U.S. FOOD & DRUG ADMINISTRATION

Biosimilar User Fee Act (BsUFA) III Regulatory Science Pilot Program

ANNUAL REPORT

CONTENTS

1. REPORT OVERVIEW 2
2. PROGRESS SUMMARY 3
   - Specific Aim 1: Forced degradation of biotherapeutics from multiple sources 3
   - Specific Aim 2: Evaluation of PQAs, including charge variants and glycosylation, using traditional methods 4
   - Specific Aim 3: Identification and relative quantitation of modifications using a MAM workflow 6
   - Specific Aim 4: Assessment of bioactivity and structure analysis of biotherapeutic products and stressed samples 6
3. RESEARCH OUTCOMES 7
   - Specific Aim 1: Forced degradation of biotherapeutics from multiple sources 7
   - Specific Aim 2: Evaluation of PQAs, including charge variants and glycosylation, using traditional methods 7
   - Specific Aim 3: Identification and relative quantitation of modifications using a MS workflow 8
   - Specific Aim 4: Assess bioactivity of biotherapeutic products and stressed samples 8
4. REGULATORY IMPACT 8
5. COMMUNICATION AND DISSEMINATION 9
6. CHALLENGES 10
7. NEXT STEPS 10
   - Specific Aim 3: Identification and relative quantitation of modifications using a MAM workflow 10
   - Specific Aim 4: Assessment of bioactivity and structure analysis of biotherapeutic products and stressed samples 10
8. REFERENCES 11
9. APPENDIX A: ADDITIONAL MATERIAL 11
10. APPENDIX B: ABBREVIATIONS 12

Check if this report is Progress or Final Report:

☒**Progress report** ☐ **Final report**

1. REPORT OVERVIEW[1]

Table 1: High-level overview of the project objective, aim(s) progress, outcomes, and timelines for communication and regulatory impact

Project Title:	Assessment of the performance of MAM vs conventional QC methods for evaluation of Product Quality Attributes of adalimumab and etanercept
Investigator:	Diane McCarthy
Organization:	United States Pharmacopeia
Grant No. (if applicable)	1U01FD007762-01
Project Objective:	To assess the performance of the MS-based MAM versus conventional QC methods to identify changes in PQAs upon forced degradation and to correlate changes in those PQAs with bioactivity, binding affinity, and structure.

Specific Aim(s)	Progress	Outcomes	Communication Timeline
1. Forced degradation of biotherapeutics from multiple sources	Complete	Adalimumab and etanercept samples from three sources (originator, locally approved biosimilar, and research grade material) were subjected to forced degradation under thermal and chemical stress conditions. Two thermal degradation time points (2 and 6 weeks), one chemical stress condition, and a control were selected for further analysis.	Conference presentations starting in August 2024 Publications pending completion of study
2. Evaluation of PQAs, including charge variants and glycosylation, using traditional methods	Complete	Control and degraded samples were analyzed for charge variants (by CEX/AEX), glycosylation, and size variants (by CE-SDS). Different profiles were observed based on the source of the material and the degradation condition, which confirms that a variety of modifications are present that can form the basis for comparison of conventional vs. MAM methods.	Conference presentations starting in August 2024 Publications pending completion of study
3. Identification and relative quantitation of modifications using a MAM workflow	In progress	Method refinement has been completed and PQAs selected for analysis, including approximately 60 PQAs for adalimumab and approximately 120 PQAs for etanercept. Sample analysis is complete and data analysis is in progress.	Conference presentations starting in August 2024 Publications pending completion of study

[1] This section will be used by program for broader research portfolio and regulatory impact analysis by the BsUFA III steering committee.

Specific Aim(s)	Progress	Outcomes	Communication Timeline
4. Assessment of bioactivity and structure analysis of biotherapeutic products and stressed samples	In progress	A second forced degradation study was completed in lab 2 and time points selected for further analysis. Sample analysis using MAM is complete and data analysis is in progress. Assessments of charge variants, function (bioassay and SPR) and structure are ongoing.	Conference presentations starting in September/October 2024 Publications pending completion of study

2. PROGRESS SUMMARY

Project Objective: The project will evaluate the performance of the mass spectrometry (MS)-based Multi-Attribute Method (MAM) compared to conventional methods in detecting changes in product quality attributes (PQAs) and their correlation with function. Adalimumab and etanercept will be used as representatives of mAbs and fusion proteins respectively, and their PQAs will be compared using both analytical approaches. This study will establish a knowledge base for mAbs and fusion proteins that facilitates the transition from conventional techniques to MAM, enabling broader adoption by biosimilar manufacturers and more efficient analysis.

Specific Aim 1: Forced degradation of biotherapeutics from multiple sources

Three samples each of adalimumab and etanercept were obtained, including originator products (Humira and Enbrel), locally approved biosimilars (from India manufacturers), and research-grade products. All samples were subjected to forced degradation to induce molecular changes that can be used to compare MAM and conventional methods in Specific Aims 2 and 3. The forced degradation conditions for adalimumab included exposure to 40 °C with varying exposure times up to 6 weeks and oxidation with H_2O_2. Etanercept was subjected to 40 °C with varying exposure times up to 6 weeks as well as alkaline stress. All samples were stored at <-65°C until analysis. Degraded samples and controls were evaluated for particulate formation, changes in concentration, change in charge variant profile, and aggregation as measures of degradation. Forced degradation study was initiated in February 2023, and the analysis of the degraded samples began in April 2023.

Appearance and concentration

Visual appearance has been conducted on all samples using an in-house procedure, referencing <790> *Visible Particulates in Injections*. Most control and stressed samples were transparent, colorless, and free of obvious particles. The only sample showing changes in visual appearance was the biosimilar adalimumab product subjected to oxidation, which appeared white (milky). On prolonged storage, the level of precipitation increased in the oxidized biosimilar. Some precipitation was also noted in the oxidized originator material after approximately 4 months in storage. Further investigation showed that addition of methionine after oxidation could mitigate precipitation upon storage. No significant changes in concentration were observed due to thermal or chemical degradation based on UV_{280} measurement using the SoloVPE System.

Aggregation

To evaluate aggregation, SEC-HPLC was performed according to USP General Chapter <129> *Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies.* Before applying, the method was evaluated using research grade adalimumab and etanercept to assess the suitability and reliability of the method. The method yielded good separation of the monomer and aggregate peaks. The evaluation also assessed the sample stability over the run and showed no differences in the profile between 0 and 25 hours.

For adalimumab, control samples from all three sources were remarkably similar. When exposed to stress conditions, the originator product and the biosimilar displayed similar responses, exhibiting increased aggregation and alterations in High Molecular Weight (HMW) and Low Molecular Weight (LMW) species profiles. In contrast, the research-grade adalimumab demonstrated significant resistance to stress temperature and a moderate response to oxidation.

For etanercept, control samples for the biosimilar and research-grade products were nearly identical, while the originator product displayed a slightly different profile. Thermal stress led to an increase in HMW species, a decrease in the main peak area, and an increase in LMW species for the originator. High-pH treatment further exacerbated these changes. The biosimilar etanercept exhibited more noticeable aggregation under thermal stress compared to the originator, and a similar profile under high-pH stress. In contrast, the research-grade etanercept product showed distinctive behavior, with a significant decrease in the main peak and increased aggregation under thermal stress, while it remained resistant to high-pH stress.

Charge variants

Changes in charge variants are typically among the earliest indications of degradation. The ProteinSimple Maurice instrument was utilized to conduct Imaged Capillary Isoelectric Focusing (icIEF) analysis, following the principles outlined in USP General Chapter <1053> *Capillary Electrophoresis*. The Maurice cIEF System Suitability Kit was used to evaluate system suitability for both adalimumab and etanercept, with USP mAb001 and research-grade etanercept as controls. An in-house method for monoclonal antibodies was tested on adalimumab and was shown to perform well with no method modification. Relative quantitation of main, acidic, and basic peaks was performed. The icIEF separation profiles of adalimumab variants showed similar main pI values at 8.9. Adalimumab research grade material has a lower amount of basic species and a higher main peak relative amount compared to originator and biosimilar products. Thermal stress caused a decrease in the main peak and an increase in acidic peaks for originator and biosimilar, while adalimumab research grade material showed a different response with an increase in the basic group. Oxidation treatment led to a decrease in the main peak and an increase in the acidic group for all samples, but the decrease in the basic group was more severe for originator and biosimilar materials compared to adalimumab research grade material.

Etanercept, required a different method due to its acidic pI and sialylated glycans. Due to the complexity of the charge variant profiles, a peak grouping strategy was used to group peaks into four distinct pI ranges. The etanercept materials tested had different icIEF separation profiles, all with peaks ranging from pI 4 to 8. Originator and biosimilar products showed similar responses to thermal and chemical treatment, with slight changes in peak group 1 and significant changes in peak group 2 and 4 upon chemical stress. Etanercept research grade material showed a significant increase in peak group 2 and decreases in other groups after thermal stress, with little impact from chemical treatment.

Based on the data from Specific Aim 1, we selected 2-week and 6-week time points from thermal degradation for adalimumab and etanercept for analysis in Specific Aim 2 along with the one-day time point for adalimumab oxidative stress and the 7-day alkaline stress for etanercept. It is worth noting that the formulation of the originator, biosimilar, and research grade products differed for both adalimumab and etanercept, so direct comparisons of products are not relevant. The forced degradation achieved the goal of generating a range of molecular variants that can be used to assess the performance of MAM vs conventional methods.

Specific Aim 2: Evaluation of PQAs, including charge variants and glycosylation, using traditional methods

Specific Aim 2.1 Evaluation of charge variants using ion exchange chromatography

An in-house CEX-HPLC method using the Thermo ProPac WCX-10 column, and a salt gradient separated charge variants of adalimumab, with samples stable at 4 °C for 2 days. System suitability was established using USP mAb001 for CEX-HPLC. The profiles of the originator and biosimilar products both before and after thermal degradation were similar. The profile of research grade product was slightly different prior to thermal degradation. For all the thermal stressed adalimumab samples, the percent of acidic isoforms are significantly increased over

time compared to their controls. Percent basic isoforms stayed relatively constant over time for all three materials. For all the oxidation stressed adalimumab samples, the main peak is mostly degraded; significant precipitation was observed for oxidized biosimilar sample, and minor precipitation observed for oxidized originator.

For etanercept, an AEX-HPLC method based on Hassett et al. (2017) was optimized using the Agilent Bio SAX column with a salt gradient. System suitability was established using research grade etanercept. Some changes were observed in the originator and biosimilar products upon thermal stress, but dramatic changes were observed in the research grade material, with an almost complete shift to the acidic isoforms. For alkaline stressed etanercept samples, percent acidic isoforms are significantly increased for originator and biosimilar compared to their controls; there is a slight increase of percent acidic isoforms for research-grade etanercept.

To mitigate precipitation observed with prolonged storage of oxidized adalimumab samples, another experiment was conducted with the inclusion of L-Methionine (300 mM) as quencher in in oxidized adalimumab samples. Although L-Methionine prevented the visible precipitation in the oxidized adalimumab samples, its impact on the CEX profile of these samples was minimal.

Specific Aim 2.2 Evaluation of N-glycosylation and sialic acid content

Adalimumab has a single *N*-glycosylation site on each heavy chain, whereas etanercept has 3 *N*-linked glycosylation sites and multiple *O*-linked glycosylation sites on each monomer. For method evaluation, research grade adalimumab and etanercept were prepared using PNGase digestion followed by labeling using the Waters Glyco-Works RapiFluor MS kit. Labeled glycans were separated by HILIC chromatography using an Acquity Premier BEH Amide column at 60 °C followed by fluorescence detection. USP mAb001 and research-grade etanercept were used to establish System Suitability for adalimumab and etanercept, respectively. As expected, releasing *N*-glycan analysis did not show significant changes of glycan profiles in stressed samples in comparison with control samples. *O*-linked glycans will be determined by MS at the peptide level under Specific Aim 3 due to the absence of robust methods for glycan release.

Sialic acid analysis was performed following USP Chapter <210> using acid hydrolysis and DAB labeling followed by RP-HPLC and fluorescence detection. For adalimumab samples, very low level of sialic acid (< 1 nmol/mg) were detected, and stress conditions did not have an impact on the sialic acid level. For etanercept, much higher level of sialic acid (>100 nm/mg) were detected in all samples. While the pH treatment did not change the sialic level for all three sample sets, thermal stress yielded different effects on the sialic acid levels for the three products. The thermal treatment had no effects on the originator samples, and little impact on biosimilar samples, but a decrease in sialic acid level was observed in the research grade product.

Specific Aim 2.3 Evaluation of size variants using CE-SDS.

The reduced and non-reduced CE-SDS (capillary electrophoresis sodium dodecyl sulfate) methods outlined in USP <129> were used as a starting point for this analysis. The <129> method provided good separation for adalimumab but was modified to include an extended run time for etanercept. Stability of the profile over the run time was also confirmed. For Adalimumab, both reduced and non-reduced CE-SDS showed that thermal stress induced consistent changes in HC (Heavy Chain) and LC (Light Chain) peak areas and led to increased fragmentation over time for all three samples (originator, biosimilar and research grade materials). Under oxidative stress, all three samples similarly showed extensive changes and increased fragmentation with non-reduced CE-SDS. Using reduced CE-SDS, the oxidation induced modifications and fragmentation was similar to that observed under thermal stress.

For Etanercept, both reduced and non-reduced CE-SDS showed increased fragmentation under both thermal and chemical stress (high pH) conditions. All samples exhibited higher susceptivity to thermal stress than chemical stress. Research-grade etanercept displayed higher sensitivity to stress conditions.

Overall, conventional methods showed that products from different sources that were subjected to stress conditions, produced varying degrees of protein degradation, fragmentation, and modifications. It is expected that the degraded materials will therefore provide a broad representation of potential modifications for assessment of the performance of MAM vs conventional methods.

Specific Aim 3: Identification and relative quantitation of modifications using a MAM workflow

Specific Aim 3.1 MAM method development/ refinement

Evaluation and refinement of the final method for MAM analysis of adalimumab and etanercept has been completed. This includes refinement of sample preparation conditions (denaturation/reduction, enzyme selection and incubation time), tuning mass spectrometer parameters, selection of PQAs for MAM monitoring, and method qualification. BioPharma Finder (5.1 processing software) was used for selecting PQAs and generating a workbook of PQAs independently for adalimumab and etanercept. These PQAs included modifications for oxidation, deamidation, succinimidation, glycosylation, glycation, and isomerization; all of which can potentially impact function or stability. A total of approximately 60 PQAs were selected for adalimumab and approximately 120 PQAs were selected for etanercept. In addition to the above selected PQAs, pyroglutamate formation, lysine clipping, and DP clipping were also included for MAM monitoring. PQAs associated with glycosylation were focused on *N*-glycosylation for adalimumab, while analysis of both *O*- and *N*-linked glycosylation were performed for etanercept. Specificity, repeatability, linearity, and robustness were evaluated to qualify the methods for adalimumab and etanercept.

Specific Aim 3.2 Analysis of Samples using the qualified MAM assay

Adalimumab and etanercept samples from the forced degradation study that were analyzed using conventional methods (under Specific Aim 2) have been analyzed using the refined and qualified MAM methods. Data analysis is currently underway.

Specific Aim 4: Assessment of bioactivity and structure analysis of biotherapeutic products and stressed samples

A second forced degradation study and analysis was performed in this phase of the study in a second laboratory to assess reproducibility. While previous analytical assessment of PQAs using conventional methods have shown high reproducibility between labs, MAM can be more difficult to replicate in a second lab due to the potential for sample preparation-induced modifications, especially deamidation and oxidation. This phase will also extend the analysis to compare changes in PTMs to function and structure.

Specific Aim 4.1 Forced degradation and physiochemical characterization

Lab 2 conducted a second forced degradation study using the same procedure as used for Specific Aim 1. Degraded samples were analyzed for visible particles, aggregation by SEC-HPLC, and changes in concentration using SoloVPE that could arise from forced degradation, as described in Specific Aim 1. The forced degradation yielded highly similar changes in profiles as that observed in Lab 1. Based on the thermal degradation results, the same time points selected in Specific Aim 1 (2 weeks and 6 weeks) were selected for subsequent analysis for both adalimumab and etanercept. The same chemical stress conditions were also selected, including the one-day time point for adalimumab oxidative stress and the 7-day alkaline stress for etanercept. Methionine was added to an additional adalimumab sample following oxidative stress to mitigate the precipitation upon storage that was observed in Lab 1.

Analysis of charge variants is in progress using the CEX-HPLC (for adalimumab) and AEX-HPLC (for etanercept) methods described in Specific Aim 2.

The MAM assays for adalimumab and etanercept developed and implemented under Specific Aim 3 have been successfully transferred to Lab 2. The tech transfer included hands-on training and method transfer by an SME from lab 1 to lab 2. MS systems between the two laboratories are similar, with lab 1 having an Orbitrap Exploris 480 MS and lab 2 having the Exploris 240 model. The difference in sensitivity between the two models will allow

for investigation of any minor differences in sensitivity for PQA detection. MAM data has been acquired from all selected adalimumab and etanercept samples and subsequent data analysis is underway.

Specific Aim 4.2 Bioactivity Assessment

To assess the impact of changes induced during forced degradation on the functionality of etanercept and adalimumab, an L929 cell-based TNF-α neutralization assay was developed and qualified. The L929 platform method was developed by optimizing cell number, actinomycin D concentration, detection reagent and volume. Once optimized, accuracy, precision, assay dilutional linearity, and range were tested for adalimumab and etanercept (separately) during qualification. Qualification data met all system suitability and acceptance criteria, and the method was considered qualified for sample analysis. Analysis of forced degradation samples is in progress.

Specific Aim 4.3 Assessment of Fc and Target binding affinity by Surface Plasmon Resonance

To assess the effect on target binding kinetics (adalimumab and etanercept) and Fc binding kinetics (high binding Fcγ RIIIa variant, adalimumab only), SPR-based assays were developed and qualified. For adalimumab and Fcγ RIIIa kinetic binding analysis, ligand (adalimumab) immobilization concentration, analyte concentration range (Fcγ RIIIa), source of ligand (three vendors were tested, and the best one selected based on reproducibility and SST criteria), association time, and disassociation time were optimized. Once optimized, qualification and validation studies were performed. During validation, accuracy, precision, dilutional linearity, range, and specificity of the method were assessed. Data met the set SST and acceptance criteria, and the method was considered suitable for the intended purpose. Similarly, TNFα and adalimumab kinetic binding analysis method was optimized and qualified by assessing the accuracy, precision, linearity, and range. Qualification data passed all set criteria and was considered suitable for intended purpose. Analysis of forced degradation samples is in progress.

---

3. RESEARCH OUTCOMES

Specific Aim 1: Forced degradation of biotherapeutics from multiple sources

Samples of adalimumab and etanercept were acquired from multiple sources, including US FDA approved products, locally approved products from India, and research-grade products. Each biotherapeutic product was subjected to forced degradation using both thermal and chemical stress. Samples were assessed for aggregates using visual inspection and SEC and for charge variants using icIEF and evaluated for changes in concentration. Two thermal stress time points (2 and 6 weeks) and one chemical stress condition were selected for subsequent analysis based on observed changes.

Specific Aim 2: Evaluation of PQAs, including charge variants and glycosylation, using traditional methods

Compendial and in-house methods were tested and refined as needed, and system suitability criteria established. Glycosylation was assessed using HILIC-FLD-MS and sialic acid content. Stress conditions that exhibited significant changes under forced degradation conditions or differences between products were analyzed using industry standard techniques. Charge variants were evaluated using CEX for adalimumab and AEX for etanercept. Size heterogeneity was assessed by CE-SDS (under both reducing and non-educing conditions). Results showed a variety of differences in the molecule associated with source and/or stability, suggesting that the degraded materials have a broad range of molecular variants that can be utilized to compare MAM vs conventional methods.

Specific Aim 3: Identification and relative quantitation of modifications using a MS workflow

MAM methods were developed for both adalimumab and etanercept and system suitability criteria established. Adalimumab and etanercept samples have been analyzed using an MAM characterization workflow to identity and determine relative quantities of post-translational changes that impact PQAs, including for oxidation, deamidation, succinimidation, glycosylation, glycation, and isomerization as well as pyroglutamate formation, lysine clipping and other product clipping. Data analysis is currently underway.

Specific Aim 4: Assess bioactivity of biotherapeutic products and stressed samples

A second forced degradation study was performed in an independent laboratory and showed similar results based on assessment of particulates, aggregation, and concentration compared to lab 1. Based on these results, two thermal stress conditions (2 and 6 weeks) and on chemical stress condition were selected for further assessment. Tech transfer of the MAM method for both adalimumab and etanercept is complete and data collection is complete for both adalimumab and etanercept samples. Assessment for changes in bioactivity, binding affinity, and structure is also underway and will be used to correlate molecular changes to changes in structure and function.

---

4. REGULATORY IMPACT

Biosimilars have the potential to save the health care system billions of dollars and improve patient access by increasing competition and reducing drug costs. Biosimilars are approved through an abbreviated 351(k) Biological License Application (BLA) which aims to establish that the biosimilar product is “highly similar to” the reference product and has no clinically meaningful differences in safety, purity, and potency. Comparative analytical assessment is a key component of the 351(k) BLA pathway for biosimilars and can require more than 15 different analytical tests, with multiple assays often used to assess similar product quality attributes (PQAs).

Over the past few years, MAM has gained traction throughout pharmaceutical development and quality control (QC) labs, due to its ability to improve the efficiency of analytical testing by replacing multiple conventional methods (e.g., peptide mapping, cation exchange chromatography, capillary electrophoresis, glycan analysis, and other methods) and to provide more detailed assessment of product quality attributes (PQAs). More widespread implementation of MAM would advance one of the goals outlined in the Research RoadMap for the BsUFAIII Regulatory Research Pilot Program: improving the efficiency of biosimilar product development.

While replacing multiple release and characterization tests with MAM during comparative analytical assessment provides an opportunity to streamline lab work and decrease development time, several challenges remain. A 2019 publication from FDA staff (Rogstad *et al*, 2019) outlined four aspects that needed to be addressed from a scientific and regulatory perspective prior to implementation of MAM, including risk assessment, method validation, new peak detection, and comparison to conventional methods. This project addresses the performance of MAM vs conventional methods, using adalimumab and etanercept as examples of the broader families of mAb and fusion protein therapeutics. Because collecting data to support bridging from conventional techniques to MAM is a significant investment that can prevent or delay implementation of MAM for assessment of biosimilars, this work will provide a publicly available dataset and a roadmap to inform transitioning to MAM.

The objective of this work is to assess the performance of the MS-based MAM versus conventional QC methods to identify changes in PQAs upon forced degradation and to correlate changes in those PQAs with bioactivity, binding affinity, and structure. Results of this study will help support transitioning from conventional techniques to MAM by creating a knowledge base that can lower the barrier to adoption of MAM and enable wider use of MAM by biosimilar manufacturers.

5. COMMUNICATION AND DISSEMINATION

USP has confirmed speaking slots at the following conferences:

- Bioprocessing Summit - August 19-22 in Boston (Li Jing to present)
    - Title: Building a Roadmap for Implementation of the Multi-Attribute Method in QC
    - This presentation will focus on the comparison of MAM vs conventional methods (portions of Aims 1, 2, and 3) for adalimumab. The presentation will also provide updates on the proposed USP General Chapter <1060> *Mass Spectrometry-Based Multi-Attribute Method for Therapeutic Proteins* and show examples of how principles from the proposed chapter were incorporated into the study.

- CASSS Mass Spec – September 11-13 in Bethesda, MD (Sheila Mugabe to present)
    - Title: Assessment of MAM vs conventional QC methods for PQA evaluation of etanercept
    - This presentation will focus on the comparison of MAM vs conventional methods for etanercept (portions of Aims 1, 2, and 3) and will provide more detail on the approach we used to establish system readiness, following principles described in the proposed USP General Chapter <1060> *Mass Spectrometry-Based Multi-Attribute Method for Therapeutic Proteins*. This presentation may also include learnings from the tech transfer of MAM between labs (Aims 3 and 4).

- Bioprocess International – September 23-26 in Boston (Diane McCarthy to present)
    - Title: Assessment of MAM *vs* conventional QC methods for PQA evaluation of adalimumab and etanercept
    - This presentation will provide an overview of results and lessons learned from the comparison of MAM vs conventional methods for both adalimumab and etanercept. This talk will focus primarily on results from Aim 4 in order to make direct comparisons of the sensitivity of MAM vs conventional methods to detect differences and link those differences to structure and function.

- AAPS 360 – October 20-23 in Salt Lake City
    - Title: A Game-Changing Quality Control Strategy: The Multi-Attribute Method
    - This presentation will provide a high-level overview of the MAM method and how MAM can be used to enhance understanding of the product and process, accelerate development timelines, and refine control strategies. The talk will incorporate elements of proposed USP General Chapter <1060> *Mass Spectrometry-Based Multi-Attribute Method for Therapeutic Proteins* as well as learning from the comparison of MAM vs conventional methods for both adalimumab and etanercept. This talk will leverage results from Aims 1 - 4 in order to assess 1) the sensitivity of MAM vs conventional methods, 2) correlation of physicochemical PQAs to changes in function and structure, and 3) the efficiency of MAM vs conventional methods.

USP also intends to make more detailed data available through webinars and publications once the data collection and analysis is complete. We envision a series of webinars (2-4) to provide a deep dive into the data and learnings. We also anticipate there will be multiple publications due to the volume of data available.

- We expect the first publication to focus on adalimumab as an example of a mAb. We expect this publication would focus primarily on the performance of the MAM method vs conventional methods and how sensitive and specific each method is with respect to monitoring changes that impact function or structure.
- We expect the second publication to cover similar aspects for etanercept as an example of a more complex molecule. This publication would also address some of the challenges associated with more complex molecules that were not encountered with adalimumab, including analysis of O-glycans and sialic acid.

- We anticipate a third publication that would provide best practices and practical advice for establishing system suitability for MAM/conventional methods and on the approach and subsequent results for tech transfer to a second lab.

6. CHALLENGES

Materials and supplies costs were greater than originally budgeted due to the need to purchase additional columns and reagents to support optimization of methods. For example, additional columns, icIEF cartridges, and reagents were needed for optimization of charge variant and MAM analysis of etanercept products, which required different methods compared to adalimumab products. Supply costs in USP India have also increased due to additional supplies needed to support optimization of SPR and bioassay methods for etanercept. Supply costs also increased in lab 2 due to price and shipping cost increases and the need to order specific supplies and reagents to match those used in lab 1. To address this, the budget was re-aligned to ensure no change to the Total Federal Award Amount.

Sample and reagent procurement was delayed in the beginning of the project, resulting in a 3–4-month delay in the start of the forced degradation study under Specific Aim 1. We also encounter an approximate 1 month delay due to mass spectrometer service issues. Additionally, delays in visa processing prevented staff from lab 2 from traveling to lab 1 to facilitate methods knowledge transfer and enable technology transfer. This was addressed by sending staff from lab 1 to lab 2 for a longer period of time to support technology transfer activities on-site, but nonetheless resulted in additional delays.

7. NEXT STEPS

We do not anticipate any major changes to the technical plan from the original proposal. Work under Specific Aims 1 and 2 is complete and the following analyses under Specific Aims 3 and 4 remain to be completed:

Specific Aim 3: Identification and relative quantitation of modifications using a MAM workflow

Data collection is complete and data analysis is currently underway. Once individual samples have been analyzed, MAM results will be compared for each molecule to identify differences in PQAs and stability between various sources and forced degradation conditions for adalimumab and etanercept. MAM results for each sample will also be compared with results from the conventional methods described in Specific Aim 2 to determine the comparability and sensitivity of each method to molecular changes.

Specific Aim 4: Assessment of bioactivity and structure analysis of biotherapeutic products and stressed samples

4.1 Forced degradation and physiochemical characterization

Forced degradation has been completed and samples selected for further analysis. Analysis of charge variants using CEX (for adalimumab) and AEX (for etanercept) is underway. The MAM tech transfer for both adalimumab and etanercept has been completed and data acquisition is complete. Subsequent data analysis will focus on comparison of results of the MAM and conventional methods to results from Specific Aim 2 and 3, and comparison of MAM results vs conventional methods.

4.2 Functional assessment: bioactivity

The methods have been optimized and sample analysis is underway to assess the impact of differences in post-translational modifications between products (innovator, biosimilar, and research-grade) or changes induced during forced degradation on the functionality of the product will be assessed using both bioassay and binding affinity for the target and Fc receptor. The changes in bioactivity will be compared to modifications detected using MAM and conventional techniques to assess their sensitivity in detecting relevant modifications that affect biotherapeutic function.

4.3 Functional assessment: binding affinity

To assess the impact of product source and forced degradation on binding affinity, surface plasmon resonance will be used to assess binding affinity of adalimumab to both FcR and TNFα. Since etanercept Fc binding functionality is not significant, only binding affinity for TNFα will be evaluated. Changes in binding affinity will be compared to modifications detected using MAM or conventional methods to identify specific modifications that may impact function.

4.4 Structural analysis

Circular dichroism (CD) will be used to analyze higher order structure. Measurements in the far ultraviolet CD region will provide information on the secondary structure elements. Conditions used previously for other mAb products will be tested and refined as needed for adalimumab and etanercept. CD spectra will be evaluated by weighted spectral difference to quantitatively compare the spectral differences. The analyzed CD spectra will be compared to changes detected using MAM and conventional biophysical characterization methods.

After completion of the study, we will provide a comparison of MAM vs conventional methods for physiochemical characterization of two different biotherapeutics, a monoclonal antibody (adalimumab) and an Fc fusion protein (etanercept).

---

8. REFERENCES

Hassett, B., Scheinberg, M., Castañeda-Hernández, G., Li, M., Rao, U. R. K., Singh, E., … Fitzpatrick, B. (2017). Variability of intended copies for etanercept (Enbrel®): Data on multiple batches of seven products. *mAbs*, *10*(1), 166–176. https://doi.org/10.1080/19420862.2017.1387346

Rogstad, S., Yan, H., Wang, X., Powers, D., Brorson, K., Damdinsuren, B., & Lee, S. (2019). Multi-Attribute Method for Quality Control of Therapeutic Proteins. *Analytical chemistry*, *91*(22), 14170–14177. https://doi.org/10.1021/acs.analchem.9b03808

---

9. APPENDIX A: ADDITIONAL MATERIAL

10. APPENDIX B: ABBREVIATIONS

This section includes all acronyms used in this document along with a corresponding definition.

ABBREVIATION	DEFINITION
AEX	Anion exchange chromatography
BLA	Biologics License Application
BsUFA	Biosimilar User Fee Act
CD	Circular dichroism
CE-SDS	Capillary electrophoresis – sodium dodecyl sulfate
CEX	Cation exchange chromatography
HC	Heavy chain
HILIC	Hydrophilic interaction chromatography
HMW	High molecular weight
HPLC	High performance liquid chromatography
icIEF	Imaged capillary isoelectric focusing
LC	Light chain
LMW	Low molecular weight
mAb	Monoclonal antibody
MAM	Multi- attribute method
MS	Mass spectrometry
PNGase	Peptide - N -Glycosidase
PQA	Product quality attribute
PTM	Post-translational modification
QC	Quality control
SAX	Strong anion exchange
SEC	Size exclusion chromatography
SPR	Surface plasmon resonance
SST	System suitability
TNF	Tumor necrosis factor
USP	United States Pharmacopeia
WCX	Weak cation exchange