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Study on an Inactivation Evaluation Method of Cleaning Processes for Biopharmaceuticals

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ABSTRACT

Currently, risk management based on a scientific approach is becoming required in the establishment of cleaning validation limits for pharmaceutical manufacturing equipment, as the acceptable daily exposure (ADE), which is set based on pharmacological and toxicological evaluation, is increasingly applied. At the early stage of development, ADE values may be set using the threshold of toxicological concern (TTC) approach due to lack of human data on toxicity. However, TTC values are estimates, so their application requires careful consideration. Especially in biopharmaceuticals (mainly proteins), whether or not the target product item is inactivated and degraded after cleaning is an important issue in evaluating the cleaning process. Therefore, a study was conducted by carrying out "CIP (clean in place) + SIP (steam in place)" and "CI (caustic immersion: alkaline treatment over a certain period of time)", which are usually processes used in the cleaning of antibody drug manufacturing equipment. The inactivation and degradation of antibody drug was evaluated from the molecular structure and physiological activity point of view, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and surface plasmon resonance. This study was successful in establishing a reliable and effective method for evaluating cleaning processes based on risk.

Arisk-based approach based on a pharmacological and toxicological evaluation is becoming perceived in the industry as a science-based approach that ensures safety of pharmaceuticals. Since the publication of International Council for Harmonization (ICH) Q9 *Quality Risk Management*, the application of this approach to the quality management of pharmaceuticals has been considered essential, and its application has also been extended to the cleaning validation of pharmaceutical manufacturing equipment. Limits such as 1/1000, 1/10,000, and 10 ppm were convention-

ally used in cleaning validation, but these limits can not be scientifically justified and are arbitrary. In September 2010, the International Society for Pharmaceutical Engineering (ISPE) published a new baseline guide called *Risk-Based Manufacture of Pharmaceutical Products* (Risk-MaPP). Risk-MaPP provides a scientific and risk-based approach, based on ICH Q9 *Quality Risk Management* principles, to manage the risk of cross-contamination to achieve and maintain an appropriate balance between product quality and operator safety (1). The basic concept of Risk-MaPP requires a consistent and science-based approach

to risk management. It also proposes the utilization of acceptable daily exposure (ADE), which is derived from a pharmacological and toxicological evaluation in setting the health-based limits. Following the publication of the Risk-MaPP in March 2015, the European Medicines Agency (EMA) revised the European Union's good manufacturing practice *Annex 15 Qualification and Validation* (EU-GMP Annex 15), and the Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S) revised the PIC/S-GMP *Annex 15 Qualification and Validation* (PIC/S-GMP Annex 15), concurrently. As a result of these revisions, residue limits for cleaning validation have become toxicologically evaluated in the EU-GMP Annex 15 and PIC/S-GMP Annex 15 (2). The cleaning of pharmaceutical manufacturing equipment is an extremely important activity in order to prevent the cross-contamination of pharmaceuticals, and it is specified in the regulatory guidelines that the cleaning activity of manufacturing processes and manufacturing support systems should be validated in the same manner as the manufacturing processes.

ADEs (or permitted daily exposures [PDEs]) are derived from various toxicological data, including clinical data, but it is difficult to exactly set product-specific ADEs at the early stages of development because human data on toxicity are not sufficient. To address this issue, the threshold of toxicological concern (TTC) approach may be used as one of the practical measures to set ADEs. The TTC approach is used to provide estimates on the safe side for chemicals that have not been subjected to extensive toxicity tests and is considered useful in the evaluation of trace contaminants in the products (3). Because TTC values are estimates, careful consideration is necessary if used for setting cleaning validation limits. When using the TTC approach, especially in biopharmaceuticals, whether the target product item is inactivated after cleaning is an important issue, and it is considered beneficial to ensure the reduction of contamination risk by supplementing the TTC with a scientific rationale on inactivation (4).

Proteins, polypeptides, and their derivatives produced by biotechnology, such as genetic recombination and high-molecular weight biopharmaceuticals that consist of these components, are known to degrade when the temperature is high or the pH is extremely acidic or basic, and may become pharmacologically

inactive. Generally, product-contact surfaces of manufacturing equipment for most biopharmaceuticals, including antibody drugs, are cleaned in environments exposed to oxidizing and alkaline agents and high temperatures; this is considered to cause the degradation of the constituent high molecule. Although there have been a few studies published on protein degradation concerning pharmaceuticals, these studies did not present detailed descriptions of the method used, and whether the degradation was complete, partial, or not degraded at all (5,6). The authors, therefore, carried out a study to establish a reliable and effective method upon considering that the inactivation of proteins by cleaning process may become a justification to support cleaning validation.

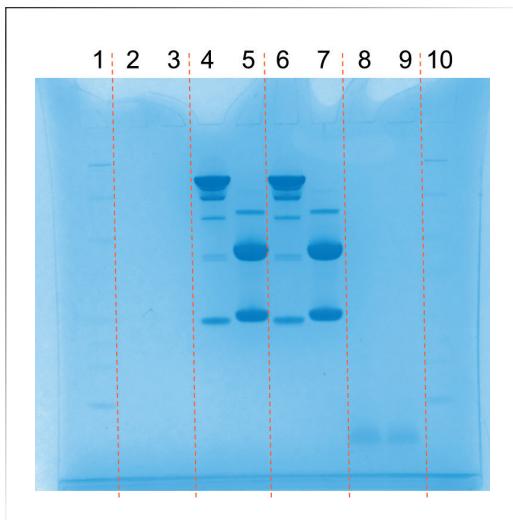
EVALUATION OF INACTIVATION AFTER THE CLEANING PROCESS

In a previous article on the series of cleaning validation for the 21st century, the authors demonstrated the effectiveness of a method for quantitatively measuring the inactivation of high-molecular proteins after cleaning, which is considered a new cleaning evaluation method for the actual production scale in biopharmaceutical manufacturing facilities (7). By modifying the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique, the authors have confirmed that oxidizing and alkaline agents, even at low concentrations, can significantly degrade monoclonal antibodies (mAbs), which are high-molecular proteins, and also have succeeded in indicating that patient exposure can be reduced to extremely low levels. In the facility, cleaning is accomplished by either a clean-in-place followed by a steam-in-place (CIP+SIP) or by immersion in a cleaning agent for more than eight hours (CI: caustic immersion). In this study, the inactivation of mAbs was evaluated using simulated conditions of these actual cleaning procedures. The CIP+SIP were simulated by treatment with alkali combined with autoclaving. The CI was simulated by treatment with alkali for eight hours only. Changes in molecular structure and biological activity were used as indicators of inactivation, and they were evaluated using methods such as SDS-PAGE and surface plasmon resonance (SPR).

EXPERIMENT METHOD

In a previous article on the statistical evaluation of cleaning processes using process capability, the

Figure 1: Effects of clean-in-place + steam-in-place. Lanes 1: marker; 2: blank (NR); 3: blank (R); 4: mAb-intact (NR); 5: mAb-intact (R); 6: mAb-control (NR); 7: mAb-control (R); 8: mAb-alkali/heat treated (NR); 9: mAb-alkali/heat treated (R); 10: marker. (R) is reduction condition, (NR) is non-reduction condition.



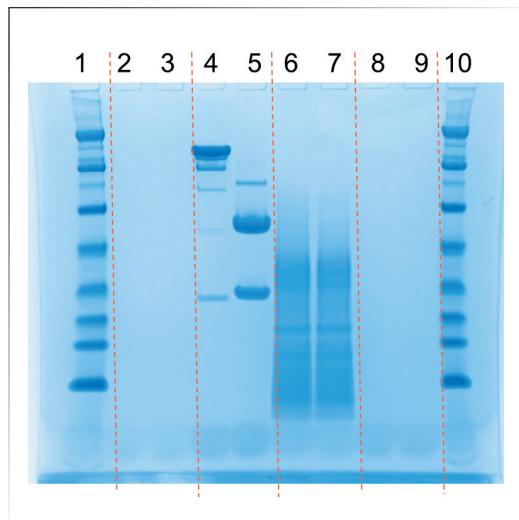
chromatography system and the purification tank indicated relatively low process capability indices (8). The evaluation of cleaning, therefore, was carried out assuming that the cleaning conditions in the actual production for these chromatography system and purification tank are the worst-case scenario. The inactivation of antibody drug was evaluated after applying these cleaning treatments against the estimated maximum residues of antibody from the purification process. The maximum residues of antibody can be derived by the following formula (**Equation 1**):

$$\text{max antibody residue} = \text{max antibody production per liter of culture} \times \text{max culture volume} \times \text{max residue rate}$$

[Eq. 1]

Many antibody drug manufacturing facilities in the world have been employing 2000L for the maximum culture volume and 3g/L for the maximum antibody production per liter of culture medium in the manufacturing of investigational products. Also, in many cases, the recovery rate of each purification process is more than 90%, so the residue of antibody drug substance in the chromatography system and the purification tank would be at maximum 10%. Taking

Figure 2. Effects of caustic immersion. Lanes 1: marker; 2: blank (NR); 3: blank (R); 4: mAb-intact (NR); 5: mAb-intact (R); 6: mAb-alkali treated (NR); 7: mAb-alkali treated (R); 8: blank (NR); 9: blank (R); 10: marker. (R) is reduction condition, (NR) is non-reduction condition.



these facts into consideration, the maximum residue was calculated by the following formula (**Equation 2**):

$$\begin{aligned} \text{max antibody residue} &= 3\text{g/L} \times 2000\text{L} \times 10\% \\ &= 600\text{g} \end{aligned}$$

[Eq. 2]

Because more than 50L of 2% sodium hydroxide (NaOH) is used as a cleaning agent in the cleaning operation of manufacturing facilities, the maximum concentration of antibody in cleaning agent will be $600\text{g}/50\text{L}=12\text{g/L}$. The sample was prepared so as to have an antibody concentration of 12g/L or more by adding a buffer solution necessary to reproduce the worst case of product concentration.

Reagent

The reagents listed below were used in this study:

- Preparation of samples: mAb is dissolved in formulation buffer (FB), which is composed of 50 mM histidine, 6% sucrose, 0.1% polysorbate 80 (pH 5.9), 1N NaOH solution, 1N hydrochloric acid
- SDS-PAGE: NuPAGE LDS Sample Buffer (4x), NuPAGE MES SDS Running Buffer

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Table I. List of samples prepared.

Sample name	Sample	Reagent A	Autoclaving	Reagent B	Note*3
Blank	FB*2	0.5N NaOH	Yes	0.5N HCl	
mAb-intact	mAb	No	No	No	Untreated
mAb-control	mAb	DDW*1	No	DDW*1	
mAb-caustic/autoclave	mAb	0.5N NaOH	Yes	0.5N HCl	NaOH treatment (8 h) and autoclave 15 min
mAb-caustic only (8 h)	mAb	0.5N NaOH	No	0.5N HCl	NaOH treatment (8 h)

*1 Sterilized water/deionized distilled water (DDW).

*2 Formulation buffer (FB).

*3 "h" is an abbreviation for hours, "min" is an abbreviations for minutes in this table.

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(20x), Marker12 unstained standard, NuPAGE 4–12% bis-tris gel 1.0x10well, NuPAGE sample reducing agent (10x), Gelcode blue safe reagent (Coomassie brilliant blue), ethanol (high-performance liquid chromatography [HPLC] grade), acetic acid. • SPR: amine coupling kit, HBS EP+ (10X) buffer, glycine 2.0, acetate 5.0, NaOH 50, human recombinant antigen.

Preparation of samples

The preparation of samples was carried out according to the following procedures to make a final concentration of at least 12g/L, considering the worst-case assumption of antibody drug residues (**Table I**). One mL of Reagent A (0.5N NaOH or DDW according to **Table I**) was added to each sample (mAb or FB) in order to simulate CIP or CI at a protein concentration of 12 g/L or more. Heat-treatment with autoclave (121 °C, 30 minutes at 15 psi) was conducted to simulate SIP only for the corresponding samples. Then, 1 mL of Reagent B was added to neutralize alkali (0.5N HCl or DDW according to **Table I**).

RESULTS

SDS-PAGE: SDS-PAGE experiments were carried out in both reduction and non-reduction conditions to grasp the degradation profile more comprehensively by observing them from the different viewpoints. In reduction conditions (R), 39 µL of each prepared samples, 15 µL of sample buffer, and 6 µL of reducing agent were mixed together, kept at 100 °C for one minute, and then cooled to room temperature.

In non-reduction conditions (NR), 45µL of each prepared samples and 15 µL of sample buffer were mixed, kept at 80 °C for three minutes, and then cooled to room temperature. Electrophoresis was conducted with the 12µL of sample loading volume and using the MES SDS running buffer under the conditions of 200V/120mA/100W/30min. Then, the gels were stained with Coomassie Brilliant Blue (CBB) reagent

CIP+SIP: To simulate the cleaning and sterilization processes (CIP + SIP) of the antibody manufacturing equipment, an alkaline treatment with NaOH solution and heat treatment by autoclave were performed. Bands of fragments were observed at the lower molecular weight positions in the gels for samples subjected to the alkaline treatment and heat treatment, and inactivation and degradation were confirmed (See **Figure 1**, Lanes 8 and 9).

CI: The effect of CI, which is carried out as part of the cleaning procedure for the chromatography system, was also studied. As a result of immersing the samples for eight hours, a smear was observed in the gels (**Figure 2**, Lane 6 and Lane 7).

Because lanes 6 and 7 showed no clear bands corresponding to the untreated protein bands, and the entire bands were smearing, this confirms that the antibody proteins are inactivated and degraded by the long alkaline treatment.

SPR: In the CIP+SIP-degraded samples subjected to alkaline treatment by NaOH solution and heat treatment by autoclave, bands of fragmented proteins were observed at the lower molecular weight positions of the gel. Further tests were conducted to compare with the non-degraded samples in order to confirm

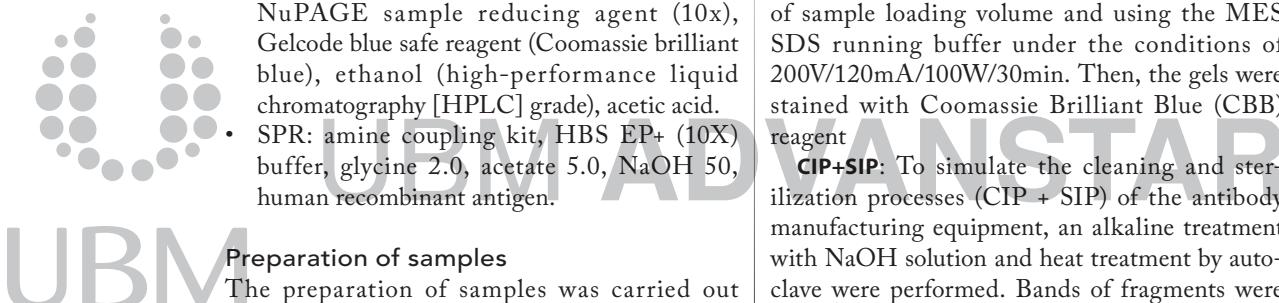
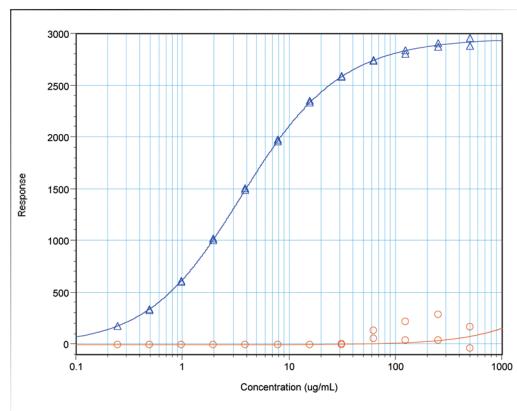


Figure 3. Binding affinity analysis of monoclonal antibodies using surface plasmon resonance. Δ is non-degraded, \circ is NA-degraded.



the impact of the fragmentation on the actual binding activity. The binding activity was measured by SPR experiments with the human recombinant antigen immobilized sensor chips at 25°C.

Immobilization of the human recombinant antigen protein to sensor chips was performed according to the amine coupling protocol under pH 5.0 conditions (10 mM acetate), and the immobilization amount was adjusted to 2500 RU (response). The binding reaction was measured by using the HBS-EP+ (1X) as a running buffer, and the injection parameters were set to a flow rate of 30 μ l/min and a contact time of 180 seconds. Data analysis for the binding profiles was conducted using the SoftMax Pro software (Molecular Devices), which is the global standard analysis software in life-science research.

The analysis of binding activity of monoclonal antibody in non-degraded samples using SPR indicated an evident binding activity to the antigen. However, in CIP+SIP-degraded samples, the binding activity was completely lost, and the toxicological risk as antibodies was not identified at all (**Figure 3**). These results revealed that mAbs lose binding activity with alkaline and heat treatments.

DISCUSSION

It is apparent that the mAbs in this study are structurally degraded and lose binding activity when subjected to alkaline and heat treatments. Even with the immersion in alkaline solution for a period of eight hours alone, the mAbs were confirmed to be inactivated. The above results

demonstrated that these mAbs can be degraded and their binding activity can be lost with the CIP and SIP that are performed in the current cleaning processes of antibody drug-manufacturing equipment.

The monoclonal antibody of 12 g/L as the worst-case scenario in this study, which is based on an assumption that the mAb titer reaches 3 g/L, was degraded and lost binding activity after alkaline and heat treatments at the final concentration. Based on this fact, it can be considered that if the titer of a mAb is 3 g/L or less, the mAb can be degraded to the same extent or more with the current cleaning method. This study, therefore, can be applied as a benchmark for manufacturing other antibody drugs in the future.

CONCLUSION

Currently, it is required to toxicologically evaluate the residue limit for cleaning validation, and the use of PDE or ADE for the determination of cleaning limits has become a requirement (1,2). It is difficult to set product-specific PDEs and ADEs at the early stage of development because human data on toxicity are not sufficient, and so the TTC approach may be used to determine the cleaning limits. TTC values, however, are estimate values for chemical substances that have not been subjected to extensive toxicity testing, so careful consideration is essential in order to determine their use as limits for cleaning validation. This study was conducted to establish a reliable and effective method to support the validity of the cleaning processes as to whether the product is inactivated or not. The evaluation of the cleaning procedures was carried out by modeling the cleaning conditions for the chromatography system and the purification tank in actual production as the worst-case scenario. The maximum residue of antibody from the purification process was treated with "CIP + SIP" and "CI" (immersion to alkaline solution for more than eight hours), which are procedures used in the actual cleaning processes of antibody drug-manufacturing equipment, and the inactivation of monoclonal antibody was evaluated by observing the changes in the structure and biological activity using SDS-PAGE and SPR. As a result of this evaluation, it was confirmed that these mAbs were inactivated by the alkaline/heat treatments or immersion in alkaline solution for at least eight hours.

Because the target subjects of this study were monoclonal antibodies, similar antibody drugs are considered to undergo similar degradation with the current cleaning method. The authors will continue these studies as a new inactivation evaluation model, which combines the inactivation evaluation method demonstrated in the present study using SDS-PAGE and SPR analysis with the analysis using process capability index based on historic cleaning data. The authors will advance this research to include application of this new evaluation model to high-molecular weight pharmaceuticals other than monoclonal antibodies.

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ABSTRACT
 Currently, risk management based on a scientific approach is becoming mandatory in pharmaceutical manufacturing. In particular, the risk-based approach, as the acceptable daily exposure (ADE) method, is increasingly applied. At the early stage of development, ADE values may be derived from the results of toxicological studies (TC) approach due to lack of toxicity data on toxicological concerns. However, their application requires careful consideration. Especially in biopharmaceuticals (mainly monoclonal antibodies), it is difficult to evaluate the risk of cleaning processes after cleaning. This is because the cleaning validation is placed + SP (steam in place) and CQ (cyclic immersion aseptic tank cleaning) over a certain period of time, which are usually processes used in the field of pharmaceuticals. In this study, we evaluated the inactivation and degradation of antibody drug was evaluated from the results of SDS-PAGE analysis and SPR analysis. The results using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and surface plasmon resonance analysis showed that the antibody drug was stable and effective method for evaluating cleaning processes based on risk.

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