U.S. FOOD & DRUG ADMINISTRATION

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

ANNUAL REPORT

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Contents

Report Overview[1]

Table 1: High-level overview of the project objective, aim(s) progress, outcomes, and timelines for communication and regulatory impact (1-2 sentence max per table cell).

Project Title:	**One-Pot Glycan - A chemoenzymatic method for simultaneous profiling and comparison of N- and O-glycans**
Investigator:	Tongzhong Ju
Organization:	FDA/CDER/OPQ/OPQR/ DPQRIII
Grant No. (if applicable)	N/A
Project Objective:	Establish and validate a chemoenzymatic method for simultaneous profiling and comparison N-and O-glycans from purified proteins and protein drugs in a One-Pot format

Specific Aim(s)	Progress	Outcomes	Communication Timeline
Aim 1: Establish a method for simultaneous profiling N-and O-glycans from purified proteins and cellular proteins in a One-Pot format.	100%	Established a method for simultaneous profiling N- and O-glycans from purified proteins in a One-Pot format. 1. A one-pot glycomic method in a single workflow has been established. 2. The one-pot method simultaneously profiles N- and O-glycans from glycoprotein drugs. 3. The one-pot method measures relative abundances of permethylated N- and O-glycans, including sialylated and PK-relevant glycan determinants.	May 1, 2023 – Sep. 30, 2024 1. *First OPQ ORISE Seminar (presentation)*: A chemoenzymatic method for simultaneous profiling of N and O-glycans in one-pot 2. *FY2023 FDA Science Forum* (June 13-14, 2023) (*Abstract & Poster*): A chemoenzymatic method for simultaneous profiling of N and O-glycans in one-pot 3. *Video Demo*: The MS&I CoE Communication Working Group presented a virtual demonstration of "a new mass spectrometry method to simultaneously profile N- and O-linked glycans from protein therapeutics" and followed by an expert panel discussion on June 20th, 2024 4. *Publication*: Ortega-Rodriguez, U., … and Ju, T. (2024) A chemoenzymatic method for simultaneous profiling N- and O-glycans on glycoproteins using a one-pot format. Cell Reports Methods, 2024, Vol 4, 100834.

[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
Aim 2: Optimize and validate the method using therapeutic proteins, and biosimilars with both N- and O-glycans	Ongoing. 60%	1. The one-pot method's reproducibility and intermediate precision using protein drugs have been demonstrated. 2. Advancement of the one-pot glycomic method is ongoing.	Oct. 1, 2024 – Sep. 30, 2025 1. Establish a standard operating procedure (SOP). 2. Publish a STAR Protocol. 3. Made a Spotlight Presentation at the OPQR All-hands Meeting, May 21, 2025.

Progress Summary

Glycosylation is generally characterized and controlled as a critical quality attribute for therapeutic glycoproteins, including biosimilars, because glycans can impact protein drug-product efficacy, half-life, stability, and safety. Analytical procedures to characterize N-glycans are relatively well established, but the characterization of O-glycans is challenging due to complex workflows and a lack of enzymatic tools. Here, we establish a simplified chemoenzymatic method to simultaneously profile N- and O-glycans from the same sample using a one-pot format by mass spectrometry (MS). N-glycans were first released by PNGase F, followed by O-glycopeptide generation by proteinase K, selective N-glycan reduction, and O-glycan release by β-elimination during permethylation of both N- and O-glycans. Glycan structural assignments and determination of the N- to O-glycan ratio were obtained from the one-pot mass spectra. The streamlined, one-pot method is a reliable approach that will facilitate advanced characterizations for biosimilars to assess their similarity to reference products regarding glycosylation. The method also ensures lot-to-lot consistency in glycosylation of glycoprotein drugs, including biosimilars.

The One-pot glycomic method for therapeutic proteins, including biosimilars has been established, and partly validated. The work has been published and communicated with the Assessors through the Method Demo organized by the OPQ MS&I CoE.

As the current procedure takes 4~5 days to complete, which is one of the major limitations, further optimization to shorten the procedure is ongoing. Furthermore, method advancement, adapting user-friendly steps and adding an internal standard for better quantification of glycan species in biosimilars is also underway. The advanced One-pot glycomic method will be validated with analysis of glycans on biosimilars.

Project Objective:

Establish and validate a method for simultaneous profiling and comparison of N-and O-glycans from purified proteins and glycoprotein drugs in One-Pot format.

Aim 1

Establish a method for simultaneous profiling N- and O-glycans from purified glycoproteins in a One-Pot format. This aim has been completed, and a manuscript has been published in *Cell reports Methods*.

Aim 2

Optimize and validate the method using therapeutic proteins, and etanercept biosimilars with both N- and O-glycans. A part of validation in aim 2, ~60% has been completed, and the rest is ongoing. The completed part includes the demonstration of method reproducibility and intermediate precision. We are also advancing the method by shortening the procedure time, adapting more user-friendly steps, and improving the quantification by including an internal standard(s) with available biosimilars.

Research Outcomes

The research outcomes include:

1. A one-pot glycomic method in a single workflow has been established.
2. The one-pot glycomic method simultaneously profiles N- and O-glycans from glycoprotein drugs.
3. The one-pot glycomic method measures relative abundances of permethylated N- and O-glycans, including sialylated and PK-relevant glycan determinants for glycoprotein drugs, including biosimilars.
4. The one-pot glycomic method reports the N- to O-glycan ratios in glycoproteins including biosimilars.
5. The one-pot glycomic method demonstrates its reproducibility and intermediate precision.

Regulatory Impact

We developed a one-pot glycomic method for simultaneous analysis of N- and-O-glycans from the same sample, and in a single workflow to facilitate the comprehensive assessment of glycosylation during the biomanufacture and release of protein-based drug products, which represents a significant advancement in the analytical assessment of N- and O-glycosylation of glycoprotein therapeutics. The regulatory impacts include:

For industry:

1. The available one-pot method will promote the development of biosimilar programs by facilitating the production cell line development to identify and develop the cell clones whose products have similar glycosylation profiles: N- and O-glycan ratio, levels of sialylation and PK-relevant glycan determinants.
2. The one-pot method can be utilized for characterization and/or release testing of the glycosylation of therapeutic proteins/biosimilars to assess the similarity of biosimilars to reference products and ensure the lot-to-lot consistency in glycosylation.
3. Advancement of the method will increase its applicability and usability in characterization and release testing of therapeutic proteins including biosimilars in industry.

For regulatory agency:

1. Structure-function assessments and comparisons related to O- and N-glycans of biosimilar products will be easier for Assessors to perform and correlate with other quality and safety attributes.
2. The data from the one-pot glycomic method can greatly assist the quality Assessors in assessing:
    a. The quality attributes of glycosylation in glycoprotein products: efficacy, PK or PD and safety;

b. The lot-to-lot comparability in glycosylation of the products/biosimilars; and

c. Glycosylation similarity of a biosimilar to their reference product.

Communication and Dissemination

Table 2: Summary of communications and dissemination of information, results, outcomes, etc. related to this study.

Title	Type of Communication (e.g., poster, manuscript, presentation)	Source	Link (if available)
A chemoenzymatic method for simultaneous profiling N- and O-glycans on glycoproteins using a one-pot format	News	OPQR News, Issue 4, March 2025	N/A
A Chemoenzymatic Method for simultaneous Profiling of N- and O-glycans in One-pot Format	Presentation	OPQR All-Hands Meeting, April 22, 2025	N/A
A chemoenzymatic method for simultaneous profiling of N- and O-glycans on glycoprotein drugs in one-pot format	Poster	OPQR Poster Day, June 17, 2025	N/A
A Chemoenzymatic Method for Simultaneous profiling of N and O-glycans in one-pot	Poster	FDA Public Meeting--BsUFA III Regulatory Science Program Interim Public Meeting, Sept. 18, 2025	N/A

March 1, 2023 – September 30, 2024

1. First OPQ ORISE Seminar, March 24, 2023 (Presentation): A chemoenzymatic method for simultaneous profiling of N and O-glycans in one-pot.
2. FY2023 FDA Science Forum, June 13-14, 2023 (Abstract & Poster): A chemoenzymatic method for simultaneous profiling of N and O-glycans in one-pot.
3. Video Demo: The MS&I CoE Communication Working Group presented a virtual demonstration of “a new mass spectrometry method to simultaneously profile N- and O-

linked glycans from protein therapeutics" and followed by an expert panel discussion on June 20th, 2024.

4. Publication: Ortega-Rodriguez, U., Bettinger, J.B., Zou, G., Falkowski, V.M., Lehtimaki, M., Matthews A.M., Biel, T.G., Pritts J.T., Wu W., Shen, R-F., Agarabi, C., Rao. V. A., Xie, H., and Ju, T., (2024) A chemoenzymatic method for simultaneous profiling N- and O-glycans on glycoproteins using a one-pot format. Cell Reports Methods, 2024, Vol 4, 100834, https://doi.org/10.1016/j.crmeth.2024.100834

October 1, 2024 – September 30, 2025

1. Establish a standard operating procedure (SOP)
2. Publish a STAR Protocol

Scientific and Technical Challenges

No scientific or technical challenges were reported for this past year.

Next Steps

1. Continue advancing and validating the one-pot glycomic method using biosimilars with N- and O-linked glycans.
2. Establish a Standard Operating Procedure (SOP) for the one-pot method.
3. Publish the method in *STAR PROTOCOLS*.

References

Ortega-Rodriguez, *et al.* A chemoenzymatic method for simultaneous profiling N- and O-glycans on glycoproteins using one-pot format, *Cell Reports Methods*, 2024, Vol 4, 100834, https://doi.org/10.1016/j.crmeth.2024.100834