Award 1U01FD007762-01: Assessment of the performance of MAM vs conventional QC methods for evaluation of Product Quality Attributes of adalimumab and etanercept

Interim Report

(progress as of June 30, 2023)

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 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.

B.1 What are the major goals of the project?

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. The project includes forced degradation studies of adalimumab and etanercept from multiple sources (Specific Aim 1), assessment of PQAs using conventional methods (Specific Aim 2), comparison of specific molecular modifications detected by MS-based MAM (Specific Aim 3), and correlation of significant PQA differences with bioactivity and structural changes (Specific Aim 4).

B.2 What was accomplished under these goals?

Specific Aim 1: Forced degradation of biotherapeutics from multiple sources

Three samples each of adalimumab and etanercept were obtained, including innovator 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 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. Degraded samples and controls were evaluated for particulate formation, changes in concentration, charge variants, and aggregation as measures of degradation.

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 which demonstrated changes in visual appearance was the biosimilar etanercept product that was subjected to oxidation, which appeared white (milky) but without any visual precipitate. No significant changes in concentration were observed due to thermal or chemical degradation based on UV 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. The impact of dilution was also assessed and a slight difference (< 0.1%) was observed between adalimumab at 50 mg/ml vs 10 mg/ml. No difference was observed in the profile of etanercept upon dilution. Based on these results, the SEC-HPLC method from <129> will be applied to undiluted samples for assessment of aggregation.

A total of 24 samples of adalimumab and 21 samples of etanercept from the forced degradation study have been analyzed by SEC-HPLC. Results showed that the adalimumab samples from three different sources exhibited remarkable similarity in terms of aggregate levels and molecular weight distributions. Under thermal stress, we observed an increase in both high molecular weight (HMW) and low molecular weight (LMW) species. Adalimumab samples subjected to oxidation showed a significant increase in aggregates in all sources, with some differences in the apparent size and percentage of aggregates. Comparison of untreated etanercept from three dissimilar sources showed slight differences among the sources, with the originator and biosimilar exhibiting closer similarity than the research-grade material. Upon thermal degradation, differences were observed between all three sources. The biosimilar product showed slightly higher levels of aggregates compared to the originator, whereas the research-grade material showed significantly higher levels of HMW species. Similarly, high pH treatment for etanercept resulted in increased HMW species and a slight increase in LMW species. Final analysis and review of quantitative data is in progress.

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*. An in-house method previously used for analysis of monoclonal antibodies was evaluated for adalimumab. While several additional conditions were tested, they did not provide improved resolution or significant differences in charge or purity. Etanercept has an acidic pI and requires development of a different method than adalimumab. A platform method recommended by the vendor was further modified based on published reports and in-house experience. The profile of etanercept is very complex due to the presence of sialylated glycans. To assess the ability of this method to detect changes upon degradation, research grade etanercept was degraded for 3 days at 50 °C. Comparison of degraded and control samples showed clear differences in the icIEF profile, despite its complexity. Desialylation was also evaluated as an option. While desialylation did simply the profile, it was not required to see differences. Finally, a strategy for grouping major peaks into acidic, main, and basic was evaluated and the grouping strategy was shown to produce consistent results across a 16-hour run and between preparations. Based on these results, we decided to perform icIEF without desialylation. The Maurice cIEF System Suitability Kit, comprised of a lyophilized peptide panel and system suitability test mix, was utilized to evaluate system suitability for both adalimumab and etanercept. USP mAb001 and research grade etanercept will also be run as controls and compared to historical data.

A total of 24 samples of adalimumab and 21 samples of etanercept from the forced degradation study have been analyzed using icIEF. The icIEF profiles of adalimumab samples obtained from diverse sources were similar and changes upon thermal stress were similar. However, the three adalimumab products showed different profiles upon chemical stress. For etanercept, the profiles were similar for all three sources, but distinct differences were observed upon thermal and chemical degradation. Final analysis and review of quantitative data is in progress.

Once the quantitative analysis is complete, we will select several time points from thermal degradation and one time point for oxidative and alkaline stress conditions for analysis in Specific Aim 2.

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

In parallel with Aim 1, we have evaluated methods for most analyses under Aim 2. Once Aim 1 is complete, we will select samples for analysis in Aim 2 to assess glycosylation, size and charge variants using conventional techniques to detect differences in the PQAs.

Charge variants

An in-house method for cation exchange chromatography (CEX-HPLC) was evaluated using control and degraded adalimumab samples (60 °C for 3 days). The in-house CEX-HPLC method demonstrated separation of the charge variants of adalimumab. The samples were stable when kept in autosampler at 4 °C for 2 days. USP mAb001 will be used to establish system suitability for this method.

For etanercept, an anion exchange (AEX) HPLC method was required for the separation of charge variants. A published AEX method (Hassett *et al.* MAbs, 2018), was evaluated for etanercept analysis. Multiple columns, mobile phase compositions, gradient slopes, and run times were evaluated and a final method selected using the Agilent Bio SAX Anion Exchange column. The results of charge variants profiles for originator and biosimilar etanercept products were similar compared to the published data. A thermally stressed sample (60 °C for up to 6 days) was also evaluated, and the method demonstrated sensitivity to detect changes in the charge variant profile of degraded etanercept samples. The samples were stable when kept in autosampler at 4 °C for 2 days. Research grade etanercept will be used to establish system suitability for this method by comparing it to historical data.

Size variants

The CE-SDS method in USP <129> was evaluated for analysis of adalimumab and etanercept. The method provided good separation for both adalimumab and etanercept. Stability of the profile over the run time was also evaluated. Adalimumab exhibited a slight decrease in the main peak area under non-reducing conditions after 20 hours. Therefore, the run time will be limited under this condition.

Because the etanercept peak was very broad due to the heterogeneity of the glycans, desialylation and deglycosylation were also evaluated. While deglycosylation yielded a sharper peak, desialylation narrowed the peak slightly. To avoid masking changes due to sample treatments, we will use untreated samples for primary CE-SDS analysis.

Glycosylation

Adalimumab has a single N-glycosylation site, whereas etanercept has 3 N-linked glycosylation sites and multiple O-linked glycosylation sites. For method evaluation, research grade adalimumab and etanercept were prepared using the Waters Glyco-Works RapiFluor MS kit. The vendor recommended column temperature of 60 °C was found to be suitable for analysis of adalimumab. For etanercept, which has a more complex glycan profile, results were compared at two different column temperatures. The results showed only minor changes to retention time and relative abundance. However, more differences were observed in resolution, where some glycan peaks merged at 40 °C, and there were also some low-level glycans that separated out distinctly. Based on the results, the column temperature for etanercept will be maintained at 60 °C. USP mAb001 and research-grade etanercept will be used to establish System Suitability for adalimumab and etanercept, respectively. O-linked glycans are typically measured by MS at

the peptide level due to the absence of robust methods for glycan release, therefore O-glycans will be measured under Specific Aim 3.

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

Evaluation of the MAM method is underway for adalimumab. Initial evaluation was performed using two temperatures for denaturation and reduction (RT and 37 C), two digestion times (30 and 60 min) and two different trypsin grades. The ion source temperature was also varied. Initial data analysis showed high sequence coverage under all conditions. Additional analysis to identify and quantitate modification is underway.

B.6 What do you plan to do during the next reporting period to accomplish the goals?

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

Selected samples from forced degradation will be analyzed for charge variants, size variants, and glycosylation. Methods have been developed and system suitability criteria established for AEX (adalimumab), CEX (etanercept), CE-SDS, and glycan analysis. The only method remaining to be evaluated is sialic acid analysis, which will be performed according to USP <210> *Monosaccharide Analysis*. While evaluation will be performed to ensure suitability, we expect the method described in <210> to be applicable to both adalimumab and etanercept without modifications.

For each of the methods used in Specific Aim 2, results from degraded samples will be compared with control samples and differences between mAb sources will be evaluated.

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

Evaluation and refinement of a final method for MAM analysis of adalimumab has been initiated and data analysis is underway. Similar evaluation and refinement of the method for etanercept will also be performed. Once the method(s) have been refined, the same samples analyzed in Specific Aim 2 will be analyzed using MAM. PQA selection will be focused on modifications that have been shown to impact function or stability (e.g., glycosylation, deamidation, succinimidation, isomerization, oxidation, and glycation). Glycosylation analysis will include N-linked glycans on all samples and O-linked glycans for etanercept samples. Other modifications like pyroglutamate formation and lysine clipping will also be included in the analysis.

The MAM results will be compared for each molecule to identify differences in PQAs and stability between various sources of adalimumab and etanercept. MAM results for each sample will also be compared with results from the conventional methods described in 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

This phase of the study will evaluate reproducibility of the MAM method in a second laboratory and will extend the analysis to compare changes in Post-Translational Modifications (PTMs) to function and structure. We will focus on the conditions that showed the greatest differences in PTMs in our previous aims.

Forced degradation and physiochemical characterization

This lab will conduct a second forced degradation study and repeat analyses using conventional and MAM methods in a second lab to assess reproducibility. Degraded samples will be analyzed for visible particles, aggregation, and changes in concentration that could arise from forced degradation, as described in Specific Aim 1. Typically, the earliest and most dramatic changes associated with degradation are observed in charge variants, so CEX-HPLC (for adalimumab) and AEX-HPLC (for etanercept) analysis of charge variants will be performed as described in Specific Aim 2. MAM analysis will also be conducted using the methods developed and implemented in Specific Aim 3.

Functional assessment: bioactivity and binding affinity

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. For bioassay, the biological activity of samples will be evaluated using a well-established L929 cell-based assay. Functional assays will be performed on selected samples from the forced degradation study, using international standards of etanercept and adalimumab as reference standards. 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. Differences in PTMs between products (innovator, biosimilar, and research-grade) or changes induced during forced degradation can impact bioactivity.

The biological activity of a monoclonal antibody or a Fc fusion protein is especially dependent on those modifications that impact Fc and/or target binding. 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.

Structural analysis

Lastly, we will assess the impact of modifications induced by forced degradation and of differences between sources on the structure of the molecule using circular dichroism (CD) to analyze higher order structure. Measurements in the far ultraviolet CD region will provide information on the secondary structure elements such as alpha helices and beta-strands, which form the core of three-dimensional structure formation of all the biomolecules. Each of the structural elements has a unique CD signal appearing at a particular wavelength in the UV spectrum. In our previous studies, structurally different mAb molecules like rituximab, bevacizumab, and a non-commercialized mAb were investigated to capture secondary structure differences. These conditions will be optimized for adalimumab and etanercept until identity of secondary structural features is clarified. CD spectra will be evaluated by weighted spectral difference or area overlap methods to quantitatively compare the spectral differences. Lysozyme, whose

structure has been extensively studied and is available in protein data bank, will be used for establishing system suitability. 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).