A novel approach for the simultaneous quantification of a therapeutic monoclonal antibody in serum produced from two distinct host cell lines

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Abbreviations: mAb, monoclonal antibody; BC, biocomparability; BE, bioequivalence; PK, pharmacokinetics; ADA, anti-drug antibody; CDR, complementarity-determining region; PTM, posttranslational modification; TOF-MS, time-of-flight mass spectrometry; LC, liquid chromatography; IAP, immunoaffinity purification; POC, proof of concept; CHO, Chinese hamster ovary cell line; Sp2/0, mouse myeloma cell line

Therapeutic monoclonal antibodies (mAbs) possess a high degree of heterogeneity associated with the cell expression system employed in manufacturing, most notably glycosylation. Traditional immunoassay formats used to quantify therapeutic mAbs are unable to discriminate between different glycosylation patterns that may exist on the same protein amino acid sequence. Mass spectrometry provides a technique to distinguish specific glycosylation patterns of the therapeutic antibody within the same sample, thereby allowing for simultaneous quantification of the same mAb with different glycosylation patterns. Here we demonstrate a two-step approach to successfully differentiate and quantify serum mixtures of a recombinant therapeutic mAb produced in two different host cell lines (CHO vs. Sp2/0) with distinct glycosylation profiles. Glycosylation analysis of the therapeutic mAb, CNTO 328 (siltuximab), was accomplished through sample pretreatment consisting of immunoaffinity purification (IAP) and enrichment, followed by liquid chromatography (LC) and mass spectrometry (MS). LC-MS analysis was used to determine the percentage of CNTO 328 in the sample derived from either cell line based on the N-linked G1F oligosaccharide on the mAb. The relative amount of G1F derived from each cell line was compared with ratios of CNTO 328 reference standards prepared in buffer. Glycoform ratios were converted to concentrations using an immunoassay measuring total CNTO 328 that does not distinguish between the different glycoforms. Validation of the IAP/LC-MS method included intra-run and inter-run variability, method sensitivity and freeze-thaw stability. The method was accurate (% bias range = −7.30–13.68%) and reproducible (% CV range = 1.49–10.81%) with a LOQ of 2.5 μg/mL.

Introduction

Monoclonal antibody (mAb) therapies provide great clinical benefit, and more than 30 are now approved for indications in various pathologic conditions including cancer, inflammation, infectious and autoimmune disease.1,2 Hundreds more are undergoing evaluation in clinical studies.1 Bioanalytical methods used to support therapeutic mAbs in clinical and non-clinical development often utilize ligand binding assays (e.g., immunoassays) that rely on highly specific reagent antibodies to assess pharmacokinetics (PK) and immunogenicity. Ligand binding assay methods are considered the standard analytical platform for regulated bioanalysis in the field of therapeutic mAb development.3,7 The assays typically employ target antigen or anti-idiotypic antibodies raised against the complementarity determining region (CDR) of the therapeutic as reagents to produce very sensitive and specific methods.8,9

Due to the fundamental design of the immunoassay format, however, such methods are unable to discriminate between distinct molecular characteristics (e.g., glycosylation) if these attributes are not recognized by the antibody reagents used in the assay. Process or manufacturing changes that result in post-translational modifications (PTMs) can occur during production from host cell lines, in post-production processing, or storage and...
Figure 1. Representative structure of the N-linked oligosaccharide located on the Fc region of human IgG (above) and analyzed glycoform structures produced in CHO and Sp2/0 host cell lines. Carbohydrate residues in red are core structural components; the green represent variable residues.

may not directly affect reagent antibody binding. Mass spectrometry (MS) analysis has provided a sensitive analytical platform that can elucidate these structural characteristics of biotherapeutics. With the development of soft ionization techniques such as electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), MS has become a fundamental methodology for comparative structural analysis of therapeutic antibodies from different production systems or formulations.

Due to their PTMs, recombinant mAbs display a much higher degree of heterogeneity than most small molecule drugs. As discussed above, these various PTMs can occur during production, processing and storage. This category of PTMs incorporates chemical modifications to the primary protein structure that may or may not affect the normal proteolytic catabolism or clearance of the molecule. The most well characterized manufacturing PTM is N-linked glycosylation of the C152 domain in the Fc region of the antibody (Fig. 1). Certain changes to individual sugar moieties located on the oligosaccharide structure, particularly the core fucose or terminal sugar residues, have been shown to influence Fc effector function, immunogenicity and clearance, which may have a direct impact on the overall therapeutic efficacy of the antibody. Therapeutic mAbs that are produced in specific cell line expression systems possess inherent PTM profiles characteristic of that host cell line. In particular, N-linked glycosylation profiles can vary greatly based on the cell line expression system used to produce the mAbs and can be analytically characterized. Common host cell lines currently used for therapeutic mAb production include Chinese hamster ovary (CHO) and mouse myeloma cells (Sp2/0). Identification and characterization of the therapeutic mAb variants produced in these different mammalian expression systems is necessary for monitoring manufacturing consistency and assessing product quality and stability. Additionally, a change in the production system of a product that is marketed or in clinical development often requires demonstration of in vitro analytical comparability and in vivo biocomparability/bioequivalence between the drug materials manufactured by different processes.

The goal of biocomparability/bioequivalence (BC/BE) testing is to demonstrate that variation between different formulations or manufacturing processes do not affect the “quality, safety and efficacy of the drug product” during development or post-marketing. Assessment for comparability of manufactured mAbs is hierarchical and commonly starts with an in vitro analysis to characterize biological, physicochemical and immunological properties and can progress to in vivo testing in animals or humans following a specific BC/BE type study design. When the in vitro analysis does not provide sufficient evidence of product comparability, the demonstration of in vivo BC/BE may be required. However, establishing BC/BE of therapeutic antibodies from different manufacturing processes in vivo remains a significant challenge. Because intact full length antibody therapeutics generally have a much longer half-life relative to small molecule drugs, the typical crossover study design employed for small molecule bioequivalence assessment becomes technically challenging to conduct. Instead, BC/BE study designs for therapeutic mAbs traditionally consist of separate treatment groups administered the test or reference products in parallel. Moreover, because of the high inter-subject PK variability that exists for therapeutic antibodies, relatively high subject numbers are required to provide sufficient statistical powering for BC/BE studies. Thus, the combination of parallel study designs with high inter-subject variability can result in group sizes exceeding 75 subjects. Lastly, immunogenicity that results in anti-drug antibody (ADA) responses may accelerate clearance that can complicate the determination of total exposure during BC/BE assessment. For example, the test and reference product treatment groups may show the same immunogenicity by virtue of an equal number of subjects within the two groups who develop anti-drug responses. However, if the anti-drug response...
and associated accelerated clearance occurs at markedly different times in the test and reference product treatment groups following administration of the drug, the difference in total drug exposure (AUC) can disproportionately impact the BC/BE assessment (Fig. 2).

Time-of-flight (TOF) mass spectrometers have become the preferred MS instrument for molecular characterization of macromolecules because of the suitable instrument resolution (≥ 10,000), high average molecular mass accuracy and broad mass-to-charge (m/z) range required for analysis of large, multiply-charged proteins. MS combined with various chromatographic techniques has proven to be a valuable analytical methodology in protein PTM characterization, particularly with regard to glycosylation profile evaluation. Key challenges in the development of mass spectrometric analysis for therapeutic proteins involve the effective separation of the compound from biological samples and the elimination of background interference due to matrix components. The complexity of these samples often necessitates the use of multidimensional chromatographic approaches, either on- or off-line, to efficiently isolate and analyze the therapeutic mAb in the presence of high concentrations of endogenous immunoglobulin. Several methods demonstrating the use of affinity purification of mAbs from complex matrices to enhance sample clean-up prior to LC-MS analysis have been reported. When combined with advanced affinity-based sample pretreatment methods, LC-MS can be used to analyze the therapeutic mAb isolated from biological matrices for in vivo structural characterization (e.g., glycosylation, oxidation, etc.)

In the present study, the therapeutic mAb CNTO 328 (siltuximab) manufactured in Sp2/0 cells typically shows an increase in terminal galactosylation (G1F and G2F glycoforms) relative to the same mAb manufactured in CHO cells. The extent of quantifiable difference in glycosylation profiles is sufficient to constitute the development of a LC-MS method based on the measurement of heavy chain mass ratios of CHO and Sp2/0-derived CNTO 328. The method incorporates a sample pretreatment (immunoaffinity purification and enrichment) step followed by glycosylation analysis of the mAb heavy chain by reverse phase LC-MS. In combination with the total CNTO 328 concentration measurement by immunoassay, this method could be applied to quantify the individual concentration of the different therapeutic mAb species (CHO vs. Sp2/0) within the same sample based on the calculated percentages of each glycoform measured. Although this method was developed for feasibility in cynomolgus monkey serum, the approach is expected to be equally applicable in human and any non-human primate species. Exploiting these distinct glycosylation profiles to differentiate and quantify therapeutic mAbs within the same sample enables the application of a novel biocomparability study design in which test and reference drug products are administered simultaneously to each subject. The primary advantage to this simultaneous administration approach resides in the much higher probability of successfully demonstrating BC/BE due to both the reference and test drug products being subject to the exact same ADME (absorption/distribution/metabolism/excretion) conditions in the same patient at the same time. This approach can substantially reduce inter-subject differences by eliminating the inter-occasion variability resulting in a reduced number of subjects needed to power the study, and decreased overall cost. It can also greatly minimize the impact of immunogenicity assuming the immunogenic mAb does not induce ADA against the otherwise non-immunogenic mAb. Furthermore, because the subjects receive the reference and test drug products simultaneously, this approach holds an additional advantage even when compared with the typical small molecule crossover design where subjects are dosed with each drug product on separate occasions.

Results

Method development of samples in PBS. Due to the heterogeneity of the glycosylation profiles produced by the CHO and Sp2/0 cell lines, several distinct glycans could be used to discriminate between the two cell line-derived CNTO 328 drug products. Initial MS method development focused on the five most prominent glycans on CNTO 328 products to differentiate the host cell lines (G0F, G1F, G2F, G0 and Mann-5) with the greatest change coming from the number of terminating...
galactose residues (Fig. 3A). Combinations of the CHO and Sp2/0 cell line-derived CNTO 328 glycoforms were used in preparation of 11 different molecular ratios in buffer according to the amount of CHO-produced CNTO 328 in each sample. Following reduction with DTT, the samples were analyzed by LC-MS in duplicate over two independent runs. The abundance of the deconvoluted peak for each glycan of interest was measured and the amounts of individual glycans were calculated as a percentage of the sum of all five different glycan forms. Statistical evaluation of the results from the five selected glycans confirmed a linear correlation between each glycan and the percentage of CHO-derived CNTO 328 in the sample (Fig. 3B). Due to the ability of each glycan to accurately predict the composition of the sample mixture (% CHO), it was concluded that the change in distribution of any single glycan could determine the CHO:Sp2/0 composition within each sample. Based upon
the LC-MS ratio calculation of the two cell line-derived CNTO 328 glycoforms and the total CNTO 328 mAb mass concentration determined by immunoassay, the mass concentration of CNTO 328 (CHO vs. Sp2/0) can be quantified from the mixture of products produced from either CHO or Sp2/0 cell lines. The data also demonstrated that for accurate assessment of the CHO:Sp2/0 mixture in adequately concentrated samples, it was sufficient to focus on the change in abundance of a single glycan. It was determined that the highly prominent glycoform consisting of a core-fucosylated species with one terminating galactose (G1F) would provide the greatest identifiable change between the two cell line products and thus, should maximize the method sensitivity. Therefore, the G1F glycoform was chosen for all subsequent method development experiments.

**Development of immunoaffinity purification of CNTO 328 from serum.** Through the use of a highly specific anti-idiotypic capture mAb raised against the CDR of CNTO 328, we developed an off-line sample pretreatment method consisting of an immunoaffinity purification and enrichment step in order to ensure the greatest removal of background interference prior to LC-MS analysis. During development of the immunoaffinity process, multiple resins were evaluated for conjugation efficiency/capacity, extraction efficiency, reproducibility, stability and purity (data not shown). The POROS®-AL activated media was selected due to the high conjugation efficiency (> 95%) and low levels of non-specific binding of matrix components. Different sample pretreatment processes were also investigated, including use of protein A/G separation and albumin depletion prior to the anti-idiotypic affinity extraction. Lower recovery of the therapeutic mAb following either of these method steps, however, outweighed any benefit in sample clean-up. Serum albumin contamination was the most prevalent sample clean-up issue, but was minimized through the anti-idiotypic specificity, high-volume non-specific washes and optimized reverse-phase LC conditions. CNTO 328 extraction efficiency was calculated by subtracting the sample concentration in the sample flow-through from the initial sample concentration. All initial and flow-through concentrations were measured using the validated MSD immunoassay. The extraction process was highly efficient with CNTO 328 sample depletion greater than 98% over the concentration range tested (250–5 μg/mL). The depletion results demonstrated that the IAP process did not contribute to the method variability, however, post-elution recovery was more difficult to assess because the acid treatment rendered the mAb non-reactive even following neutralization. Although the immunoaffinity purification and enrichment method was developed off-line, the optimization process required additional development (i.e., sample volume, concentration, elution, pre-LC reduction) for successful sample pretreatment prior to LC-MS analysis. Specificity for CNTO 328 was established relative to other cross-reactive immunoglobulins and serum protein components through the analysis of unspiked serum blanks during the immunoaffinity purification and enrichment steps (data not shown). The sample pretreatment (IAP and enrichment) effectively removed cross-reactive substances and resulted in a lowered background during subsequent MS detection.

**Development of LC-MS analysis of CNTO 328 glycosylation.** For method development, CNTO 328 was extracted by immunoaffinity purification from serum samples spiked with CHO and Sp2/0 cell line-derived mAb materials alone or mixed in an equal 1:1 ratio. The method was tested over a concentration range representative of that expected from an in vivo proof of concept CNTO 328 PK study in cynomolgus monkeys (1.25–250 μg/mL). Deconvoluted peak intensity values (abundance) of the G0F and G1F glycans from CNTO 328 were compared for each cell line-derived glycoform at three control concentrations: 5, 50 and 250 μg/mL (Fig. 4). In order to verify the linear relationship between the glycosylation profiles of CNTO 328 samples produced from each cell line, the G0F and G1F peak intensity values for each cell line derived product in the mixture were graphed against the three concentrations analyzed (Fig. 5A). The slopes for each of the glycans analyzed (G0F/G1F) across the IAP pretreated serum concentration range were nearly identical and were independent of the different product mixture from the two cell lines. Evaluation of the G0F:G1F peak intensity ratio for each serum concentration and different product mixture were compared with the G0F:G1F ratio of the non-pretreated PBS buffer controls to assess any impact of the purification process on the potential alteration of glycosylation profile of the therapeutic antibody. The peak intensity ratio for each cell line mixture was calculated against the CNTO 328 buffer control ratio, which was normalized to one, to determine the % bias for each concentration of IAP pretreated sample (Fig. 5B). The G0F:G1F peak intensity ratio values for the IAP pretreated serum samples did not exceed ± 15% bias of the buffer control with mean % bias of 2.80%. The data suggests that there is no analytical bias for either cell line-derived glycosylation profile and, additionally, confirms that no preferential interaction exists between the two CNTO 328 glycoforms and the anti-idiotypic antibody used for the IAP procedure. The summary of the data can be found in Table 1. The linearity of the peak intensity values for G0F and G1F and the highly correlated results of the IAP pretreated samples compared with non-pretreated buffer controls demonstrate that CNTO 328 drug product mixtures at any concentration behave comparably within the analytical method.

**IAP/LC-MS method validation in cynomolgus monkey serum.** CNTO 328 drug products with distinct glycoform profiles derived from CHO and Sp2/0 cell lines were combined in a 1:1 ratio (w/w) in pooled cynomolgus monkey serum and used for the LC-MS method validation analysis. Validation parameters assessed included inter-run variability (performed over 3 d), intra-run variability, IAP pretreated and non-pretreated method sensitivity and freeze/thaw stability (1 cycle). CNTO 328 serum control concentrations of 10, 50 and 250 μg/mL were used for inter-run, intra-run and freeze/thaw stability, while 1.25, 2.5, 5 and 10 μg/mL concentrations were tested to establish method sensitivity following IAP purification and enrichment (Fig. 6). For every analysis, the G1F glycan percent abundance for all CNTO 328 serum validation samples was assessed against the IAP pretreated CNTO 328 PBS buffer control. The required on-column injection amount needed to provide acceptable MS peak response for non-pretreated CNTO 328 buffer samples was
determined to be 800 ng with an injection volume of 100 μL. The use of an in-line concentration trap or higher pressure LC system (UPLC) was not explored, but may improve the chromatographic resolution and peak shape and help to enhance the sensitivity of the method. The capacity of the method to detect and accurately measure the spiked G0F:G1F peak ratio following IAP pretreatment provided sensitivity at a sample concentration of 2.5 μg/mL. Although the G0F glycan was present following analysis of the IAP pretreated sample at 1.25 μg/mL, no G1F glycan peak could be identified. Demonstrating the accuracy and precision of the method, the mean % bias across all validation samples analyzed was 3.46% and the mean % CV for intra and inter-run samples was 4.74%. The intra-run and inter-run sample accuracy resulted in G1F % bias values < 10% with mean % bias values of 2.14% and 0.69%, respectively. The average observed mass for the deconvoluted heavy chain G1F glycan from the CNTO 328 non-pretreated buffer control was 50773.65 daltons with a standard deviation of 0.60. Results from all method validation samples are listed in Table 2. The observed mass for all QC validation serum samples was within 100 ppm of the average mass of the non-pretreated buffer control indicating no effect on the therapeutic antibody or the N-linked oligosaccharide through the IAP pretreatment.

While the method represents a useful approach, this bioanalytical technique is not without its challenges. Although the IAP/LC-MS method provides structural information not obtainable by standard analytical methods for therapeutic antibody analysis, e.g., immunoassay, it is not likely to achieve the sensitivity levels comparable to that of the immunoassay (LOQ = 40 ng/mL). Furthermore, due to the labor intensive off-line immunoaffinity sample enrichment step needed to minimize matrix interference and improve the limit of detection prior to LC-MS analysis, the low throughput of the method can be a limiting factor. With the greatly decreased number of subjects, and therefore number of samples, required for the biocomparability assessment using this approach when simultaneous dosing administration is employed, the method may be suitable regardless of the lower sample throughput. Improvement through incorporation of an online sample pretreatment step or automated sample processing would greatly increase throughput and sample processing efficiency.
Figure 5. LC-MS analysis of G0F/G1F glycans from CHO, 1:1 ratio, or Sp2/0 spiked serum samples following immunoaffinity purification and enrichment. G0F and G1F peak intensities plotted across the range of serum concentrations of CNTO 328 displayed very high method linearity (A). The ratio of G0F:G1F peak intensity for each cell line mixture in serum samples correlated highly to the non-pretreated CNTO 328 buffer controls (B).
A method to simultaneously quantify the serum concentrations of a therapeutic mAb produced in two different host cell lines (CHO vs. Sp2/0) has been developed centered around identifying and analyzing differences in the mAb glycosylation profiles. The varying amount of terminal galactose residues on the N-linked oligosaccharide produced by each cell line was exploited to determine the relative percentage of the CHO or Sp2/0-produced CNTO 328 mAb contributing to the total sample concentration. The ability to discriminate product glycoforms within the same sample allows an entirely new approach for designing biocomparability/bioequivalence studies for mAb therapeutics. By employing the analytical capability of the LC-MS method to calculate the relative percentage of CNTO 328 per sample derived from the individual host cell lines combined with the total measurement of CNTO 328 mass concentration by immunoassay, the concentration of each CHO and Sp2/0 cell line reference standard could be indirectly quantified. Thus, the mixture of test and reference drug products with different glycoform profiles could be administered simultaneously to the same subject and monitored for distinct ratios of the two drug forms on the same occasion. Such a design would provide powerful advantages to the currently employed parallel study designs for long-lived large molecule biotherapeutics and even small molecule crossover designs through the elimination of inter-occasion differences. The reduced number of subjects required to power the study would significantly lower the cost and complexity, while the elimination of the parallel study design should greatly reduce the inter-subject variability, increasing the probability of successfully and accurately establishing biocomparability. Finally, such an approach could reduce the impact of anti-drug antibodies (immunogenicity) and their accelerated clearance on the assessment of large molecule biocomparability.


discussion

Materials and Methods

Table 1. Method linearity per cell line mixture (CHO/50:50/Sp20)

| Sample | Cell line mixture | G0F intensity | G1F intensity | G0F:G1F intensity ratio | % Bias |
|--------|------------------|---------------|---------------|------------------------|--------|
| Non-pretreated Buffer control | CHO | 266385 | 21643 | 12.31 | N/A |
| | Sp2/0 | 143655 | 131381 | 1.09 | N/A |
| 250 µg/mL | CHO | 396324 | 29328 | 13.51 | 9.79 |
| | Sp2/0 | 268182 | 240090 | 1.12 | 2.16 |
| 50 µg/mL | CHO | 96199 | 6889 | 13.96 | 13.45 |
| | Sp2/0 | 75560 | 31408 | 2.41 | -3.26 |
| 5 µg/mL | CHO | 57330 | 53185 | 1.08 | -1.42 |
| | Sp2/0 | 7820 | 667 | 11.72 | -4.74 |

Data represents peak intensity values from G0F and G1F glycans from three CNTO 328 cell line mixtures: CHO only, CHO:Sp20 (50:50) and Sp20 only. The G0F:G1F peak intensity ratios for IA pretreated serum samples (250, 50 and 5 µg/mL) were compared against non-pretreated CHO, 50:50, or Sp2/0 buffer controls to calculate % bias. Mean % bias across the serum concentrations was 2.80%.

Materials. CNTO 328, a recombinant monoclonal IgG1 antibody, produced in either Chinese hamster ovary (CHO) or Sp2/0 mouse myeloma cells, was produced in-house at Janssen R&D LLC. Anti-idiotypic antibodies against CNTO 328 were produced in-house at Janssen R&D LLC. Poros®-AL activated media used for conjugation was purchased from Applied Biosystems (Life Technologies, 1-6029-06). Glycine (G7403), sodium sulfate (239313), sodium cyanoborohydride (296945) and dithiothreitol (DTT; D5545) were purchased from Sigma. Acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA; 9835-03), H2O with 0.1% TFA (9836-03), sodium...
containing the therapeutic as well as a master mix containing a biotinylated anti-idiotypic CNTO 328 capture antibody and a ruthenium labeled anti-idiotypic CNTO 328 detection antibody, binding to a different epitope on CNTO 328 from the capture antibody, were added to a streptavidin-coated MSD plate and incubated for 90 min. The plate was washed and MSD Read Buffer was diluted 1:4 and added to the plate. The plate was read on the MSD sector imager. This validated method had previously demonstrated equivalent reactivity to and recovery of CHO and Sp2/0-derived products (Table S1).

Immunoaffinity support generation and pretreatment procedure. A CNTO 328 anti-idiotypic mAb was concentrated using a 10 kD molecular weight cut-off centrifugation spin filter (EMD Millipore, UFC901024) prior to coupling. The concentrated mAb was conjugated to POROS®-AL activated media at a ratio of 1:40 (w/w) media to antibody. Heated 2 M Na₂SO₄ was added to the anti-idiotype mAb before combining with the activated POROS®-AL media. The final conjugation solution was brought up to a volume of 1 mL per 100 mg of media in 1× PBS, pH 6.0. NaCNBH₃ was added at 100 mM to the final volume of conjugate resin. The conjugation reaction was allowed to proceed overnight at room temperature with end-over-end mixing. Unbound anti-idiotype mAb from the conjugation was collected.

Figure 6. Deconvoluted MS data from method sensitivity of G0F/G1F glycans from CHO:Sp20 (1:1) spiked serum samples following immunoaffinity purification and enrichment. Concentrations from 10 (C), 5 (D) and 2.5 (E) μg/mL were IAP extracted from pooled cyno serum and analyzed for relative %G1F based on peak intensity. Serum samples were assessed against the relative %G1F of the non-pretreated (A) and IAP pretreated buffer controls (B).

hydroxide (5672-02) and hydrochloric acid (5619-02) were purchased from J.T. Baker. TRIS-HCl 1M (51238) was purchased from Lonza. Phosphate buffered saline (1×) was purchased from Cellgro® (Mediatech Inc., 21-030-CM). Pooled cynomolgus monkey serum (CYNSRM) was purchased from Bioreclamation.

Preparation of samples for method development. CNTO 328 controls for analytical method development were spiked in 1x PBS buffer at 11 different CHO:Sp2/0 ratios as %CHO values of 0, 25, 33.3, 40, 44.4, 50, 55.6, 60, 66.7, 75 and 100%. For method development of controls in a biological matrix, CHO and Sp2/0-produced CNTO 328 drug products were spiked alone or combined in equivalent amounts in cynomolgus monkey serum. The CNTO 328 drug products were also spiked in 1x PBS buffer in equivalent amounts only. CNTO 328 concentrations in buffer and serum controls were verified by an electrochemiluminescence-based immunoassay platform (Mesoscale Discovery, SECTOR® Imager 6000). Alternatively, CNTO 328 concentrations in spiked buffer controls were verified using a Nanodrop ND-1000 (Thermo Scientific) at 280 nm. Aliquots of the spiked samples were stored at −70°C and thawed prior to analysis.

Therapeutic mAb immunoassay. The CNTO 328 immunoassay employed a sandwich format with electrochemiluminescence detection. Briefly, standards, quality controls and samples containing the therapeutic as well as a master mix containing a biotinylated anti-idiotypic CNTO 328 capture antibody and a ruthenium labeled anti-idiotypic CNTO 328 detection antibody, binding to a different epitope on CNTO 328 from the capture antibody, were added to a streptavidin-coated MSD plate and incubated for 90 min. The plate was washed and MSD Read Buffer was diluted 1:4 and added to the plate. The plate was read on the MSD sector imager. This validated method had previously demonstrated equivalent reactivity to and recovery of CHO and Sp2/0-derived products (Table S1).
Table 2. Method validation data

| Assay parameter       | Sample | Replicate | G1F mass | % G1F abundance (% of G0F) | % Bias | % CV |
|-----------------------|--------|-----------|----------|-----------------------------|--------|------|
|                       |        |           |          |                             |        |      |
|                       | 250 μg/mL | 1        | 50774.40 | 43.50                        | 0.23   |      |
|                       |         | 2        | 50774.33 | 42.30                        | −1.56  | 1.93 |
|                       |         | 3        | 50774.64 | 43.90                        | −1.57  |      |
|                       | 50 μg/mL | 1        | 50775.80 | 46.10                        | 6.22   |      |
|                       |         | 2        | 50773.89 | 44.30                        | 3.10   | 2.10 |
|                       |         | 3        | 50774.40 | 44.70                        | 0.22   |      |
|                       | 10 μg/mL | 1        | 50771.11 | 40.90                        | −4.82  |      |
|                       |         | 2        | 50775.04 | 48.20                        | 8.07   | 10.81|
|                       |         | 3        | 50776.80 | 39.60                        | −3.65  |      |
|                       |         |           |          |                             |        |      |
|                       | 250 μg/mL | 1        | 50774.42 | 43.50                        | 1.16   |      |
|                       |         | 2        | 50773.32 | 44.00                        | 2.33   | 1.49 |
|                       |         | 3        | 50774.69 | 44.80                        | 4.19   |      |
|                       | 50 μg/mL | 1        | 50775.73 | 46.30                        | 7.67   |      |
|                       |         | 2        | 50774.96 | 41.60                        | −3.26  | 5.37 |
|                       |         | 3        | 50774.66 | 44.50                        | 3.49   |      |
|                       | 10 μg/mL | 1        | 50776.80 | 39.60                        | −3.65  |      |
|                       |         | 2        | 50776.29 | 40.40                        | −1.70  | 6.73 |
|                       |         | 3        | 50775.13 | 44.80                        | 9.00   |      |
|                       |         |           |          |                             |        |      |
|                       | 10 μg/mL | N/A      | 50778.33 | 38.10                        | −7.30  |      |
|                       | 5 μg/mL  | N/A      | 50776.48 | 43.60                        | 6.08   | N/A  |
|                       | 2.5 μg/mL| N/A      | 50776.04 | 43.80                        | 6.57   |      |
|                       | 250 μg/mL| N/A      | 50775.10 | 44.50                        | −0.22  |      |
|                       | 50 μg/mL | N/A      | 50774.54 | 45.60                        | 2.24   | N/A  |
|                       | 10 μg/mL | N/A      | 50775.10 | 50.70                        | 13.68  |      |

1Deconvoluted mass of the G1F peak was ± 100 ppm of the non-pretreated buffer control; 2The % G0F peak abundance for all samples was normalized to 100%; 3The % bias was calculated against the % G1F abundance of the IAP pretreated PBS buffer control for each assay; the mean % bias for all validation samples was 3.46%; 4The % CV was calculated per concentration for each inter-run and intra-run triplicate set; the mean % CV for all inter-run and intra-run samples was 4.74%.

and concentrated to assess the efficiency of coupling. The % unbound was calculated by A280 measurement of the concentrated conjugation solution. Conjugated resin was quenched for 2 h with 0.5 M TRIS-HCl + 100 mM NaCNBH₃. The resin was washed and stored in 1× PBS, pH 6.

For the purification procedure, resin containing the equivalent of 300 μg anti-CNTO 328 mAb was added to a 2 mL centrifuge column (Pierce Biotechnology, 89896) and washed in 1× PBS, pH 3.0 followed by 1× PBS, pH 6.0 prior to analysis. A volume of 500 μL of sample (serum or 1× PBS) was combined with 500 μL of PBS in a centrifuge column, capped, mixed and incubated at room temperature for 20 min. Following sample incubation, the column was centrifuged at 2500× g and the flow-through fraction was collected and analyzed by immunoassay for extraction efficiency. Resins were washed with 1× PBS, pH 6.0 then eluted with 100 mM glycine, pH 2.0. The eluate was neutralized by adding 500 mM Tris, pH 10.0 at a 5:1 ratio (v/v). The purified sample was concentrated and buffer exchanged into 1× PBS, pH 6.0 with a 10 kD MWCO centrifuge filter. A final sample volume of 200 μL was kept for LC-MS analysis. Each analysis included CNTO 328 spiked PBS buffer samples: (A) IAP purified/enriched buffer sample (pretreatment) and (B) a non-pretreated buffer sample for direct LC-MS analysis. The CNTO 328 PBS buffer samples were used as experimental controls for the immunoaffinity purification and sample enrichment method (A) and for the deconvoluted CNTO 328 heavy chain mass identification (B), respectively. Unspiked pooled cynomolgus monkey serum was also included in every analysis and used as a background control for MS detection.

LC-MS analysis of CNTO 328 glycosylation. CNTO 328 glycosylation analysis was performed by reverse phase separation on an Agilent HPLC 1100 Series (Agilent Technologies) equipped with a 150 × 1.5 mm I.D., 3 μm Intrada WPRP column (Imtak Corp., WPR75). Following the LC separation, an Agilent TOF mass spectrometer (Agilent Technologies, LC/MCD) equipped with an electrospray ionization (ESI) source was used for sample analysis. All samples were reduced for 30 min at 37°C with 100 mM DTT prior to analysis. A total of 100 μL of purified and reduced sample was injected into 80:20 initial condition of mobile phase A (0.1% TFA in water): mobile phase B (0.1% TFA in ACN:water, 80:20). The gradient separation was
performed at a flow rate of 200 μL/min from 20% B to 60% B over 15 min at a column temperature of 60°C. The column was washed at 100% B with a saw tooth gradient and allowed to equilibrate to initial conditions before starting the next injection. The UV (UV) detection wavelength was set at 280 nm. The mass spectra were acquired in positive ion mode with an m/z acquisition range from 1,000–3,200. The ESI-MS conditions were as follows: capillary voltage: 4,000 V, fragmentor voltage: 300 V, drying gas temp: 360°C, drying gas flow: 8 L/min and nebulizer pressure: 45 psi. Early development and feasibility assessment of the MS method using CNTO 328 PBS buffer controls without pretreatment was performed on a LTQ Orbitrap XL ETD (Thermo Scientific).

**Data analysis.** The spectral data of the reduced CNTO 328 heavy chains were deconvoluted (maximum entropy) with Agilent MassHunter Qualitative Analysis software. The deconvolution parameters were: S/N (signal/noise) threshold: 30, m/z range: 1,400–3,000, peak width: 2.0 m/z, average mass (%): 90 and isotope width: unspecified. The peak abundance, a measure of peak height intensity, of the deconvoluted profile was used to determine the relative ratio between the selected glycosylation peaks of interest (G0F and G1F). The G0F peak abundance was normalized to 100% and the % abundance of the G1F peak was compared against the IAP pretreated CNTO 328 control samples in the PBS buffer. The observed mass for the glycosylated heavy chain for all IAP pretreated samples was confirmed within 100 ppm of the G1F peak of the non-pretreated CNTO 328 buffer control.

**Validation parameters.** For validation of the method, the following analyses were performed: intra-run variability, inter-run variability, IAP pretreated and non-pretreated sensitivity and freeze/thaw stability. QC consisted of CHO and Sp2/0-derived CNTO 328 drug products spiked at a 1:1 ratio in pooled cyno serum and were analyzed at 250, 50 and 10 μg/mL for intra-run, inter-run and freeze/thaw analysis. CNTO 328 spiked in pooled cyno serum at 1.25, 2.5, 5 and 10 μg/mL was analyzed for IAP pretreated method sensitivity. Non-pretreated MS method sensitivity was evaluated by direct LC-MS analysis of CNTO 328 PBS buffer controls at the following injection concentration: 100, 200, 400 and 800 ng. MS data for all samples was determined by calculating the % GIF deconvoluted peak abundance against the G0F peak abundance (normalized to 100%). The acceptable limit for all IAP pretreated validation samples was ± 20% of the % GIF peak abundance of the IAP pretreated CNTO 328 PBS control.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Materials**

Supplemental materials may be found at: www.landesbioscience.com/journals/mabs/article/22773
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