How stable are new biologics?

Insights into the stress profiles of an antibody-based biopharmaceutical drawing on the example of Adalimumab

Bettina Katterle

Eurofins BioPharma Product Testing Munich GmbH, Munich

Correspondence: Dr. Bettina Katterle, Eurofins BioPharma Product Testing Munich GmbH, Behringstr. 6/8, 82152 Planegg/Munich, Germany

ABSTRACT

The interest in biopharmaceuticals, mostly recombinant proteins and/or peptides, is increasing worldwide in healthcare [1]. To ensure long-term stability and safety of proteins in the drug life-time from release through to product shelf life and finally patient use, the drug formulations were developed by biopharmaceutical companies to keep drug properties stable even after unexpected periods of storage temperature variations. In comparison to most chemically synthesised products, biopharmaceuticals are particularly sensitive to changes in temperature and humidity, light and oxygen exposure, and shear forces. If a therapeutic protein cannot be stabilized adequately, it will partially or totally lose its therapeutic properties or even cause immunogenic reactions thus potentially further endangering patients' health. In order to understand and monitor changes in the complex structure of biopharmaceuticals during their production and lifetime, various validated physicochemical, biochemical and immunochemical analytical test methods should be employed.

ZUSAMMENFASSUNG

Wie stabil sind biologische Wirkstoffe? Stressprofile von Antikörperwirkstoffen am Beispiel Adalimumab

Biopharmazeutika, meist rekombinante Proteine und/oder Peptide, sind weltweit von steigendem Interesse für das Gesundheitswesen [1]. Um die Langzeitstabilität und Sicherheit der biopharmazeutischen Arzneimittel von der Freigabe über die Produktlagerung bis hin zum Patientengebrauch zu gewährleisten, werden Arzneimittelformulierungen von biopharmazeutischen Unternehmen speziell entwickelt, um die Eigenschaften auch nach unerwarteten Lagertemperaturschwankungen stabil zu halten.

Im Vergleich zu den meisten chemisch synthetisierten Produkten sind Biopharmazeutika besonders empfindlich gegenüber Temperatur- und Feuchtigkeitsschwankungen, Licht- und Sauerstoffbelastung sowie Scherkräften. Wenn ein therapeutisches Protein nicht ausreichend stabilisiert werden kann, verliert es seine therapeutischen Eigenschaften ganz oder teilweise oder verursacht sogar immunogene Reaktionen, die die Gesundheit der Patienten weiter gefährden können. Um Veränderungen in der komplexen Struktur von Biopharmazeutika während ihrer Herstellung und Lebensdauer zu verstehen und zu überwachen, sollten verschiedene validierte physikalisch-chemische, biochemische und immunochemische analytische Testmethoden eingesetzt werden.

KEY WORDS

- Stability of Biopharmaceuticals
- Stress Conditions
- Analytical Testing
- Chromatographic and Electrophoretic Methods

Pharm. Ind. 80, Nr. 11, 1557–1563 (2018)

1. Introduction

This article describes the exposure of the commercially available therapeutic antibody Humira® (Adalimumab) to the various stress conditions in order to detect any possible change of the monoclonal antibody sequence and structure (monoclonal antibody – mAB).

Quality control (QC) testing of protein biologics is one of the critical parts of biopharmaceutical manufacturing. The analytical results of various QC testing methods help to define and monitor the batch to batch variations [2]. Any profound structural changes in the protein will affect activity and safety. In this case, actions have to be taken. Formulated biopharmaceuticals, the final drug product, are developed to preserve the drug substance stability and the formulation has become a key aspect of the pharmaceutical development.

In general, stability studies must be performed on both the drug substance and the final drug product. The goal of a stability study is to ensure that the product quality remains the same over a certain time period. This is used, among other things, to establish recommended storage conditions and shelf-lives. Correspondent International Conference on Harmonisation (ICH) guidelines define the types of studies, analytical testings and their specifications [2–5].

The ICH guideline Q1A(R2), chapter 2.1.2, emphasizes that the stress testing on the drug substance should be employed for the assessment of parent drug stability, the inherent stability characteristic and the setting of the drug shelf life. This includes the development of test studies to ascertain the stability of the drug substance with the suitable stability-indicating analytical procedures.

Table 1

Assessment of the purity / impurity profile of a monoclonal antibody by a combination of orthogonal methods.

Attributes	Aim of the test	Analytical technique
Structure/post-translational modifications	Deamidation/oxidation N/C-terminal modifications	Peptide mapping high-performance liquid chromatography (HPLC) / ultra high performance liquid chromatography (UPLC) (UV, MS)
	Assessment of disulfide bridges	
	Intact LC/HC, HMW/LMW	CE-SDS/HPLC (UPLC) (UV; MS)
Product related substances and impurities	Charge heterogeneity/Isoforms	iCIEF (UV)
	Aggregates	SE-HPLC/UPLC (UV)
	Carbohydrate profiling	HPLC/UPLC (FL, MS)

Therefore, during the development of the analytical procedures as well as their validation, it is critical to show that the methods are capable of detecting modified protein forms or degradation products of drug product samples resulting from product instability. In case that degradation standards (impurity standards) are not available, chapter 1.2.2 of the ICH guideline Q2 (R1) recommends including samples in the validation program that are stored under relevant stress conditions such as light, heat, humidity, acid/base hydrolysis and oxidation. Therefore, controlled force-degraded experiments under various conditions have to be set up in order to show if the analytical independent, orthogonal methods are capable of detecting those formed impurities.

Commonly, even an unstressed recombinant (monoclonal) antibody displays already a complex heterogeneous profile caused by its sequence and structure and the possibility of post translational modifications, for instance C-terminal lysine processing, aggregates and N-linked glycosylation deamidation and oxidation sites (fig. 1). The sites of possible post translational modifications are known for well characterized antibodies. Here, the characterization work of the different modifications is predominately performed by using highly advanced mass spectrometry methods. The different modifications are regarded as product-related substances or impurities (terminology depends on their effects on activity and safety). Table 1 shows the selected physicochemical analytical techniques used for assessing the product–related substances or impurities in the quality control process.

Various and independent (orthogonal) analytical methods as listed in Table 1 are typically used for release and stability testing in order to determine identity and impurity. They are mainly chromatographic and electrophoretic separation techniques. Activity testing using cell-based bioassay and/or binding assays completes the release and stability testing panel.

In short-term accelerated studies, typically a protein sample is exposed to elevated temperatures to accelerate the degradation (truncation) and/or aggregation. Acidic or basic pH can promote aggregation, hydrolytic degradation or deamidation. Peroxide or other oxidizing agents might be used to induce oxidation of methionine [8]. This "worst case" approach reflects sample alterations that might be detected in the drug substance and drug product samples during long-term and short-term storage.

The ICH guideline Q2 (R1) describes the validation of analytical procedures. The validation of product-related substances or impurities aims to validate either quantification or % limit [3 and table therein]. If the % limit is evaluated, parameters such as specificity precision (intermediate precision, repeatability) and limit of detection should be included in the validation. The collected validated data ensure ruggedness or robustness and reliability of the state-of-the-art analytical methods.

A stress study of Adalimumab, a commercially available formulated monoclonal antibody, has been designed to demonstrate that the independent, state-of-the-art sensitive testing methods are able to detect the various modifications to the structure of the protein. Stress testing is desirable as "worst case scenario" because degradation pathways and reaction kinetics will be able to be identified or at least degradation products will be able to be characterized. Different stress conditions were chosen to induce degradation and/or aggre-

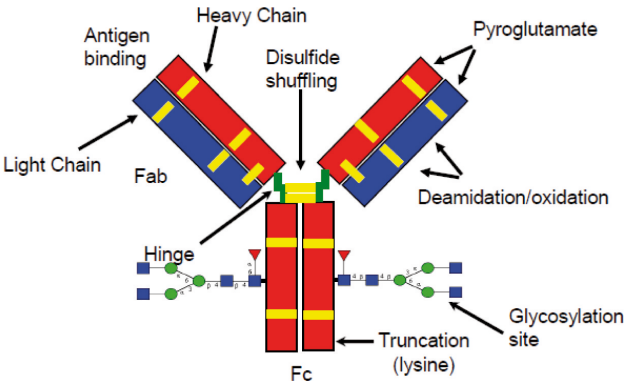

Figure 1: General structure of an antibody with 450 to 550 amino acids. The heavy chain (HC), the light chain (LC) as well as the common sites prone to posttranslational modifications are depicted (Source: Eurofins BioPharma Product Testing Munich GmbH [10]).

Pharm. Ind. 80, Nr. 11, 1557–1563 (2018)

gation as well as post translational modifications, e.g., oxidation of methionines or deamidation.

2. Materials and Methods

2.1 Stability and degradation at accelerated conditions

The commercially available recombinant monoclonal antibody was subjected to various stress conditions. For this series of stress experiments for this publication it was decided to perform only one experimental set up per condition. The reason is that robustness validation is generally performed with a similar set-up. The aim was to monitor new or disappearing peaks in order to understand which kind of effects can be expected if the protein had been stressed. Hence, evaluation of precision (repeatability and intermediate precision) was not in the scope of the experiments presented here. The chromatographic and electrophoretic runs are only evaluated semi-quantitatively (e.g., in (limit) %) for decreasing or new peak area(s). The resulting qualitative stress profiles are regularly observed in client-specific validations of monoclonal antibodies.

Here, the impact of thermal stress, 55 °C for a time period of up to 72 h, acidic (0,1 N HCl), basic (0,1 N NaOH) and oxidative stress (3 % H_2O_2) was investigated. The acidic and basic stress conditions could not be measured since the protein was denatured and could not be subjected to the different analytical methods. The remaining samples were analyzed by two different capillary electrophoresis methods (CE – SDS), imaged capillary isoelectric focusing (iCIEF), and by size exclusion chromatography (SEC). Aggregation amount, charge variants, level of high-molecular-weigth (HMW) and low-molecular-weight (LMW) variants were evaluated. The obtained data were compared to the data of the unstressed antibody.

Adalimumab drug product samples (50 mg/mL) were subjected to thermal stress by incubating at 55 °C for a time period of 24 h, 48 h and 72 h. Prior to analysis, samples were diluted with water to 10 mg/mL.

In order to subject Adalimumab to oxidative stress, H_2O_2 in a final concentration of 3 % (v/v) was added to 50 mg/mL drug product samples. The samples were then diluted to a concentration of 40 mg/mL by adding purified water and incubated for 24 h at room temperature. Finally, samples were diluted to 10 mg/mL with purified water before analysis. The unstressed samples were processed in the same way, but the stress agent was excluded. They were analyzed in parallel. The stressed samples were aliquoted and analyzed by the techniques described below. Only one chromatographic and electrophoretic run per stress condition has been evaluated.

2.2 System suitability of the chromatographic and capillary electrophoretic methods

Internally validated methods were employed to detect degradation profiles of Adalimumab drug product samples. Defined system suitability criteria were applied. For the electrophoretic and chromatographic methods described below, the relative standard deviation (RSD) should be less than 1 % for retention/migration time and for peak area evaluated for controls/marker proteins.

2.3 Reduced and non-reduced Capillary Electrophoresis – Sodium Dodecyl Sulfate

All experiments were performed on the PA 800 *Plus* Pharmaceutical Analysis System (SCIEX) controlled by Empower3 Chromatography Data Software (Waters). A bare fused-silica capillary of 50 µm i.d. x 360 µm o.d. x 30.2 cm total length were used for the separation.

Capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) was used for separation of denatured protein size variants under non-reduced or reduced conditions. For non-reduced CE-SDS (nrCE-SDS), samples were denatured with sodium dodecyl sulfate at 70 °C for 5 min in the presence of iodoacetamide (Sigma). For reduced CE-SDS (rCE-SDS), 2-mercaptoethanol (Bio-Rad) was added to the protein denaturation step to reduce the disulfide bonds. After denaturation, both non-reduced and reduced samples were injected into a bare fused-silica capillary (Beckman Coulter, Brea, CA, USA) and separated based on hydrodynamic size resulting from an applied electric field in which migration of smaller sized proteins is inversely related to overall size. Analytes were monitored by UV absorbance at 220 nm. Purity was evaluated by determining the corrected peak area (CPA) of each species as a percentage of the total peak area. A 10 kDa marker protein (Beckman Coulter, Brea, CA, USA) was used.

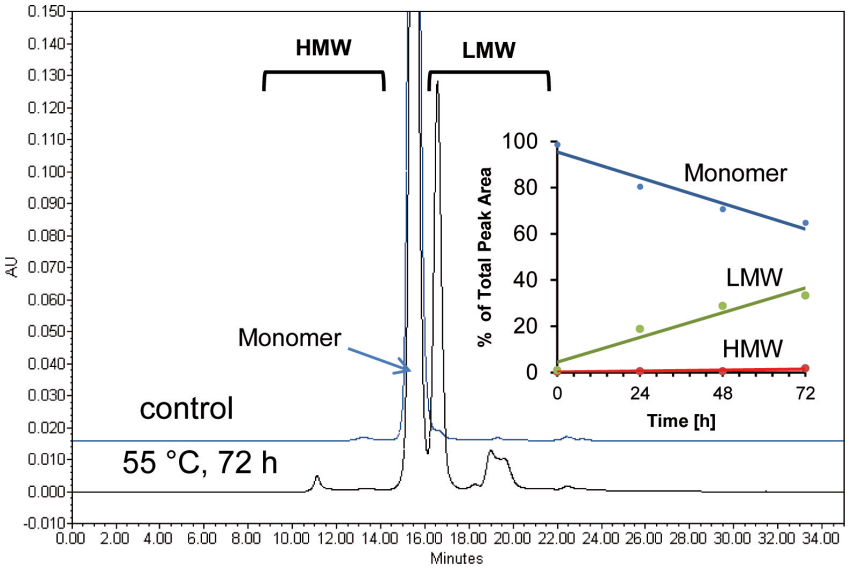

Figure 2: Analysis of mAB Monomer, aggregates (HMW) and degradation products (LMW) by Size Exclusion Chromatography before and after thermal stress; mAB incubated for 72 h at 55 °C (black line), untreated MAB control sample (blue line); AU (280 nm) absorbance units. Inset: The evaluation of % total peak area of the monomer (main peak) and the HMW and LMW species is shown. The decrease in monomer and increase in LMW and HMW with time (h) was detected.

2.4 Imaged capillary isoelectric focusing

Charge profiles/isoelectric points (pI) of the analytes were determined by imaged capillary isoelectric focusing (icIEF) analysis using an iCE3 system (ProteinSimple) equipped with a 5 cm x 100 µM ID fluorocarbon coated capillary. Samples were separated according to their pI and detected by using a whole column UV absorption detector (280 nm) that avoids disturbing focused protein zones. The iCE CFR Software (ProteinSimple) were used for automated pI calibration and data conversion. The sample peaks were integrated with Empower 3 Software (Waters) in order to determine the distribution between acidic, basic and neutral forms in percent. pI markers with pI 8.18 and pI 9.46 were used.

2.5 Size Exclusion High Performance Liquid Chromatography with UV detection

Aggregates were analyzed by size exclusion chromatography (SEC) with UV absorbance (280 nm). SEC measurements were made on

a Waters Acquity H-class Bio UPLC system with a Tosoh Biosciences (Minato, Tokyo, Japan) TSK-GEL G3000SWXL column (5 µm, 7.8 x 300 mm). 100 µg samples were injected into the UPLC system. The levels of high molecular weight (HMW) species, monomer and low molecular weight species (LMW) were detected and their distribution in percent of total peak area evaluated.

3. Results

3.1 Formation of aggregates and degraded products detected by SE chromatography under native conditions

Aggregates of monoclonal antibodies can be already induced during the manufacturing process due to many parameters such as pH, temperature, reactive oxygen species or shear forces and contribute to the heterogeneity profile of the drug substance and drug product. They are regarded as product-related impurities since they are known to affect drug activity and safety. In the authors' experience, the acceptance criteria (specifications) for release and stability are set to around 2 % or even less in quality control testing . Protein aggregates can be categorized in soluble/insoluble, covalent/non-covalent, reversible/non-reversible and native/denatured [6]. Non-covalent aggregates of the monoclonal antibody can be separated and detected under native conditions by size exclusion chromatography. As seen in fig. 2 the monomeric peak of the antibody of 147 kD (retention time 16 min) and aggregates (HMW) as well as the degraded fragments (LMW) were separated and quantified as % of monomer peak area.

Formulated Adalimumab showed a slightly increased formation of higher order aggregates at 55 °C over time (maximum 72 h) than the unstressed formulation (fig. 2) but not higher than 1–2 % in total. This is expected since the formulated antibody was stressed. However, the harsh conditions led to a pronounced degradation (inset of fig. 2) of the monomer (decrease of the main peak at the retention time of 16 min, increase of LMW fraction).

3.2 Formation of aggregates and degraded products detected by CE electrophoresis under denaturing conditions

Product-related substances and impurities including different size or modified variants can be analyzed by reduced capillary electrophoresis (rCE) and non-reduced capillary electrophoresis (nrCE; fig. 3 and 4) to identify covalently associated aggregates and incomplete disulfide – linked fragments. Fragment species are considered a critical quality attribute and are routinely monitored to assess the antibody integrity. The unstressed antibody shows the glycosylated heavy chain (HC) and the light chain (LC) as well as a low level of non-glycosylated heavy chain (NG) under reduced conditions (fig. 3). After thermal stress the proteins starts to degrade as already detected by SEC and peaks with lower molecular weight as HC were detected. The amount of HC decreases whereas the impurities increase. The amount of light chain (LC) was not significantly affected (inset of fig. 3).

The nrCE shows the arising degradation profile under thermal stress more clearly (fig. 4). The fragments (considered as impurities) can be separated from the IgG monomer. The IgG monomer decreased to around 60 %.

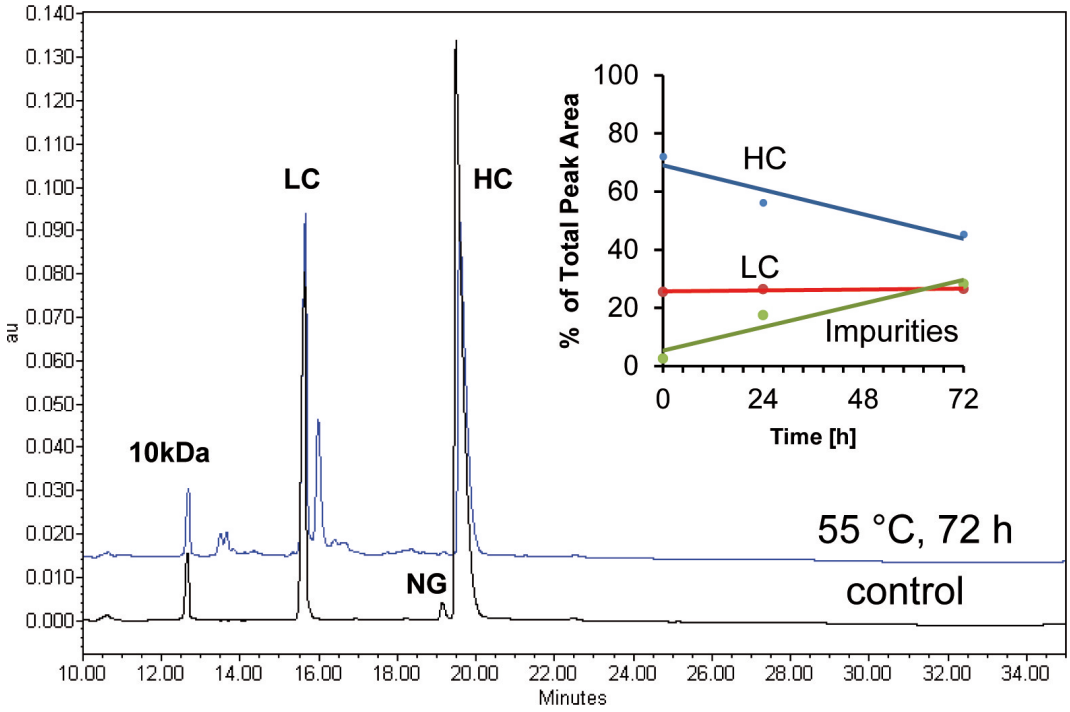

Figure 3: Analysis of thermal stressed mAB by reduced CE-SDS; mAB incubated for 72 h at 55 °C (blue line), untreated MAB control sample (black line); AU (220 nm) absorbance units; NG – non-glycosylated species; LC – light chain, HC – heavy chain; 10 kDa marker. Inset: The evaluation of % total peak area of the heavy chain peak (HC), the light chain (LC) and impurities is shown. The decrease in HC and increase in impurities with time (h) was detected.

Pharm. Ind. 80, Nr. 11, 1557–1563 (2018)

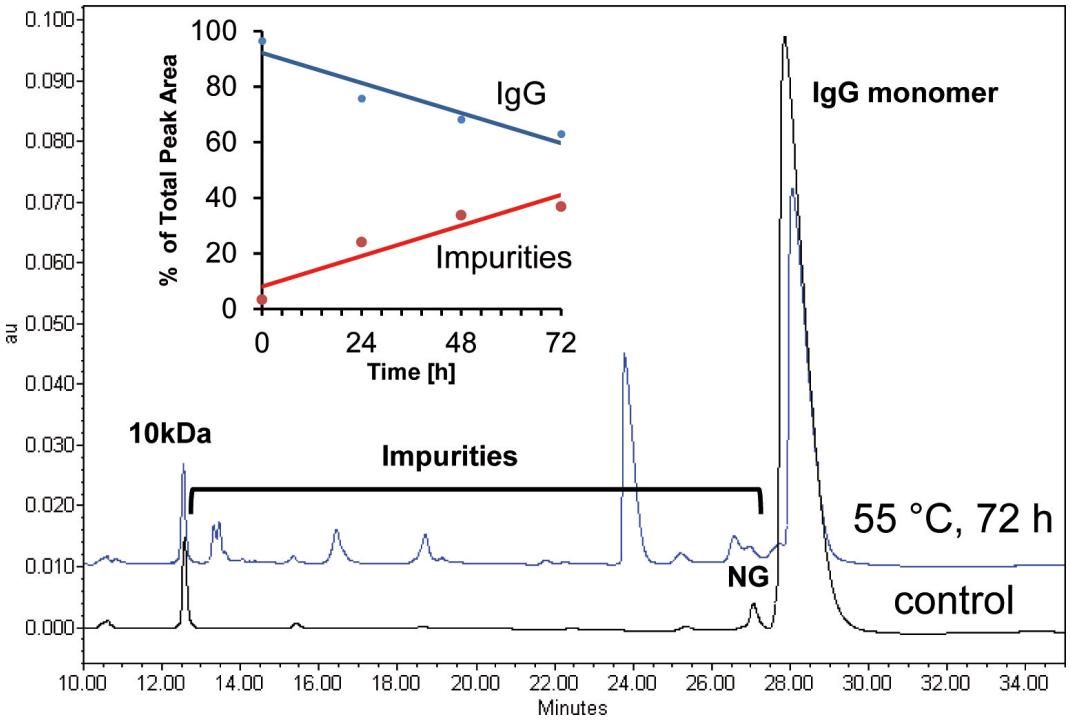

Figure 4: Analysis of thermally stressed mAB by non-reduced CE-SDS; mAB incubated for 72 h at 55 °C (blue line), untreated MAB control sample (black line), IgG monomer (main peak) at 28 min; AU (220 nm) absorbance units; NG – non-glycosylated species; 10 kDa marker. Inset: The evaluation of % total peak area of the IgG monomer and impurities is shown. The decrease in IgG monomer and increase in impurities with time (h) was detected.

The amount of monomer or LC/HC has been evaluated in % of total corrected peak area. The corrected peak area is calculated by peak area multiplied by the capillary length; the result is divided by the migration time.

Altogether, the results contribute to the understanding of potential impurity profiles of a monoclonal antibody after exposure to the stress conditions.

3.3 Charge variants detected by imaged capillary isoelectric focusing

Imaged capillary isoelectric focusing (iCIEF) for the analysis of therapeutic antibodies and antibody drug conjugates provides a fast separation and resolution of the acidic (A), neutral (MI) and basic (B) variants of an antibody as shown in fig. 5 and 6). The dominant neutral major isoform (MI) does not contain structures with C-terminal lysine(s). It consists primarily of the heavy chain domain glycosylated via the asparagine with biantennary structures with 0, 1 or 2 terminal galactoses (see general antibody structure in fig. 1).

The charged isoforms are caused by heterogeneity in the glycosylation, i.e., content of sialic acids, truncation, mannose but also by common post translational modifications such as deamidation, oxidation and fragmentation. For instance, methionine oxidation may be a post translational modification that can impact the bioactivity of the antibody and potentially induce an immunogenic response [8, 9]. Most IgG1 antibodies have two conserved heavy chain (HC) methionine residues located at the FcRn binding interface of the constant heavy chain domains. It was reported that oxidation of these two methionine residues decreased thermal stability, protein A binding, FcRn binding, and circulation half-life of IgG1 antibodies [8].

Table 2 summarizes examples of chemical changes for formation of acidic and basic variants [6]. During stress conditions the variants can be formed. The yielding iCIEF profile can be used for the control during release and stability testing. In fig. 5 it is shown that the analyte is separated according to its pI, a low pI compared to the main peak comprises the acidic variants while a high pI compared to the main peak represents the basic variants (fig. 4, control). Due to the thermal stress (55 °C, 72 h) the basic variants increase. Here, mainly the induced fragmentation as already shown by the capillary electrophoresis can be assumed to be the reason. The acidic variants are only slightly increased.

Figure 6 shows the separation of a sample treated with 3 % hydrogen peroxide. Under oxidative stress, the acidic species increased predominately whereas the major isoform and the basic isoform decreased. This is different to the results of the thermal stress. Here, the increase of the acidic forms may be due to fragmentation of the oxidized protein. It has been described that the variety of acidic forms may be based on multiple modifications [6].

4. Conclusion

State-of-the-art analytical testing methods for assessing the heterogeneity profile of proteins have been used in order to analyze the main quality attributes of antibody products such as aggregation amount, charge variants, level of low-molecular-weight (LMW) variants. For instance, the phenomenon of protein aggregation (e.g., covalent/non-covalent) is a common issue that compro-

mises the quality, safety, and efficacy of antibodies and can happen at different steps of the manufacturing process, including fermentation, purification, final formulation, and storage.

The stress study performed on a commercially available formulated antibody yielded in different impurity profiles that were monitored with size exclusion chromatography (SEC), reduced and non-reduced capillary electrophoresis (rCE and nrCE) and imaged capillary isoelectric focusing (iCIEF). It showed that the protein degrades under thermal and oxidative stress exhibiting truncations and different charge distribution as the unstressed reference samples. The results indicate the importance of a stress study as described in the ICH guideline Q2 (R1) in order to confirm the ability of the method to detect different drug modifica-

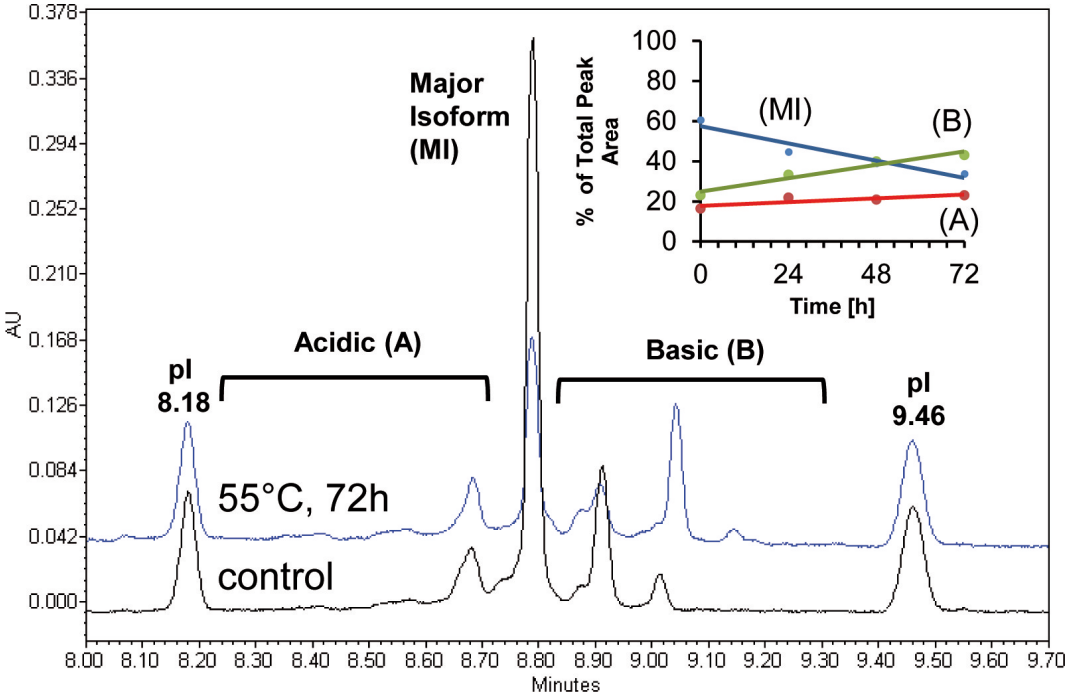

Figure 5: Charge profiles of thermally stressed mAB measured by imaged capillary isoelectric focusing; mAB incubated for 72 h at 55 °C (blue line), untreated mAB control sample (black line); pI markers at 8.18 and 9.46; AU (280 nm) absorbance units; pI – isoelectric points. Inset: The evaluation of % total peak area of the acidic species (A), the major isoform (MI) and the basic species (B) is shown. Predominately, the percentage of the basic species increased, and the major isoform decreased whereas the acidic percentage is mainly unaffected.

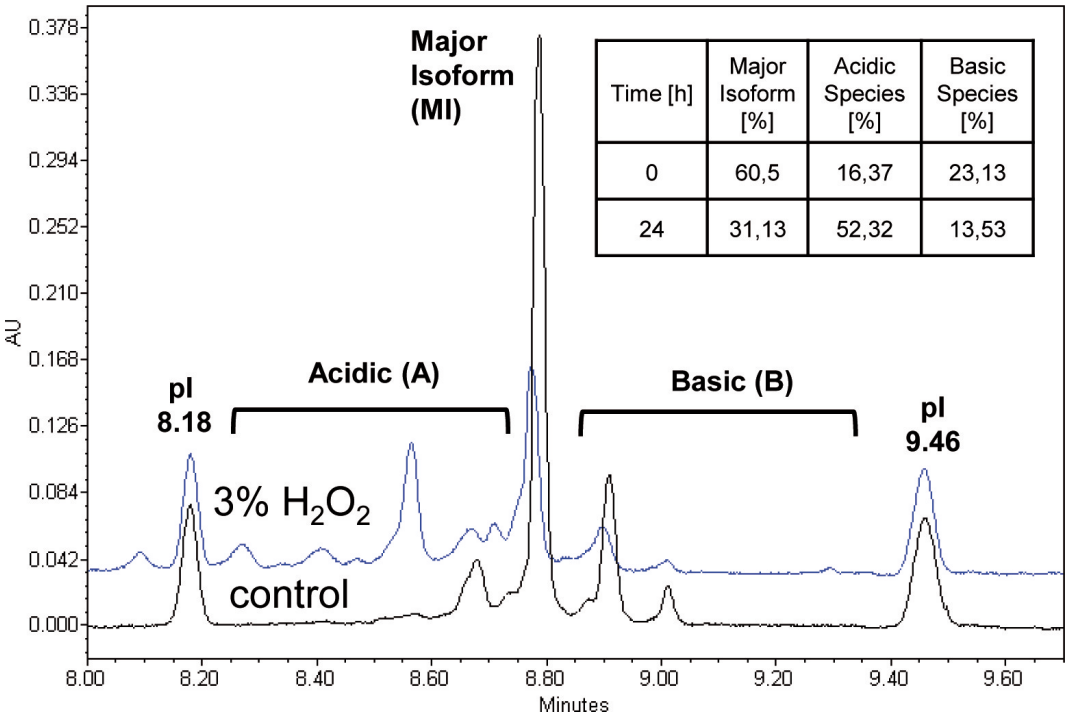

Figure 6: Charge profiles of mAB after oxidative stress measured by imaged capillary isoelectric focusing; mAB incubated for 24 h with 3 % H_2O_2 (blue line), untreated MAB control sample (black line); pI markers at 8.18 and 9.46; AU (280 nm) absorbance units; pI – isoelectric points. Inset: The evaluation of % total peak area of the acidic species (A), the major isoform (MI) and the basic species (B) is shown. The percentage of the basic species and the major isoform decreased whereas the acidic increased.

Pharm. Ind. 80, Nr. 11, 1557–1563 (2018)

Table 2

Various post-translational modifications that may result in charge heterogeneity. Examples are listed in [6].

Acidic Variants	Basic Variants
de-amidation (Asn residue)	C-terminal Lys
fragments	methionine oxidation
reduced disulfide bonds	incomplete disulfide bonds
glycosylation – e.g., sialic acids; mannoses	de-glycosylation
non-classical disulfide linkage	fragments

tions as well as to assess the robustness of the used analytical technique.

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