stability only by looking at one or a few methods but have to be certain that we can see all possible stability issues. These methods we have evaluated here seems to be stability-indicating and could be used for examining concentration, purity, and degradation in time. For more in-depth analysis regarding glycosylation etc. other more sophisticated techniques such as mass spectrometry might be needed, however, is not needed in the stability study of pharmaceutical products.

Regarding microbiological safety, this has previously been developed and described[19]. In this previous study, 631 vials from 18 different mAb products were tested. The integrity of the already opened vials was tested using the dye ingress method with a VDT/S Vacuum Leak Tester with a 300-mm diameter container (Erweka GmbH, Heusenstamm, Germany). It was found that if a needle (BD Microlance™ 3 18 G 1½00 1.2" 40 mm, Beckton, Dickinson, and Company) was used for the extraction of the content in the vial, the integrity of the vial remained. The aseptic handling of the preparation method was also monitored. The risks of contamination were within the accepted limits; >1% with 95% confidence interval according to The Dutch Hospital Pharma guideline: GMP-z (ziekenhuisfarmacie), Annex Z3 concerning process validation of aseptic compounding[27].

In conclusion, the combination of the methods shows that we can detect physiochemical and biological instabilities of mAbs with the complementing methods that are described in this research paper (see Table 9). The previous microbiological contamination research explains how the vials should be handled to maintain the microbiological stability[19].

Conclusion

The methods CAC, SEC, DLS, T_m and T_{agg} curves, pH measurements, vacuum leak test, and aseptic processing validation make up the panel of methods that we used in our hospital pharmacy to examine the prolonged stability of the mAbs nivolumab and pembrolizumab. These methods all have stability indicating capability and compliments each other to get a full evaluation of the stability within the mAb solution. All the methods can be generically applied for other mAb, with the exception that the ELISA needs to be revalidated when a different antigen is needed. These methods are now applied to investigate the use of nivolumab and/or pembrolizumab concentrate and infusion solution beyond the timeframe as indicated by the manufacturer and under relevant clinical conditions from a hospital pharmacy perspective.

Acknowledgements

The authors would like to thank the people at Phenomenex (Utrecht, The Netherlands) for their expertise in the development of the SEC. We would also like to thank Alexander Fish for his help with the thermal denaturation and helpful input.

The authors declare no conflict of interest related to this study. This research did not receive any specific grant or financial support from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. European Medicines Agency (EMA): Opdivo® - Summary of Product Characteristics (SmPC). Published 2015; last updated 2020. Available at http://ema.europa.eu. Accessed March 2021.
2. European Medicines Agency (EMA): Keytruda® - Summary of Product Characteristics (SmPC). Published 2015; last updated 2020. Available at http://ema.europa.eu. Accessed March 2021.
3. Glover, Z. W. *et al.* Compatibility and stability of pertuzumab and trastuzumab admixtures in i.v. infusion bags for coadministration. *J Pharm Sci* 102, 794–812 (2013).

4. Navas, N. *et al.* Quantification of an intact monoclonal antibody, rituximab, by (RP)HPLC/DAD in compliance with ICH guidelines. *Anal. Bioanal. Chem.* 405, 9351–9363 (2013).
5. Qi, P. *et al.* Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form. *J. Pharm. Sci.* 98, 3117–3130 (2009).
6. Paul, M., Vieillard, V., Jaccoulet, E. & Astier, A. Long-term stability of diluted solutions of the monoclonal antibody rituximab. *International Journal of Pharmaceutics* 436, 282–290 (2012).
7. Young, B. L. *et al.* Evaluation of the physicochemical and functional stability of diluted REMSIMA(R) upon extended storage--A study compliant with NHS (UK) guidance. *Int J Pharm* 496, 421–431 (2015).
8. Tokhadze, N., Chennell, P., Le Basle, Y. & Sautou, V. Stability of infliximab solutions in different temperature and dilution conditions. *J Pharm Biomed Anal* 150, 386–395 (2018).
9. Hernández-Jiménez, J. *et al.* Study of aggregation in therapeutic monoclonal antibodies subjected to stress and long-term stability tests by analyzing size exclusion liquid chromatographic profiles. *Int. J. Biol. Macromol.* 118, 511–524 (2018).
10. Kaiser, J. & Kramer, I. Physicochemical stability of diluted trastuzumab infusion solutions in polypropylene infusion bags. *Int J Pharm Compd* 15, 515–520 (2011).
11. Cordoba, A. J., Shyong, B. J., Breen, D. & Harris, R. J. Non-enzymatic hinge region fragmentation of antibodies in solution. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 818, 115–121 (2005).
12. AZO Nano. Dynamic Light Scattering (DLS) - Understanding the Basics. (2013). Available at: https://www.azonano.com/article.aspx?ArticleI D=3662.
13. Hernandez-Jimenez, J. *et al.* Study of aggregation in therapeutic monoclonal antibodies subjected to stress and long-term stability tests by analyzing size exclusion liquid chromatographic profiles. *Int J Biol Macromol* 118, 511–524 (2018).
14. Hsieh, S. R. *et al.* Exploring the behavior of bovine serum albumin in response to changes in the chemical composition of responsive polymers: Experimental and simulation studies. *Polymers (Basel).* 8, (2016).
15. Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* 185, 129–188 (1999).
16. Zhang, S., Garcia-D'Angeli, A., Brennan, J. P. & Huo, Q. Predicting detection limits of enzyme-linked immunosorbent assay (ELISA) and bioanalytical techniques in general. *Analyst* 139, 439–445 (2013).
17. Konstantinou, G. N. Enzyme-linked Immunosorbent Assay (ELISA). in *Methods in Molecular Biology* 1592, 79–94 (2017).
18. Schneider, Z. Importance of isoelectric point (pI) of antibodies. *Antibody Society* (2017). Available at: https://www.antibodysociety.org/new-articles/importance-isoelectric-point-pi-antibodies/.
19. Arnamo, A. H., Huitema, A. D. R., Beijnen, J. H. & Nuijen, B. Use of leftovers of monoclonal antibody products after partial extraction – A microbiological safety study. *J. Oncol. Pharm. Pract.* (2019). doi:10.1177/1078155219850297
20. ICH Expert Working Group. ICH Topic Q2(R1) Validation of Analytical Procedures : Text and Methodology. *International Conference on Harmonization* 1994, 17 (2005).
21. Stetefeld, J., McKenna, S. A. & Patel, T. R. Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys. Rev.* 8, 409–427 (2016).
22. Pluim, D. *et al.* Enzyme linked immunosorbent assay for the quantification of nivolumab and pembrolizumab in human serum and cerebrospinal fluid. *J. Pharm. Biomed. Anal.* 164, 128–134 (2019).
23. McGinley, M., Wang, Y. & Rustamov, I. Relevant Parameters in Developing Protein Aggregation Methods Using Yarra ™ GFC columns. *Phenomenex Appl.* 1–4 (2012).
24. Rivera, B. Transform Your Mindset for HPLC and UHPLC Method Development for Analyzing Proteins— Including Monoclonal Antibodies! @ Phenomenex.
25. Yu, C. M., Mun, S. & Wang, N. H. L. Phenomena of insulin peak fronting in size exclusion chromatography and strategies to reduce fronting. *J. Chromatogr. A* 1192, 121–129 (2008).
26. Armstrong, J. K., Wenby, R. B., Meiselman, H. J. & Fisher, T. C. The Hydrodynamic Radii of Macromolecules and Their Effect on Red Blood Cell Aggregation. *Biophys. J.* 87, 4259–4270 (2004).

27. Dutch Hospital Pharmacist guideline. GMP-Z Z3: Aseptisch Handelen. 1–16 (2013). Available at: http://nvza.nl/wp-content/uploads/2016/04/Z3-GMPZ_Herziening-2013-Z3-Aseptische-handelingen-def-IGZ.pdf.
28. Alvarez Martin, T. *et al.* Study of biological activity of infliximab. *Eur. J. Clin. Pharm.* 19, 374–378 (2017).
29. Ikeda, R. *et al.* Stability of infliximab in polyvinyl chloride bags. *Am J Heal. Syst Pharm* 69, 1509–1512 (2012).
30. Zhang, Y., Vermeulen, L. C. & Kolesar, J. M. Stability of stock and diluted rituximab. *Am J Heal. Syst Pharm* 70, 436–438 (2013).
31. Kim, S. J. *et al.* In-Use Stability of the Rituximab Biosimilar CT-P10 (Truxima ®) Following Preparation for Intravenous Infusion and Storage. *BioDrugs* 33, 221–228 (2019).
32. Roos, A. & Wieslander, J. *Evaluation of Complement Function by ELISA*. (2013).
33. Chen, Y. H. *et al.* Evaluation of the sterility, stability, and efficacy of bevacizumab stored in multiple-dose vials for 6 months. *J Ocul Pharmacol Ther* 25, 65–69 (2009).
34. Ikesue, H., Vermeulen, L. & Kolesar, J. Stability of cetuximab and panitumumab in glass vials, plastic syringes, and polyvinyl chloride bags. *J. Oncol. Pharm. Pract.* 1), 22 (2010).
35. Dasso, J., Lee, J., Bach, H. & Mage, R. G. A comparison of ELISA and flow microsphere-based assays for quantification of immunoglobulins. *J. Immunol. Methods* 263, 23–33 (2002).
36. Goldspiel, J. T., Goldspiel, B. R., Grimes, G. J., Yuan, P. & Potti, G. Stability of alemtuzumab solutions at room temperature. *Am J Heal. Syst Pharm* 70, 439–442 (2013).
37. Pabari, R. M., Ryan, B., Ahmad, W. & Ramtoola, Z. Physical and structural stability of the monoclonal antibody, trastuzumab (Herceptin(R)), intravenous solutions. *Curr Pharm Biotechnol* 14, 220–225 (2013).
38. Particulate Contamination: Visible Particles. European Directorate for the Quality of Medicines and Healthcare Council of Europe. *European Pharmacopeia* 10.1, 2.9.20 (2020).
39. Mahler, H. C., Friess, W., Grauschopf, U. & Kiese, S. Protein aggregation: Pathways, induction factors and analysis. *J. Pharm. Sci.* 98, 2909–2934 (2009).
40. Panchal, J., Kotarek, J., Marszal, E. & Topp, E. M. Analyzing subvisible particles in protein drug products: A comparison of Dynamic Light Scattering (DLS) and Resonant Mass Measurement (RMM). *AAPS J.* 16, 440–451 (2014).
41. Wang, W. Protein aggregation and its inhibition in biopharmaceutics. *Int. J. Pharm.* 289, 1–30 (2005).
42. Fekete, S., Beck, A., Veuthey, J. L. & Guillarme, D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *J. Pharm. Biomed. Anal.* 101, 161–173 (2014).
43. Berkowitz, S. A. Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. *AAPS J.* 8, 590–605 (2006).
44. Size-Exclusion Chromatography. European Directorate for the Quality of Medicines and Healthcare Council of Europe. *European Pharmacopeia* 10.1, 2.2.30 (2020).
45. Lahlou, A., Blanchet, B., Carvalho, M., Paul, M. & Astier, A. Mechanically-induced aggregation of the monoclonal antibody cetuximab. *Ann. Pharm. Fr.* 67, 340–352 (2009).
46. Hawe, A., Kasper, J. C., Friess, W. & Jiskoot, W. Structural properties of monoclonal antibody aggregates induced by freeze-thawing and thermal stress. *Eur. J. Pharm. Sci.* 38, 79–87 (2009).
47. Katayama, D. S. *et al.* Solution behavior of a novel type 1 interferon, interferon-τ. *J. Pharm. Sci.* 94, 2703–2715 (2005).
48. An Introduction to Antibodies: Antibody-Antigen Interaction @ www.sigmaaldrich.com. Available at: https://www.sigmaaldrich.com/technical-documents/articles/biology/antibody-antigen-interaction.html.
49. Potentiometric Determination of pH. European Directorate for the Quality of Medicines and Healthcare, Council of Europe. *European Pharmacopeia* 10.1, 2.2.3 (2020).
50. Shintani, H. Rapid Assay of Bioburden, Endotoxin and Other Contaminnation. *J. Chromatogr. Sep. Tech.* 04, (2013).
51. Kim, S. J. *et al.* In-Use Physicochemical and Biological Stability of the Trastuzumab Biosimilar CT-P6 Upon Preparation for Intravenous Infusion. *BioDrugs* (2018). doi:10.1007/s40259-018-0314-1
52. Sandle, T. Bioburden determination. in *Pharmaceutical Microbiology* 81–91 (Elsevier, 2016). doi:10.1016/b978-0-08-100022-9.00007-4

53. Bioburden Testing | Laboratory Testing Services | STERIS AST. Available at: https://www.steris-ast.com/services/bioburden-testing/. (Accessed: 26th February 2021)

54. Arnamo, A. H., Huitema, A. D., Beijnen, J. H. & Nuijen, B. Use of leftovers of monoclonal antibody products after partial extraction – A microbiological safety study. *J. Oncol. Pharm. Pract.* 1078155219850297 (2019). doi:10.1177/1078155219850297