Abstract
Product and process related impurities of biopharmaceuticals have serious implications on product safety and efficacy in clinical use. Low molecular weight (LMW) impurities are generated during process and stability studies are routinely analyzed using non-reducing capillary electrophoresis with SDS during different stages of product development and release. The current sample processing methodology with heat denaturation is known to induce fragmentation and interfere with the LMW impurity analysis. In this study, we compared different sample processing buffers with different compositions and pH and finally found a solution to the problem of sample artifacts generated during heat denaturation step of sample processing which interferes with the LMW impurity analysis. In this study, we compared different sample processing buffers with different compositions and pH and finally found a solution to the problem of sample artifacts generated during heat denaturation step of sample processing which interferes with the LMW impurity analysis. This study suggests that, the sample processing with 25mM Citrate buffer does not require heat denaturation at higher temperatures and hence is the most appropriate buffer for sample processing in LMW impurity analysis. The 25mM Citrate buffer has shown better drug product stability and integrity compared to other buffers. Hence, we recommend the 25mM Citrate buffer with 8M Urea for sample processing in LMW impurity analysis by CE-SDS method.

Keywords: Sample buffer, capillary electrophoresis, anti-CD20 monoclonal antibody, pH, Low molecular weight impurities

Introduction
Therapeutic monoclonal antibodies (mAbs), which are produced with recombinant DNA technology constitute for majority of bio-pharmaceutical approvals in recent times (1). Therapeutic mAbs are commonly produced in the cells of murine/mammalian origin and their purification involves a variety of chromatography and filtration steps in the harsh conditions which may influence the structural and functional integrity of the antibodies and may generate process and product related impurities. Hence, the purity and quality of these antibodies is utmost critical for them to be used in the patients. To ascertain the purity of these antibodies, the process and product development as well as product release involves full structural and functional characterization using a battery of analytical methods. Analytical characterization of purity is crucial for the safety and efficacy of therapeutic monoclonal antibodies and for their commercial release (2). Testing the monoclonal antibody drug products for their stability is an important parameter for evaluating the product shelf life and is also a regulatory

A simple and novel sample preparation approach for effective characterization of antibody low molecular weight impurities by CE-SDS method

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requirement. The product-related impurities such as high and low molecular weight impurities and process-related impurities must be well characterized and analyzed during in-process, stability and lot-release stages of therapeutic mAb production. Most importantly stability studies at high temperature induce product degradation and these low molecular weight impurities such as degradation products of therapeutic mAbs must be thoroughly characterized.

High performance size exclusion chromatography (HP-SEC) is a commonly employed method for protein characterization based on its molecular weight (3). However, HP-SEC is low in sensitivity and resolving power for low molecular weight species. The other method, SDS-PAGE although has better sensitivity, it has some limitations such as manual operation, long run time, and inaccurate quantification (4). Non-reducing capillary electrophoresis- SDS (Non-reducing CE-SDS) is considered one of the best method to analyze low molecular weight impurities in therapeutic monoclonal antibody samples. Because of its automation, quantitative nature, capillary electrophoresis (CE) technology is the new benchmark for therapeutic antibody-purity analysis.

In the biopharmaceutical industry, currently CE-SDS is applied at all stages of the product development, which includes analysis of structural isoforms, analysis of size variants, carbohydrate occupancy, and in process development, and product release (5-8). For the product degradation or stability analysis, non-reducing CE-SDS is the most preferred method, which offers great aptitude of low molecular weight impurities detection. In the non-reduced CE-SDS analysis, the native protein is treated with SDS prior to CE separation to mask the protein native charges (9).

Sample processing and the choice of sample processing buffers are the most critical factors in the CE-SDS based impurity analysis as sample preparation itself known to induce antibody fragmentation and contribute to the increased LMW impurities in the samples (10,11). Some studies have also reported that the free thiol groups in the antibody itself induce disulfide bond catalysis, resulting in increased antibody degradation. To arrest the antibody degradation, some studies have suggested the addition of alkylating agents to the CE-SDS sample buffer to prevent artifacts generated due to harsh sample processing which involves heat denaturation and alkaline conditions of buffer (10-14). These alkylating agents have minimal impact on the sample artifacts generated due to heat denaturation as samples with constant concentration of Iodoacetamide (IAM) has shown lesser fragmentation at lower temperatures compared to the samples incubated at higher temperatures (15). Hence, heat-induced antibody fragmentation and generation of low molecular weight (LMW) impurities during sample processing interferes with the CE-SDS based LMW impurity analysis and pose a greater challenge for non-reducing CE-SDS to be used in the analysis of low molecular weight impurities. Avoiding the sample artifacts during sample processing is highly recommended for accurate analysis of low molecular weight impurities. Unfortunately, with the existing sample processing buffers this cannot be achieved completely. Zhang et al. (16) used Citrate buffer in the pH range of 5.5 to 6.5 for sample processing to minimize sample artifacts. However, they are only marginally successful, because still heating at high temperature (65°C) is required and heating is known induce fragmentation. The only solution is to achieve effective sample processing at lower temperatures with pH maintained around neutral conditions. In this study we used Urea in combination with Citrate buffer with pH 6 to achieve good separation with lesser degradation products.

**Materials and Methods**

**Preparation of reagents and buffers:** The Tris-Cl sample buffer containing 100 mM Tris-Cl and 1% SDS with pH 9 was prepared by dissolving 605 mg of Tris base and 0.5 gm of SDS in 50 ml water for injection (WFI) and pH was adjusted...
using 1N HCl. The 25mM Citrate phosphate sample buffer pH was prepared as per previous literature (16). The 25mM citrate + 8M Urea buffer was prepared by dissolving 48 gm of Urea in 100ml 25mM citrate sample buffer with pH 6. The alkylation agent, 250 mM IAM was prepared by dissolving 23 mg of Iodoacetamide in 500 μl of filtered WFI. The other reagents like SDS-MW Gel buffer and 10kDa internal marker was prepared as per manufacturers recommendations.

Preparation of monoclonal antibody samples: Both the reference standard (Reference Medicinal Product from innovator) and the test samples were treated simultaneously. Test samples were processed in duplicates and injected individually while the standard was processed as singlet and injected in duplicates. The standards and test samples were diluted to 1 mg/ml with filtered WFI from the nominal concentration. To 45 μl aliquots of standard and test samples 50 μl of sample buffer, 2 μl of 10 kDa internal marker and 5 μl of 250 mM IAA solution were added and mixed thoroughly by vortexing in Eppendorf tubes followed by centrifugation at 300 g for 1 min at 25°C. The samples and standard were incubated at the respective temperatures (at 70°C and 90°C as per the experimental protocol) for 10 minutes followed by centrifugation at 300 g for 1 min at 25°C and taken 100 μl aliquots of samples and standard. The anti-CD20 mAb (Chimeric) sample and other anti-VEGF (Humanized) and anti-Her2 (humanized) mAbs samples (1 mg/ml) were incubated with 50 μl of Tris-Cl buffer pH 9, Citrate buffer pH 6 and Citrate buffer with 8M Urea pH 6 were processed as mentioned previously.

Non-reducing capillary electrophoresis – sodium dodecyl sulfate (NR CE-SDS): Capillary electrophoresis system (PA 800 plus) from Beckman coulter with bare fused silica capillary (Total length – 30.2 cm, Effective length – 20.2 cm, Inner diameter – 50 μm, Outer diameter – 375 μm) containing Photodiode array (PDA) detector was used for performing all the experiments. Reagents were aliquoted into the vials and were placed in the inlet and outlet trays. The Inlet and outlet and sample trays were placed on the arms of the instrument. Capillary cartridge was installed and method was created as per manufacturer’s recommendation. System suitability was tested using reference standard and bracketing reference standard. Standard and samples (0.5mg/ml) were injected in duplicates with 7 kV voltage for 30 seconds. During separation, a voltage of 15kV with reverse polarity maximum current of 300μA was applied. The PDA detector was set with electropherogram channel 1 wavelength of 214nm and channel 2 wave length of 220nm and reference channel wavelength of 350nm containing band width of 10nm and the sampling rate of 2Hz for data collection. All experiments were carried with bare fuse silica capillaries and the capillary temperature was maintained at 25°C throughout the experiments. All the calculations were performed using corrected peak areas and migration times.

Capillary preconditioning: All the new capillaries were preconditioned through a rinse procedure starting with 0.1N NaOH for 10 min for cleaning the capillary surface, 0.1N HCl for 5min for neutralizing the capillary surface, water for 5min for removing the residual acid, using 20 psi pressure. A voltage of “15 kV was applied with reverse polarity to the capillary filled with running buffer for 5 min after the rinse.

Results and Discussion

Effect of heating, sample buffer composition and pH on the LMW profile of anti-CD20 monoclonal antibody: We found significant additional degradation induced by Heating in anti-CD20 mAb samples at 90°C compared to 70°C (Fig. 1 a & b). The sample preparation involving heat denaturation is contributing to the increased low molecular weight impurities, which are interfering the analysis. Interestingly, we found that the increased degradation at higher temperature (90°C) was primarily due to higher pH. The Tris-Cl buffer with pH 6 shown lesser degradation products and more intact mAb portion at both the temperatures (Fig.1 a & b). Zhang et al.16 also observed the similar results with undisclosed
antibody, and our results second their results. We have also evaluated the effect of Tris-Cl buffer pH (6 to 9) on LMWs% and intact mAb peak area (A %) at 70 °C for 10 mins incubation time. We found that the degradation pattern is linear in the range of pH 9 to 7 with intact mAb A% increased and LMW% decreased with decrease in pH proves that pH independently effect the product degradation during denaturation procedure (supplementary data set 1).

Although, Zhang et al. (16) reported the citrate buffer pH 6 shown better intact mAb peak area, they have not disclosed the antibody. Our results specifically suggested that, the temperature has a significant impact on fragmentation of anti-CD20 chimeric antibody, only at higher pH. Since, the Tris-Cl buffer with pH 6 is not stable for analysis, we tested the 25mM Citrate phosphate buffer with pH 6 for further experiments. We also compared the degradation profiles (LMWs) of both Chimeric and Humanized monoclonal antibodies in both 25 mM Citrate phosphate buffer and 100mM Tris-Cl buffer at 70°C and 90°C temperatures. To find whether the observed effect is mAb specific, we have also evaluated the effect of sample buffer and its pH on different therapeutic monoclonal antibodies with different engineering patterns such as chimeric and humanized antibodies at both the temperatures. Although both mAbs have exhibited more degradation in Tris-Cl buffer compared to citrate buffer, the extent of degradation was more in case of chimeric anti-CD20 monoclonal antibody suggesting antibody type may also influence the extent of degradation at both the temperatures (Fig. 2 a & b). The addition of alkylating agent IAM does not seem to have much effect on the heat induced degradation of anti-CD20 monoclonal antibody, as we observed similar LMW profile in with and without IAM (data not shown). Zhu et al. (15) also reported that the alkylating agents have minimal effect on fragmentation due to the variations in incubation times and concentration of IAM and due to variation in heating times of samples (15).

Table 1. Comparison of Degradation profile of three different antibodies in all three buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Chimeric anti-CD20 mAb</th>
<th>Humanized anti-VEGF mAb</th>
<th>Humanized anti-HER2 mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrate Buffer, pH 6.0</td>
<td>Citrate Buffer, pH 6.0 + 8M Urea</td>
<td>Citrate Buffer, pH 6.0 + 8M Urea</td>
</tr>
<tr>
<td>Time</td>
<td>10 min.</td>
<td>10 min.</td>
<td>10 min.</td>
</tr>
<tr>
<td>Temp.</td>
<td>70 °C</td>
<td>70 °C</td>
<td>70 °C</td>
</tr>
<tr>
<td>LC</td>
<td>2.1</td>
<td>0.77</td>
<td>0.49</td>
</tr>
<tr>
<td>HC</td>
<td>0.14</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>1H1L</td>
<td>0.41</td>
<td>0.42</td>
<td>0.39</td>
</tr>
<tr>
<td>2HC</td>
<td>1.25</td>
<td>1.95</td>
<td>1.86</td>
</tr>
<tr>
<td>2H1L</td>
<td>5.59</td>
<td>1.95</td>
<td>1.55</td>
</tr>
<tr>
<td>Intact mAb</td>
<td>90.3</td>
<td>94.48</td>
<td>95.32</td>
</tr>
</tbody>
</table>
| LC – Light chain, HC – Heavy chain, 1H1L – 1 heavy and 1 Light chain, 2HC – 2 Heavy chains, 2H1L – 2 Heavy chains and 1 Light chains

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Effect of sample buffer composition and pH on LMW impurities analysis

**Fig. 1a & 1b.** Effect of temperature and pH on product degradation profile in the Tris-Cl buffer

**Fig. 2a & 2b.** Effect of sample buffer on product degradation profile of two different therapeutic monoclonal antibodies

**Effect of sample buffer composition and pH on LMW impurities analysis**
Urea as denaturing agent in sample preparation: Since, we observed heat denaturation increase the product degradation further and interferes with the analysis of inherent LMWs, we have evaluated the possibility of substituting heat denaturation with chemical denaturation using chaotropic agents such as Urea. The samples were incubated with 25 mM Citrate phosphate Buffer containing 8M Urea (pH 6.0) at 37°C for 10 mins and compared with the samples subjected to routine heat denaturation at 70°C for 10 minutes in both Tris-Cl and Citrate buffers. We found the addition of urea to the citrate buffer marginally decreased the product degradation further at 37°C and improved the intact mAb peak area percentage at lower temperatures suggesting that Citrate buffer with Urea is better alternative to the Citrate buffer and Tris-Cl buffers which require heat denaturation during sample processing. However, Urea concentration (range 4 to 10M) does not seems to have any effect on degradation profile (See supplementary data set 2). This suggests the heat denaturation can be replaced by chemical denaturation with Urea for the analysis of LMW impurities by non-reducing CE-SDS. These results suggests that, the chemical denaturation by Urea has exhibited the similar resolving power and separation with better product integrity at 37°C compared to the 25mM Citrate buffer and Tris-Cl buffer run at 70°C.

Batch to batch variation of anti-CD20 chimeric mAb product degradation in all three buffers: We have compared this effect using the anti-CD20 monoclonal antibody product of innovator and in-house generated anti-CD20 antibody and found that the rate of degradation is more in the in-house generated antibody which may be attributed to quantity of other critical quality attributes of the antibody which may have an effect on the degradation profile (Fig. 3 a & b).

Variation in degradation profile of different antibodies with all three buffers: All three buffers including Citrate buffer containing urea are compared with three different classes of antibody such as chimeric, humanized and fully human
Effect of sample buffer composition and pH on LMW impurities analysis

Antibodies. All three antibodies shown better intact mAb A% in Citrate buffer with Urea and the extent of degradation was more in the case of chimeric anti-CD20 monoclonal antibody compared to the other two antibodies (Table 1) (Fig. 4 a, b & c). The peak heights in Citrate Buffer and Citrate Buffer with urea are nearly half when compared to Tris-Cl Buffer, which may be due to the conductivity variations in the buffers. The amount of protein entering the capillary is less due to conductivity and ionic strength of buffer. However, these variations does not show any effect on the results (data not shown). We found, the peaks irrespective of their molecular weight enter the capillary in a similar fashion both in both Tris-Cl buffer and Citrate Buffer.

Evaluation of urea sample preparation: We have also evaluated the role of Urea concentration (4, 6, 8 and 10 M) and incubation times (0, 5, 10, 15 min) at 37 °C and found that there is no significant impact of Urea concentration and incubation times in the specified ranges on the product degradation profile. However, we have observed base line drift and decreasing peak heights with increasing molarity of urea. IAM peak was not observed in samples treated with urea buffer.

Conclusion
Sample buffer composition, Temperature and pH affects the product degradation of therapeutic monoclonal antibodies. Antibodies with different engineering patterns such as chimeric and humanized show variations in the product degradation with chimeric having the highest degradation and humanized with low degradation profiles. The 25mM citrate sample buffer containing 8M urea is the most appropriate buffer for sample processing for LMW impurity analysis by CE-SDS method as heat denaturation step is not required for this buffer and hence the sample artifacts generated during heat denaturation which interferes with the LMW impurity analysis can be avoided with this buffer.

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Conflicts of interest : The authors declare there is no conflict of interest.

References

Fig. 4a, 4b & 4c. CE-SDS electrophoretograms of three different antibodies in all three butters


