A rapid HPLC-based method for a comparative assessment of biosimilar structural heterogeneity and biological activity

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Introduction

Glycosylation is a post-translational modification (PTM) of the crystalized fragment (Fc) of monoclonal antibodies (mAbs) or immunoglobulin (IgG) sub-classes. The variety of glycosylation profiles feasible on the Fc region is costly to identify. The TSKgel FcR-IIIA-NPR (FcR) Column is a novel affinity column that exploits the affinity of different glycan profiles of the FcγRIIIa receptor purported for use in mAb glycosylation characterization[1].

The FcR column has a nonporous stationary phase with a modified recombinant non-glycosylated FcγRIIIa ligand that will bind to the FcγRIIIa binding site found on the N-linked glycosylation of the Fc region with a higher affinity for the greater amount of terminal galactose units[2].

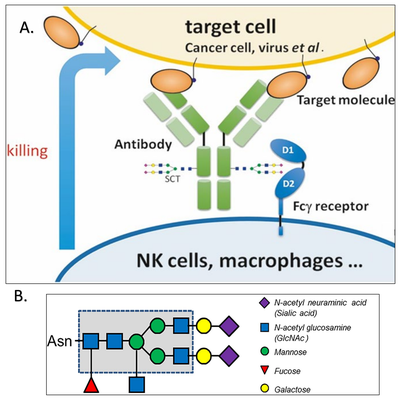


Figure 1: A. ADCC schematic[3]. Antibody equates to drug product throughout this research while the Fcγ receptor equates to the ligand immobilized on the FcR column. The antibody is labeled with asparagine 297 "N-Glycans" on the Fc region. B. Structure of N-Glycans with grey box specifying core structure and possible structure variation components[4]. These modifications to the core Fc glycans comprise ~90% of human IgG1 Fc glycoforms.

Glycosylation is a well-defined critical quality attribute (CQA) with significant impact on the Fc effector mediated antibody-dependent cellular cytotoxicity (ADCC) mechanism of action (MoA) for many mAb drug products. CQAs are commonly evaluated using forced degradation studies to identify degradation pathways when assessing product stability[5]. Biosimilars need to establish that they have similar stability profiles to their reference product[6].

We present research that interrogates the feasibility of the FcR HPLC affinity column to identify differences in glycosylation PTMs between proposed biosimilars and their reference products as part of the comparative analytical data required in biosimilar development[7].

Materials and Methods

Using an Agilent HPLC system:

- Mobile phase A (MPA): 20mM ammonium acetate, 400 mM sodium chloride, pH 6.5 in water
- Mobile phase B (MPB): 50mM ammonium acetate, 400 mM sodium chloride, pH 4.5 in water
- 1 mL/min for 25 minutes, 5 minutes post run
  - 1 min 0% MPB – 15 min 100% MPB

Drug products used are outlined in Table 1.

Drug Products

Control: NIST mAb			
Remicade	Infliximab	Rituxan	Rituximab
Inflectra	Infliximab- dyyb	Truxima	Rituximab- abbs
Renflexis	Infliximab- adba	Ruxience	Rituximab- pvvr
Avsola	Infliximab- axxq	Riabni	Rituximab- arrx

Table 1. Drug products included in this study with the reference product highlighted in grey, and the respective biosimilars following below . All drug products (250 µL) and control (25 µL) were at a concentration of 10 mg/mL with 5 µL injection volume for a total of 50 mg being injected into the HPLC system.

Forced Degradation Studies

Temperature Stability

- Freeze/ Thaw Cycling -80°C / 2 hrs @ RT, 5x, 24 hrs cycles
- Dry Heat 60°C, 70°C, 80°C 2 Hours

Physical Stress

- Agitation Shaker, 24 hrs @ 300 rpm

pH Stability

- Acidic pH Adjustment w/ HCl or H_3PO_4 pH 5, pH 3, pH 2

Oxidative

- 30% Hydrogen Peroxide 1%, 5%, 10% H_2O_2 by volume

Figure 2. Method outline for forced degradation conditions applied to both control and drug products outlined in Figure 1. These conditions were previously reported to affect glycans[6] with freeze/thaw conditions with the least impact on overall N-glycan profile. Freeze/Thaw and agitation did not show significant difference from the unstressed, and will not be covered throughout the rest of this poster.

Peak area was determined using Agilent ChemStation CDS data analysis to integrate and quantify the flow through, low affinity, mid affinity, and high affinity peaks specified in Figure 3.

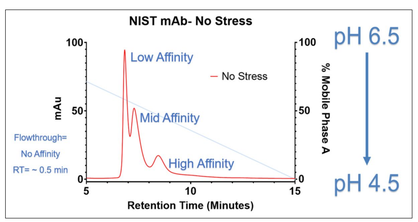


Figure 3. Example chromatogram of well characterized NIST mAb glycan profile resulting from the TSKgel FcR-IIIA-NPR HPLC column with retention time (x-axis) from minute 5 to 15, absorbance (y-axis, left), and percent mobiles phase A (y-axis, right) including labels for the flowthrough, low affinity, mid affinity, and high affinity peaks.

Drug products were monitored for each stress condition for precipitation. If precipitation occurred, the sample was briefly centrifuged, and the supernatant was used for testing.
Columns were monitored for integrity throughout the study with unstressed NIST mAb bookending HPLC sequences. H2O2 caused column degradation. Once confirm (data not shown), this stress condition was no longer expanded.

Results and Discussion

Unstressed Control

- Similarity in peak pattern is seen between the reference products and their respective biosimilars included in this study.
- Between the two drug product groups, differences in overall peak profile shape and intensity is seen in Figure 4.

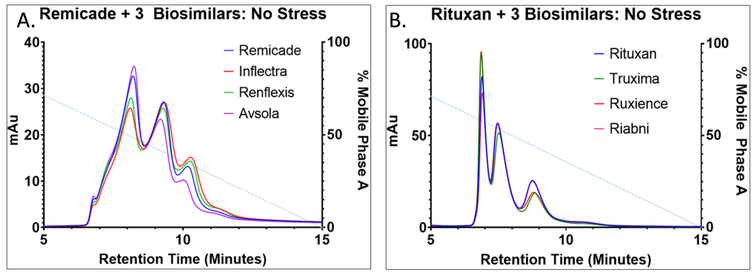
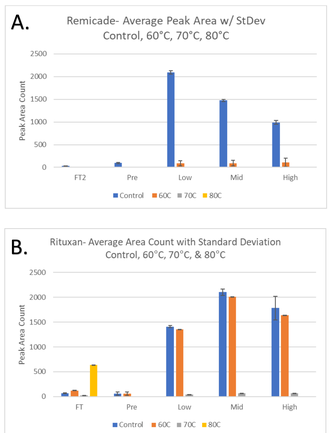


Figure 4. Glycan peak profile resulting from the FcR Column method. Descending light blue line indicates the mobile phase gradient using the right y-axis. A. Unstressed Remicade and its three biosimilars (Inflectra, Renflexis, Avsola). B Unstressed Rituxan and its three biosimilars (Truxima, Ruxience, Riabni). C. Average peak percentage for unstressed drug products with reference product highlighted in blue, including total peak area.

C.

	Average Peak Percentage (%)					Total Peak Area
	Flowthrough	Pre	Low	Mid	High	
Remicade	0.6 ± 0.1	2.0 ± 0.3	44.7 ± 0.5	31.5 ± 0.2	21.1 ± 0.9	4690 ± 60
Inflectra	1.1 ± 0.1	1.6 ± 0.2	39.3 ± 1.8	34.5 ± 0.4	23.5 ± 1.6	4648 ± 100
Renflexis	1.0 ± 0.3	1.6 ± 0.2	36.4 ± 1.3	34.9 ± 1.0	26.1 ± 0.6	4558 ± 70
Avsola	2.0 ± 0.6	2.2 ± 0.2	46.7 ± 1.3	30.7 ± 1.5	18.4 ± 0.4	4260 ± 53
Rituxan	1.2 ± 0.3	1.0 ± 0.7	26.1 ± 2.0	38.8 ± 1.1	32.8 ± 2.7	5420 ± 315
Truxima	2.3 ± 0.4	1.4 ± 0.8	33.2 ± 2.1	37.8 ± 1.0	25.3 ± 2.6	5128 ± 282
Ruxience	3.2 ± 0.4	1.3 ± 0.8	30.9 ± 1.6	38.8 ± 1.6	25.9 ± 2.0	5546 ± 383
Riabni	1.6 ± 0.3	1.0 ± 6	25.4 ± 1.2	39.9 ± 1.0	32.1 ± 1.8	5279 ± 302

Temperature Stability: Dry Heat



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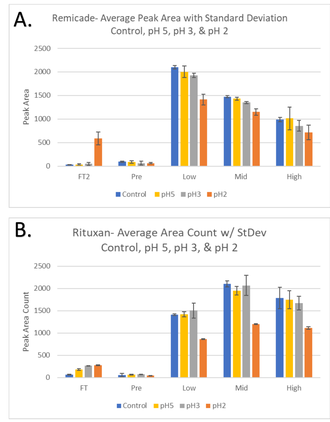
	Delta Average Peak Percentage					Total Peak Area
	Flow through	Pre	Low	Mid	High	
Stress Condition: 60 °C						
Remicade	-3.4	1.6	12.2	-1.3	-9.0	294 ± 219
Inflectra	-2.3	1.0	14.3	2.1	-15.3	306 ± 211
Renflexis	-0.4	1.1	11.9	2.7	-15.2	654 ± 462
Avsola	-1.8	1.1	12.3	-0.1	-11.9	807 ± 779
Rituxan	-1.1	0.0	0.0	0.0	1.2	5237 ± 169
Truxima	-1.6	0.2	0.4	-0.9	2.0	4853 ± 93
Ruxience	0.3	-0.3	1.4	-0.7	0.6	5267 ± 237
Riabni	-1.3	0.0	-2.4	0.0	3.7	4899 ± 80
Stress Condition: 70 °C						
Remicade	-37.6	2.0	12.3	8.7	14.6	12 ± 1
Inflectra	-24.3	1.6	11.4	1.2	10.1	20 ± 1
Renflexis	-99.0	1.6	36.4	34.9	26.1	3 ± 0
Avsola	-95.0	2.2	43.7	30.7	18.4	5 ± 0
Rituxan	-10.9	1.0	3.7	5.1	1.0	200 ± 5
Truxima	-14.3	1.8	7.8	6.3	-1.2	146 ± 1
Ruxience	-52.7	1.7	16.5	20.7	14.3	2388 ± 13
Riabni	-10.4	1.4	4.3	6.0	-0.9	194 ± 3
Stress Condition: 80 °C						
Remicade	-27.5	2.0	-27.2	31.5	21.1	22 ± 6
Inflectra	-66.4	1.6	6.8	34.5	23.5	10 ± 1
Renflexis	-89.4	1.6	26.7	34.9	26.1	9 ± 0
Avsola	-98.0	2.2	46.7	30.7	18.4	4 ± 1
Rituxan	-98.8	1.0	26.1	38.8	32.8	23 ± 0
Truxima	-97.7	1.8	33.2	37.8	25.3	27 ± 2
Ruxience	-96.8	1.7	30.9	38.8	25.9	115 ± 2
Riabni	-98.4	1.4	25.4	39.9	32.1	23 ± 0

Figure 5. A. Average peak area with standard deviation for 60°C (n=4), 70°C (n=1), and 80°C (n=1) and unstressed control for Remicade. B. Average peak area with standard deviation for 60°C (n=4), 70°C (n=1), and 80°C (n=1) and unstressed control for Rituxan. C. Difference between peak percentages of the unstressed drug products (values outlined in Figure 4.C) and stress conditions of 60°C, 70°C, and 80°C including total peak area.

pH Stability



C.

	Delta Average Peak Percentage					Total Peak Area
	Flow through	Pre	Low	Mid	High	
Stress Condition: pH 5						
Remicade	-0.2	-0.3	-1.1	0.1	1.5	4573 ± 151
Inflectra	-0.1	-0.3	-1.8	0.4	1.8	4625 ± 215
Renflexis	0.0	-0.1	-0.3	-0.9	1.3	4532 ± 98
Avsola	0.2	-0.2	0.7	-0.4	-0.5	4223 ± 121
Rituxan	-2.2	-0.2	-1.4	1.1	-1.0	5364 ± 377
Truxima	-2.3	0.4	-0.1	0.6	1.8	5625 ± 1020
Ruxience	0.3	0.4	-0.7	0.0	0.4	5259 ± 221
Riabni	-1.4	0.3	-1.4	-0.5	3.4	6060 ± 1400
Stress Condition: pH 3						
Remicade	-0.3	-0.1	-0.9	-0.6	1.8	4248 ± 158
Inflectra	-0.4	-0.1	-1.0	0.1	1.4	4414 ± 151
Renflexis	-1.0	0.1	1.1	-0.6	0.5	4288 ± 119
Avsola	0.0	-0.1	0.8	-0.6	-0.1	3972 ± 98
Rituxan	-3.5	-0.3	-0.9	1.8	2.9	5574 ± 553
Truxima	-5.9	0.3	2.7	2.9	0.4	4245 ± 431
Ruxience	-1.6	0.3	2.0	1.5	-1.8	4883 ± 422
Riabni	-5.1	0.2	0.9	2.7	1.6	5093 ± 1660
Stress Condition: pH 2						
Remicade	-15.6	0.2	8.4	2.2	4.8	3932 ± 156
Inflectra	-12.0	0.0	5.4	2.4	4.2	3958 ± 124
Renflexis	-68.4	0.8	26.3	23.0	18.3	2529 ± 266
Avsola	-9.4	0.0	5.4	1.3	2.6	3726 ± 143
Rituxan	-6.7	-0.2	1.5	4.6	0.9	3496 ± 49
Truxima	-11.9	0.0	5.6	6.4	-0.2	3754 ± 841
Ruxience	-96.8	1.3	30.9	38.8	25.9	3358 ± 390
Riabni	-16.2	0.0	4.9	8.4	3.2	4004 ± 93

Figure 6. A. Average peak area with standard deviation for pH stress conditions (n=4) and unstressed control for Remicade. B. Average peak area with standard deviation for pH stress conditions (n=4) and unstressed control for Rituxan. C. Difference between peak percentage of the unstressed drug products (values outlined in Figure 4.C) and stress conditions of pH 5, pH 3, and pH 2, including total peak area.

Conclusion

Freeze/ thaw cycling and agitation did not show a significant difference compared to the unstressed control. This was expected, however, as it is known that these conditions minimally impact mAb glycan profiles. Hydrogen peroxide degraded the column rapidly thus oxidative stress was not expanded beyond n=4. Dry heat and acidic pH adjustment resulted in observable changes to drug products' glycan peak profile that will be further elucidated.

Initial studies of the TSKgel FcR-IIIA-NPR Column conclude that the column is able to identify:

- **Differences between a reference product and their biosimilars**
- **Changes induced by degradation in glycan peak profile**

Future work for the TSKgel FcR-IIIA-NPR column:

- Sample size expansion across multiple columns
- Identify specific glycan profile within each peak area and the specific glycan species contributing to peak profile differences in degradation conditions
- Link Fc effector activity to the resulting glycan profile identified within the chromatogram peak patterns

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The opinions discussed in this presentation are those of the authors and do not necessarily reflect FDA policy.

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