USP Reference Standard Monoclonal Antibodies: Tools to Verify Glycan Structure

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Abstract: The glycan profile is a critical quality attribute for pharmaceutical monoclonal antibodies due to the potential physiological impact of the glycan composition when used as a drug product. Monoclonal antibody reference standards are useful as system suitability samples for glycan profile testing. The development of future glycan profiling techniques could be better evaluated by testing well-characterized reference standards. The USP has introduced monoclonal antibody reference standards (i.e., USP mAb 001 RS, USP mAb 002 RS, and USP mAb 003 RS) with the glycan profiles reported herein that can be used to assess the analytical testing of monoclonal antibody glycan profiles. Comparison of the USP reference standards to other available reference standards (NISTmAb) is presented. The glycan profile of the USP monoclonal antibody reference standards covers a range of glycan species that complements other available reference standards. The USP mAb reference standards are a valuable tool that can be used to verify the glycan structure and provide the system suitability of analytical methods.

Keywords: glycan analysis; monoclonal antibody; NISTmAb; USP

1. Introduction

Monoclonal antibodies have become a valuable biologic that the clinician may use to treat a myriad of illnesses. It is the specificity of binding and mode of action of the monoclonal antibody toward its target that allows these therapeutics to precisely treat the illness. There are numerous structural features that contribute to the specific actions of a given monoclonal antibody, including the glycan structure associated with the monoclonal antibody [1–9]. The glycan structure of a monoclonal antibody is typically heterogeneous with a predominance of a particular structure(s) that can contribute to stability, bioactivity, pharmacokinetics and pharmacodynamics (PK/PD), and immunogenicity of the molecule [4–6,9]. For these reasons, the glycan structure of a monoclonal antibody product is a critical quality attribute that must be evaluated through analytical characterization during the development and commercialization of a monoclonal antibody product.

Analytical characterization of the glycan structure of a monoclonal antibody requires scientifically sound analytical procedures that are accurate, precise, and reproducible [1–3,7,8]. To demonstrate that an analytical procedure is scientifically sound often requires characterizing a reference standard in parallel with a test sample to demonstrate that the analytical procedure was performing properly as expected. For the determination of glycan structure, this requires a reference standard that has been highly characterized, using one or more orthogonal methods, to fully understand the glycan structure of the molecules in the reference standard.

A monoclonal antibody reference standard, with extensive glycan characterization [1], is available from the National Institute of Standards and Technology designated as NIST Reference Material 8671, NISTmAb. The United States Pharmacopeia (USP) developed three monoclonal antibody reference standards (i.e., USP mAb 001 RS, USP mAb 002 RS,
and USP mAb 003 RS) that may be used as control materials to demonstrate whether
glycan characterization procedures provide an accurate result. The USP mAb reference
standards are different proteins of the same IgG1 subclass yet provide sufficient variability
to examine a broad spectrum of glycan structures. Glycan characterization is commonly
performed by analysis of glycans released from the protein backbone. As a result of lacking
UV absorption chromophores, the release glycans are usually derivatized by introducing
fluorescence tags such as 2-aminobenzamide (2-AB) and 2-aminanthranilic acid (2-AA)
to facilitate High-Performance Liquid Chromatography—Fluorescent Detection (HPLC-
FLR) [10]. HPLC-FLR is suitable for profiling and quantitative analysis but is limited
for structure identification [10,11]. In recent years, Mass Spectrometry (MS) has been
increasingly utilized with FLR for glycan identification and providing structure information.
In this study, we used a rapid labeling kit-RapidFluor-MS (RFMS) which provides good
sensitivity for FLR and MS detections of N-glycans [3,10,11]. The glycan structures of
these three USP mAb reference standards are provided in this report to further their use in
supporting glycan structure characterization studies.

2. Results and Discussion
2.1. Glycan Profiling and Proposed Identifications

The USP reference standard antibodies glycan profiles were determined using methods
previously described [2,3]. The highest normalized glycan abundance in all three antibodies
was F(6)A2 at 44.46% (mAb 001, Table 1), 67.81% (mAb 002, Table 2), and 49.56% (mAb 003,
Table 3). A normalized abundance cutoff was set to 0.1%. Minor glycans with normalized
abundance below 0.1% were not included in this article. This does not mean that minor
glycans are not important. Some minor glycans may have even stronger bioactivities
and be responsible for immune responses. This report is to present the major glycans
readily detected in the three USP reference standard antibodies. The identification of very
minor glycans in these reference standards is beyond the scope of this study. We would
encourage the readers to identify vary minor glycans if more sensitive instrumentation or
methodologies are accessible. The number of structural elements within the glycan profile
of each monoclonal antibody is summarized in Table 4. The co-elution of glycans and
unknown glycans, glycans that were not included in the database library, were observed
for all three antibodies as shown in Tables 1–3. Each of the co-eluting species was mass-
confirmed through manual inspection of the mass spectrometry data. The normalized
abundance is expressed as the total area of the FLR peak and includes the contribution of
coenzyme species in Tables 1–3, if they are present. In some cases, more than one glycan
isomers were proposed under the same FLR peak. These isomers may coexist, or it is
possible that only one of them dominates, as they cannot be distinguished with the current
approach. This indicates the limitation of the current approach, although it is widely
used currently in industry [3,8]. A combination of this approach with automatic analysis
of LC-MSMS data and ion mobility data may help to differentiate the glycan isomers
precisely [9].

The chromatogram observed with USP mAb 001, USP mAb 002, and USP mAb 003
(Figure 1) resembles that of other mAbs [3]. A comparison of the major glycans observed
among these USP monoclonal antibody reference standards is shown in Figure 2. The low
standard deviation of glycan abundance in Figure 2 indicates a good reproducibility of the
assay. Most of the characteristic structural elements observed in the glycan profile of mAbs
are summarized for the three USP monoclonal antibody reference standards in Table 4.
Table 1. A list of the mass-confirmed N-linked glycans derived from the USP mAb 001. The table also includes the observed retention times, normalized peak area percentage, expected and observed GU values, expected and observed masses and m/z values, and the mass error expressed in parts per million (ppm). In the case of co-eluting glycans, total peak area percentages are reported. In case of glycan isomers proposed under the same glycan peak, the isomers may coexist or one of them dominates, as they cannot be distinguished with the current approach.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|---------------|--------------------|--------------------|--------------|-----------------|
| F(6)M3         | 9.92              | 0.0           | 4.77                   | 4.79          | 1367.5603          | 1367.5689          | 684.7923     | 6.31            |
| A1             | 10.52             | 0.3           | 4.96                   | 4.99          | 1424.5818          | 1424.5943          | 713.3049     | 8.83            |
| F(6)A1         | 11.72             | 2.0           | 5.31                   | 5.36          | 1570.6397          | 1570.6523          | 786.3340     | 8.04            |
| A2             | 12.15             | 1.2           | 5.49                   | 5.53          | 1627.6611          | 1627.6721          | 814.8439     | 6.76            |
| F(6)A2         | 13.31             | 44.5          | 5.82                   | 5.86          | 1773.7190          | 1773.7381          | 887.8769     | 8.82            |
| M5 Isomer      | 14.30             | 0.1           | 6.19                   | 6.18          | 1545.6080          | 1545.6171          | 773.8164     | 5.91            |
| F(6)A2B        | 14.60             | 4.7           | 6.19                   | 6.18          | 1976.7984          | 1976.8105          | 989.4131     | 6.14            |
| F(6)A1G(4)1    |                   |               | 6.22                   | 6.27          | 1732.6925          | 1732.7011          | 867.3584     | 4.99            |
| A2[6]G(4)1     |                   |               | 6.26                   |              | 1789.7139          | 1789.7197          | 895.8677     | 3.23            |
| A2[3]G(4)1     | 15.08             | 0.2           | 6.38                   | 6.43          | 1789.7139          | 1789.7269          | 895.8713     | 7.25            |
| F(6)A2[6]G(4)1 | 15.67             | 27.2          | 6.53                   | 6.62          | 1935.7719          | 1935.7857          | 968.9007     | 7.17            |
| F(6)A2[3]G(4)1 | 16.08             | 9.36          | 6.69                   | 6.76          | 1935.7719          | 1935.7847          | 968.9002     | 6.65            |
| F(6)M5A1       | 16.44             | 0.1           | 6.91                   | 6.87          | 1894.7453          | 1894.7571          | 948.3864     | 6.41            |
| F(6)M4A1G(4)1  |                   |               | 7.12                   |              | 1894.7453          | 1894.7551          | 948.3854     | 5.35            |
| F(6)A1G(4)1Ga(3)1 |           |               | 7.12                   |              | 1894.7453          | 1894.7551          | 948.3854     | 5.35            |
| M6             |                   |               | 7.11                   |              | 1707.6609          | 1707.6687          | 854.8422     | 4.61            |
| M6D3           |                   |               | 7.12                   |              |                    |                    |              |                 |
| Unknown 1      | 17.25             | 0.8           | 7.15                   |              | 2023.7993          | 1012.907          |              |                 |
### Table 1. Cont.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|---------------|-------------------|-------------------|--------------|-----------------|
| M6D1           | 17.35             | 0.3           | 7.14                   | 7.18          | 1707.6609         | 1707.6701         | 854.8429     | 5.43            |
| A2G(4)1Ga(3)1  |                   |               |                        |               |                   |                   |              |                 |
| A2G(4)2        |                   |               | 7.10                   |               |                   |                   |              |                 |
| F(6)A3G(4)1    |                   |               |                        |               |                   |                   |              |                 |
| F(6)A2[3]BG(4)1 | 17.72           | 0.2           | 6.97                   | 7.29          | 2138.8512         | 2138.8661         | 1070.4410    | 7.08            |
| F(6)A3G(4)1 iso |                 |               |                        |               |                   |                   |              |                 |
| F(6)A3G(4)3S(3,3,3)3 | 17.80 | 0.3           | 7.41                   | 7.34          | 2226.8673         | 2226.8789         | 1114.4770    | 5.24            |
| F(6)A2[6]G(4)1Ga(3)1 | 18.31     | 5.9           |                        |               |                   |                   |              |                 |
| F(6)A2G(4)2    |                   |               | 7.43                   | 7.51          | 2097.8247         | 2097.8369         | 1049.9263    | 5.84            |
| F(6)A2[3]G(4)1Ga(3)1 |             |               |                        |               |                   |                   |              |                 |
| F(6)A2[3]G(4)1S(3)1 |             |               |                        |               |                   |                   |              |                 |
| M4A1G(4)1Ga(3)1 | 18.64             | 0.2           | 7.42                   | 7.63          | 1910.7402         | 1910.7541         | 956.3849     | 7.28            |
| M5A1G(4)1      |                   |               | 7.43                   |               |                   |                   |              |                 |
| F(6)M5A1G(4)1  | 19.04             | 0.2           | 7.74                   | 7.77          | 2056.7981         | 2056.8119         | 1029.4138    | 6.71            |
| F(6)M4A1G(4)1Ga(3)1 |    |               |                        |               |                   |                   |              |                 |
| M7D3           | 19.56             | 0.2           | 7.80                   | 7.93          | 2056.7981         | 2056.8123         | 1029.4140    | 6.90            |
| M7             | 20.03             | 0.1           | 8.0                    | 8.14          | 1869.7137         | 1869.7283         | 935.8720     | 7.84            |
| M7D1           |                   |               |                        |               |                   |                   |              |                 |
| F(6)A3G(4)1Ga(3)1 iso | |               | 7.88                   |               | 2300.9041         | 2300.9221         | 1151.4689    | 7.88            |
| F(6)A2G(4)2S(3)1 | 20.16           | 0.5           | 8.12                   | 8.19          | 2388.9201         | 2388.9355         | 1195.4756    | 6.46            |
| F(6)A2G(4)2S(6)1 | 20.37           | 0.3           | 8.55                   | 8.26          | 2388.9201         | 2388.9361         | 1195.4759    | 6.71            |
Table 1. Cont.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|---------------|-------------------|-------------------|--------------|-----------------|
| F(6)A2G(4)2Ga(3)1 | 20.72             | 0.1           | 8.25                   | 8.39          | 2259.8775         | 2259.8925         | 1130.9541    | 6.65            |
| F(6)M4A1G(4)1Sg(6)1 | 8.58              |               |                        | 8.90          | 2031.7665         | 2031.7833         | 1016.8995    | 6.79            |
| F(6)A2G(4)2Ga(3)1 iso | 8.30             |               |                        |               | 2259.8775         | 2259.8925         | 1130.9541    | 6.65            |
| M8             |                   |               |                        |               | 22.03             | 8.84              |              |                 |
| M8D1D3         | 22.03             | 0.3           | 8.82                   | 8.90          | 2680.0155         | 2680.0299         | 1341.0228    | 5.38            |
| M8D2D3         |                   |               |                        |               | 8.76              |                   |              |                 |
| F(6)A2G(4)2S(3,3)2 | 22.25             | 0.3           | 8.85                   | 8.99          | 2680.0155         | 2680.0299         | 1341.0228    | 5.38            |

Nomenclature: F: fucose; G: galactose; Sg: N-glycolyneuraminic acid; Sx, number (x) of sialic acids linked to galactose; Ga: α1,3-linked galactose; A1: monoantennary; A2: biantennary; Mx: number (x) of mannose on core GlcNAcs; D1 indicates that the α1,2 mannose is on the Manα1,6Manα1,6 arm; D2 indicates that the α1,2 mannose is on the Manα1,3Manα1,6 arm; D3 indicates that the α1,2 mannose is on the Manα1,3 arm of M6 and on the Manα1,2Manα1,3 arm of M7 and M8; B: bisecting GlcNAc linked _1,4 to 1,3 mannose; Numbers with parentheses indicate the preceding monosaccharide’s linkage and those in brackets define to which core mannose is extended, if it needed to be defined. Numbers not in parentheses indicate the amount of the preceding feature. For example, F(6)A2[3]G(4)1Ga(3)1 represents a core fucosylated (α1,6-linked) bianntennary glycan with a β1,4-linked galactose directly attached to the α1,3-linked core mannose and an α1,3-linked galactose attached to the β1,4-linked galactose.

Table 2. A list of the mass-confirmed N-linked glycans derived from the USP mAb 002. The table also includes the observed retention times, normalized peak area percentage, expected and observed GU values, expected and observed masses and m/z values, and the mass error expressed in parts per million (ppm). In the case of co-eluting glycans, total peak area percentages are reported. In case of glycan isomers proposed under the same glycan peak, the isomers may coexist or one of them dominates, as they cannot be distinguished with the current approach.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|---------------|-------------------|-------------------|--------------|-----------------|
| M3             | 8.74              | 0.1           | 4.36                   | 4.41          | 1221.5024         | 1221.5113         | 611.7635     | 7.31            |
| A1             | 10.52             | 0.1           | 4.96                   | 4.99          | 1424.5818         | 1424.5911         | 713.3034     | 6.55            |
| F(6)A1         | 11.7              | 1.7           | 5.31                   | 5.36          | 1570.6397         | 1570.6483         | 786.3320     | 5.50            |
| A2             | 12.24             | 0.2           | 5.49                   | 5.53          | 1627.6611         | 1627.6797         | 814.8427     | 5.30            |
| F(6)A2         | 13.28             | 67.8          | 5.82                   | 5.85          | 1773.7190         | 1773.7337         | 887.8747     | 8.30            |
| M5 Isomer      | 14.25             | 0.1           | 6.19                   | 6.16          | 1545.6080         | 1545.6187         | 773.8172     | 6.94            |
Table 2. Cont.

| Component Name          | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|-------------------------|-------------------|---------------|------------------------|---------------|-------------------|-------------------|--------------|------------------|
| F(6)A2B                 | 14.52             | 3.3           | 6.19                   | 6.25          | 1976.7684         | 1976.8115        | 989.4136     | 6.64             |
| M5                      |                   |               |                        |               | 1545.6080         | 1545.6217        | 773.8187     | 8.88             |
| F(6)A1G(4)1             |                   |               |                        |               | 1732.6925         | 1732.7069        | 867.3613     | 8.33             |
| A2[6]G(4)1              |                   |               | 6.26                   |               | 1789.7139         | 1789.7231        | 895.8694     | 5.16             |
| F(6)A3                  | 15.32             | 0.1           | 6.27                   | 6.53          | 1976.7984         | 1976.8125        | 989.4141     | 7.15             |
| F(6)A2[6]G(4)1          | 15.60             | 16.0          | 6.53                   | 6.60          | 1935.7719         | 1935.7871        | 968.9014     | 7.87             |
| F(6)A2[3]G(4)1          | 15.99             | 6.5           | 6.69                   | 6.73          | 1935.7719         | 1935.7845        | 968.9001     | 6.52             |
| F(6)M5A1                | 16.34             | 0.1           | 6.89                   | 6.87          | 1894.7453         | 1894.7569        | 948.3863     | 6.14             |
| F(6)M4A1G(4)1           |                   |               |                        |               | 1894.7453         | 1894.7607        | 948.3882     | 8.14             |
| F(6)A1G(4)1Ga(3)1       | 17.00             | 0.1           | 7.02                   | 7.05          | 1707.6609         | 1707.6737        | 854.8447     | 7.46             |
| M6                      |                   |               | 7.11                   |               |                   |                   |              |                  |
| M6D3                    |                   |               | 7.12                   |               |                   |                   |              |                  |
| Unknown 1               | 17.04             | 0.1           |                        | 7.08          |                   | 2023.8045        | 1012.9101    |                  |
| M6D1                    |                   |               | 7.14                   |               | 1707.6609         | 1707.6703        | 854.8430     | 5.47             |
| A2G(4)1Ga(3)1           | 17.27             | 0.3           | 7.05                   | 7.15          | 1951.7668         | 1951.7771        | 976.8964     | 5.20             |
| A2G(4)2                 |                   |               | 7.10                   |               | 1951.7668         | 1951.7771        | 976.8964     | 5.20             |
| F(6)A3G(4)1             |                   |               | 6.91                   |               | 2138.8512         | 2138.8667        | 1070.4412    | 7.36             |
| F(6)A2[3]BG(4)1         | 17.6              | 0.1           | 6.97                   | 7.27          | 2138.8512         | 2138.8667        | 1070.4412    | 7.36             |
| F(6)A3G(4)1 iso         |                   |               | 7.02                   |               |                   |                   |              |                  |
| F(6)A3G(4)3S(3,3,3)     | 17.59             | 0.2           |                        | 7.41          |                   |                   |              |                  |
| F(6)A2[3]G(4)1S(3,3)    |                   |               |                        | 7.53          |                   |                   |              |                  |
Table 2. Cont.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|---------------|-------------------|-------------------|--------------|-----------------|
| F(6)A2G(4)1Ga(3)1 | 18.20             | 2.2           |                        | 7.38          | 2097.8247         | 2097.8395        | 1049.9276    | 6.93            |
| F(6)A2G(4)2     |                   |               |                        | 7.43          | 2097.8247         | 2097.8395        | 1049.9276    | 6.93            |
| F(6)A2[3]G(4)1Ga(3)1 |               |               |                        | 7.53          | 2226.8673         | 2226.8859        | 1114.4508    | 8.51            |
| M4A1G(4)1Ga(3)1 | 18.51             | 0.1           |                        | 7.42          | 1910.7402         | 1910.7521        | 956.3873     | 6.35            |
| M5A1G(4)1       |                   |               |                        | 7.43          | 1869.7137         | 1869.7283        | 835.8720     | 7.67            |
| M7              | 19.89             | 0.1           |                        | 8.0           | 2388.9201         | 2388.9409        | 1195.4783    | 8.77            |
| M7D1            | 20.05             | 0.1           |                        | 8.12          | 2388.9201         | 2388.9409        | 1195.4783    | 8.77            |
| M8              | 22.23             | 0.1           |                        | 8.80          | 2462.9569         | 2462.9725        | 1232.4941    | 6.35            |

Nomenclature: F: fucose; G: galactose; Sg: N-glycolylneuraminic acid; Sx, number (x) of sialic acids linked to galactose; Ga: α1,3-linked galactose; A1: monoantennary; A2: biantennary; Mx: number (x) of mannose on core GlcNAcs; D1 indicates that the α1,2 mannose is on the Manα1,6Manα1,6 arm, D2 indicates that the α1,2 mannose is on the Manα1,3Manα1,6 arm, D3 indicates that the α1,2 mannose is on the Manα1,3 arm of M6 and on the Manα1,2Manα1,3 arm of M7 and M8; B: bisecting GlcNAc linked 1,4 to 1,3 mannose; Numbers with parentheses indicate the preceding monosaccharide’s linkage and those in brackets define to which core mannose is extended; Numbers not in parentheses indicate the amount of the preceding feature. For example, F(6)A2G(4)1Ga(3)1 represents a core fucosylated (α1,6-linked) biantennary glycan with a β1,4-linked galactose directly attached to the α1,3-linked core mannose, and an α1,3-linked galactose attached to the β1,4-linked galactose.
Table 3. A list of the mass-confirmed N-linked glycans derived from the USP mAb 003. The table also includes the observed retention times, normalized peak area percentage, expected and observed GU values, expected and observed masses and m/z values, and the mass error expressed in parts per million (ppm). In the case of co-eluting glycans, total peak area percentages are reported. In case of glycan isomers proposed under the same glycan peak, the isomers may coexist or one of them dominates, as they cannot be distinguished with the current approach.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|---------------|-------------------|-------------------|--------------|-----------------|
| A1             | 10.46             | 0.5           | 4.96                   | 4.97          | 1424.5818         | 1424.5891         | 713.3024     | 5.01            |
| F(6)A1         | 11.62             | 2.0           | 5.31                   | 5.33          | 1570.6397         | 1570.6483         | 786.3320     | 5.49            |
| A2             | 12.15             | 1.3           | 5.49                   | 5.50          | 1627.6611         | 1627.6713         | 814.8435     | 6.29            |
| F(6)A2         | 13.19             | 49.6          | 5.82                   | 5.83          | 1773.7190         | 1773.7303         | 887.8730     | 6.39            |
| M5 Isomer      | 14.25             | 0.1           | 6.19                   | 6.13          | 1545.6080         | 1545.6153         | 773.8155     | 4.74            |
| F(6)A2B        | 14.52             | 3.7           | 6.19                   | 6.22          | 1976.7984         | 1976.8133         | 989.4145     | 7.56            |
| M5             | 14.93             | 0.1           | 6.38                   | 6.38          | 1789.7139         | 1789.7243         | 895.8700     | 5.83            |
| F(6)A1[3]G(4)1 | 15.50             | 26.2          | 6.53                   | 6.57          | 1935.7719         | 1935.7879         | 968.9018     | 8.28            |
| F(6)A2[6]G(4)1 | 15.90             | 8.9           | 6.69                   | 6.70          | 1935.7719         | 1935.7909         | 968.9033     | 9.83            |
| F(6)M5A1       | 16.19             | 0.1           | 6.89                   | 6.79          | 1894.7453         | 1894.7575         | 948.3866     | 6.45            |
| F(6)M4A1G(4)1  | 17.00             | 0.1           | 7.02                   | 7.02          | 1894.7453         | 1894.7559         | 948.3858     | 5.61            |
| F(6)A1G(4)1Ga(3)1 | 17.04          | 0.3           | 7.11                   | 7.12          | 1707.6609         | 1707.6699         | 854.8428     | 5.24            |
| Unknown 1      | 17.13             | 0.1           | 7.14                   | 7.11          | 1951.7668         | 1951.7773         | 976.8965     | 5.40            |
Table 3. Cont.

| Component Name | Observed RT (min) | Peak Area (%) | Expected Glucose Units | Glucose Units | Expected Mass (Da) | Observed Mass (Da) | Observed m/z | Mass Error (ppm) |
|----------------|-------------------|---------------|------------------------|--------------|-------------------|-------------------|-------------|-----------------|
| F(6)A3G(4)3S(3,3,3)3 | 17.59 | 0.2 | 7.41 | 7.26 | 2226.8673 | 2226.8785 | 1114.4471 | 5.04 |
| F(6)A2[6]G(4)1Ga(3)1 | 18.11 | 5.5 | 7.43 | 7.44 | 2097.8247 | 2097.8311 | 1049.9234 | 2.93 |
| F(6)A2[3]G1Ga(3)1 | 7.53 | 2226.8673 | 2226.8857 | 1114.4507 | 8.28 |
| F(6)A2[3]G(4)1S(3)1 | 18.44 | 0.3 | 7.42 | 7.56 | 1910.7402 | 1910.7535 | 956.3846 | 7.09 |
| F(6)M5A1G(4)1 | 18.84 | 0.1 | 7.74 | 7.70 | 2056.7981 | 2056.8093 | 1029.4125 | 5.51 |
| F(6)M4A1G(4)1Ga(3)1 | 19.35 | 0.1 | 7.80 | 7.89 | 2056.7981 | 2056.8149 | 1029.4153 | 8.24 |
| M7D3 | 19.96 | 0.4 | 8.12 | 8.11 | 2388.9201 | 2388.9299 | 1195.4728 | 4.16 |
| M7 | 20.15 | 0.2 | 8.55 | 8.18 | 2388.9201 | 2388.9329 | 1195.4743 | 5.42 |
| F(6)A2G(4)2S(3)1 | 20.51 | 0.1 | 8.25 | 8.32 | 2259.8775 | 2259.8989 | 1130.9573 | 9.27 |
| F(6)M4A1G(4)1Sg(6)1 | 20.96 | 0.1 | 8.58 | 8.58 | 2201.8356 | 2201.8505 | 1101.9331 | 6.79 |
| M8 | 21.8 | 0.2 | 8.84 | 8.89 | 2031.7665 | 2031.7779 | 1016.8968 | 5.88 |
| M8D1D3 | 22.00 | 0.2 | 9.32 | 8.89 | 2680.0155 | 2680.0289 | 1341.0223 | 5.01 |

Nomenclature: F: fucose; G: galactose; Sg: N-glycolyneuraminic acid; Sx, number (x) of sialic acids linked to galactose; Ga: α1,3-linked galactose; A1: monoantennary; A2: biantennary; Mx: number (x) of mannose on core GlcNAcs; D1 indicates that the α1,2 mannose is on the Manα1,6Manα1,6 arm, D2 indicates that the α1,2 mannose is on the Manα1,3Manα1,6 arm, D3 indicates that the α1,2 mannose is on the Manα1,3 arm of M6 and on the Manα1,2Manα1,3 arm of M7 and M8; B: bisecting GlcNAc linked 1,4 to 1,3 mannose; Numbers with parentheses indicate the preceding monosaccharide’s linkage and those in brackets define to which core mannose is extended, if it needed to be defined. Numbers not in parentheses indicate the amount of the preceding feature. For example, F(6)A2[3]G(4)1Ga(3)1 represents a core fucosylated (α1,6-linked) biantennary glycan with a β1,4-linked galactose directly attached to the α1,3-linked core mannose, and an α1,3-linked galactose attached to the β1,4-linked galactose.
Figure 1. Annotated FLR trace depicting the N-linked glycans derived from USP mAb 001, mAb 002, and mAb 003. Peaks for glycans A1, F6A1, A2, F6A2, F6A2B, F(6)A2[6]G(4)1, and F(6)A2[3]G(4)1 are identified. A peak representing a mixture of F(6)A2[6]G(4)1Ga1, F(6)A2G(4)2 = G2F, F(6)A2[3]G1Ga1, and F(6)A2[3]G(4)1S(3)1 was observed.

Figure 2. Abundance level of various glycans found in USP mAb 001, mAb 002, and mAb 003. The error bars indicate three standard deviations of the averaged abundance from four injections.

Table 4. Number of structural elements found in USP mAb reference standards.

| Structural Element | USP mAb 001 | USP mAb 002 | USP mAb 003 |
|-------------------|-------------|-------------|-------------|
| Total Glycans     | 47          | 45          | 40          |
| High Mannose      | 12          | 12          | 10          |
| Hybrid            | 11          | 10          | 11          |
| Complex           | 23          | 22          | 18          |
| Unknown *         | 1           | 1           | 1           |
| Biantennary       | 22          | 25          | 26          |
2.2. Applications for Verification Glycan Structures and Assessing Critical Quality Attributes of mAbs

Glycans associated with protein products are a diverse group of molecules that are challenging to characterize. N-glycosylation of the Fc region of an IgG molecule has been identified as a critical quality attribute for some monoclonal antibodies due to the recognized effector functions (e.g., ADCC, CDC, and PK) of certain glycan species [4,5]. Monoclonal antibody N-linked glycans with terminal sialic acid can result in antibodies with longer half-lives in the body than non-sialylated antibodies, monoclonal antibodies with a high content of glycans with terminal mannose are cleared more quickly from the blood, and the absence of core fucose on monoclonal antibody glycans can enhance the affinity for the Fc region with the FcyRIIIa receptor, increasing the ADCC [6]. Each of the glycan elements including sialylated, and many more, can be found in the USP mAb reference standards.

The five most abundant glycans that have been reported for USP mAb 001, mAb 002, mAb 003, and NIST mAb are shown in Table 5. The results indicate that the USP monoclonal antibody reference standards and the NISTmAb are similar with regard to glycan profiles, with the notable exception of USP mAb 002. USP mAb 002 shows a higher level of F(6)A2 and lower levels of F(6)A2[6]G(4)1, F(6)A2[3]G(4)1, and F(6)A2G(4)2 when compared to other glycan reference standards (Table 5). These differences observed with USP mAb 002 make it valuable as a unique sample when evaluating the system suitability of analytical techniques to determine monoclonal antibody glycan profiles. Comparison of the glycan profiles beyond the five most abundant glycans as shown in Table 5 becomes difficult given that some of the identified glycans that are less abundant co-elute. A further complication with the quantitative comparison of low abundance glycans is possible with 47, 45, and 40 glycans observed with USP mAb 001, USP mAb 002, and USP mAb 003, respectively. Fifty-seven glycans were reported for NIST mAb [1], and in another study, 35 glycans were reported for NIST mAb [3]. This variation may have an impact on the comparison of relative glycan abundance determination between reference standards based on the sum of the total peak area. The low abundance glycans are useful when evaluating if a particular analytical method can detect the entire glycan profile.

| Glycan          | USP mAb 001 | USP mAb 002 | USP mAb 003 | NIST mAb * | NIST mAb ** |
|-----------------|-------------|-------------|-------------|------------|------------|
| F(6)A2          | 44.46       | 67.81       | 49.56       | 39.09      | 39         |
| F(6)A2[6]G(4)1  | 27.17       | 16          | 26.2        | 28.12      | 37.8       |
| F(6)A2[3]G(4)1  | 9.36        | 6.51        | 8.93        | 10.18      | -          |
| F(6)A2G(4)2     | ≤5.89       | ≤2.22       | ≤5.49       | 7.51       | 7.3        |
| F(6)A1          | 1.98        | 1.71        | 1.99        | 2.127      | 2.5        |

* Results reported in Reference [1]; ** Result reported in Reference [3].

There is value in having multiple well-characterized monoclonal antibodies that can serve as reference standards to verify the various analytical glycan profiling techniques now...
and in the future. Although quantitative comparison of glycan profiles among the monoclonal antibody reference standards may be difficult for less abundant glycans (i.e., those glycans less than the five most abundant), their use in the evaluation of the analytical methods with regard to resolution and sensitivity is significant. Discrepancies observed with multiple glycosylation analytical methods and multiple laboratories highlight the value in having available multiple reference standards to use for analytical method verification and control [7].

As previously reported [8,9], without validated reference standards, manufacturers cannot create an in-depth glycan profile that includes the minor glycans that may be present or validate the suitability of their equipment for such an analysis. The major glycans present in the USP monoclonal antibodies cover a quantitative range (Table 5) which gives added benefit in their use in evaluating the suitability of an analytical method. Therein lies the value of the USP mAb 001, mAb 002, and mAb 003 reference standards available from the United States Pharmacopeia.

3. Materials and Methods

3.1. Reagents and Materials

The mAb 001, mAb 002, and mAb 003 reference standards were obtained from the United States Pharmacopeia. RFMS-labeled human IgG (RFMS Glycan Performance Test Standard, product number 186007983) and mouse IgG (Intact mAb Mass Check Standard, product number 186006552) were purchased from Waters Corp (Milford, CT, USA). Ammonium formate solution, Glycoworks® RapiFluor-MS Performance Test Standard, Glycoworks Rapi-Fluor-MS dextran calibration ladder, and a Glycoworks Rapi-Fluor-MS 24-sample N-Glycan Kit were obtained from Waters Corp. The complete kit consists of three modules: deglycosylation, labeling, and clean-up. When combined, the kits contain all of the supplies needed to complete the sample preparation, and all three were utilized in this project. The Glycoworks Deglycosylation Module contained Intact mAb Mass Check Standard, Glycoworks Rapid PNGase F and Buffer, and RapiGestTM SF. The Glycoworks® RapiFluor-MSTM Labeling Module contained the Glycoworks® RapiFluor-MSTM Reagent and Glycoworks® Reagent Solvent Anhydrous Dimethylformamide (DMF). The Glycoworks® RapiFluor-MSTM Clean-up Module contained a Glycoworks® HILIC µ-Elution Plate, Glycoworks® SPE elution buffer (200 mM ammonium acetate, 5% acetonitrile (ACN)), and Glycoworks® Sample Diluent (DMF). The Glycoworks® RapiFluor-MSTM Sample Collection Module contained sample collection tubes, a waste tray, and a collection tray. The Amicon spin concentrator (0.5 mL, 10 K), LC-MS grade water, and acetonitrile were purchased from Thermo Fisher Scientific.

3.2. PNGase Digestion to Remove Glycans from mAbs

N-linked glycans derived from the mAb 001, mAb 002, and mAb 003 were prepared in duplicate. The samples were diluted and buffer-exchanged to a concentration of 2 mg/mL in a phosphate-buffered saline solution (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). In brief, in an Amicon spin concentrator, add 10 µL of 10 mg/mL sample and 40 µL PBS solution, and then centrifuge at 7500 × g at 8 °C for 15 min. After washing 3 times with 100 µL of PBS and centrifugation, collect the sample and bring the volume to 50 µL. In a 1 mL Eppendorf tube, add 7.5 µL (15 µg) buffer-exchanged sample, 5.3 µL of water, and 6 µL of a 5% RapiGest solution. The proteins were denatured at 95 °C for 3 min and then cooled to the ambient temperature. Next, add 1.2 µL of Rapid PNGase F, aspirate to mix well, and then incubate at 55 °C for 5 min to release the N-linked glycans as their glycosylamines.

3.3. RFMS Labeling of Released Glycans

Following the digestion, the amino group of the released glycosylamines was labeled with RFMS. A 24-reaction kit was used, and the RFMS reagent (9 mg) was dissolved in 131 µL of anhydrous DMF. A 12 µL aliquot of this solution was added to each glycan
sample and allowed to react at the ambient temperature for 5 min. Then, the reaction was quenched with the addition of a 358 µL aliquot of ACN, which also adjusted the solution to an appropriate organic solvent concentration for Hydrophilic Interaction Liquid Chromatography (HILIC)-based purification.

3.4. Glycan Purification

The derivatized N-linked glycans were purified using a HILIC µ-Elution plate. The medium was first washed with three 200 µL aliquots of water, which was followed by three 200 µL of 85% ACN in water. Then, the samples (400 µL) were loaded onto the medium and washed with two 600 µL of washing solution containing 90% ACN and 1% water in water. The glycans were eluted by three 30 µL aliquots of an SPE Elution Buffer (200 mM ammonium acetate in 5% ACN). The eluate was diluted with 310 µL of GlycoWorks SPE Diluent and transferred into autosampler vials for Liquid Chromatography Mass Spectrometry (LC-MS) analysis.

3.5. UPLC-MS Analysis

A Waters ACQUITY H-class Ultra-Performance Liquid Chromatography (UPLC) system, consisting of a quaternary solvent manager, a sample manager, set to 5 °C, a column manager, operating at 60 °C, and a fluorescence (FLR) detector (excitation wavelength 265 nm, emission wavelength 425 nm, data collection rate of 2 Hz) was used for the separations. The glycans were separated with a Waters ACQUITY UPLC Glycan BEH Amide column (2.1 × 150 mm, 1.7 µm particle size, 130 Å pore size). Mobile Phase A was a 50 mM ammonium formate solution (pH 4.4), and Mobile Phase B was neat ACN. Analyte separation was accomplished by gradient elution using a gradient running from 75 to 54% Mobile Phase B over 35 min at a flow rate of 0.4 mL/min. For the mAb 001, mAb 002, and mAb 003 analysis, the UPLC system was coupled to a Waters Synapt-G2S QT of MS and was operated in its positive sensitivity mode to monitor the m/z range from 600 to 2500 at a scan rate of 2 Hz. The capillary voltage was set to 3 kV, and a cone voltage of 40 V was used. The source temperature was 120 °C, and the desolvation temperature was set at 350 °C. For all Mass Spectrometry (MS) analysis, a 100 pmol/µL solution of [Glu1]-fibrinopeptide B in 50%/50%/0.1% water/ACN/formic acid was used for Lockspray calibration, and the 2+ ion at m/z 785.8427 was used for calibration. Along with sample injections, RFMS-labeled dextran was injected at the beginning and end of the sequence as standards for determining GU values (Glucose Units) of all detected glycans. The Glycoworks® RapiFluor-MS Performance Test Standard was also injected prior to the samples and used for system suitability tests. Duplicate injections were performed for each of the duplicate sample preparations.

3.6. Data Analysis

HILIC-UPLC/FLR/MS data were processed and analyzed using the Glycan Assay (FLR with MS confirmation) workflow within UNIFI. This workflow first converted the retention times of the labeled glycans samples to Glucose Units (GU) based on a cubic spline calibration curve against a dextran ladder labeled with RFMS. Then, these GU values were searched against the RFMS Glycan GU Scientific Library housed within UNIFI for glycan structural identification. The library searches used a GU tolerance of 0.3 GU and a mass error of 10 ppm. For relative quantitation, the FLR peak area for each glycan was expressed as a percentage of the total summed peak area for all the glycans identified. In the case of co-eluting glycans, mass-confirmed through manual inspection of the data, the total peak area and percentage amounts are reported. The relative abundance of glycans is from an average of four injections of the duplicate sample preparations.

4. Conclusions

In this study, we have presented the glycan profiles and proposed identifications of all major and most minor glycans released from the three recently released monoclonal
antibody reference standards (i.e., USP mAb 001 RS, USP mAb 002 RS, and USP mAb 003 RS). The glycan profile of the USP monoclonal antibody reference standards covers a range of glycan species that complements other available reference standards. The USP mAb reference standards can be used as a valuable tool to verify glycan structures and provide the system suitability of analytical methods for glycan profile testing of pharmaceutical mAb products.

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Abbreviations

ACN: Acetonitrile; DMF, Dimethylformamide; ESI, Electrospray Ionization; FA, Formic Acid; FLR, Fluorescence; Gal, Galactose; gal-a1,3-gal, Two galactoses linked via an a1–3 bond; GlcNAc, N-acetylglucosamine; GU, Glucose Unit; GUH, b-N-acetylhexosaminidase; HILIC, Hydrophilic Interaction Liquid Chromatography; hr, Hour; IgG, Immunoglobulin G; kV , Kilovolt; M5, Mannose-5 glycan; mAb, Monoclonal antibody; min, Minute; mg, Milligram; mM, Millimolar; MS, Mass Spectrometry; ppm, Parts per million; QTOF, Quadrupole Time-of-Flight; RFMS, RapiFluor-MS; RM, Reference Material; SPE, Solid-phase extraction; mL, Microliter; UPLC, Ultra-Performance Liquid Chromatography; LC-MS, Liquid Chromatography Mass Spectrometry; V, Volt.

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