ARTICLE

Predicting the HILIC Retention Behavior of the N-Linked Glycopeptides Produced by Trypsin Digestion of Immunoglobulin Gs (IgGs)

Majors J. Badgett,1 Emily Mize,1 Tyler Fletcher,1 Barry Boyes,2 and Ron Orlando1,*

1Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602, USA; and 2Advanced Materials Technology, Wilmington, Delaware 19810, USA

The prediction of the retention behavior/time would facilitate the identification and characterization of glycoproteins, particularly the analytical challenges, such as the characterization of low-abundance glycoforms. This task is essential in the biotherapeutics industry, where the type and amount of glycosylation on recombinant IgG alter the efficacy, function, and immunogenicity. Models exist for the prediction of the hydrophilic interaction liquid chromatography retention of peptides and glycans. Here, we have devised a unified model to predict the retention behavior of glycopeptides from human IgGs and applied this to the analysis of glycopeptides from rabbit IgGs. The combined model is capable of accurately predicting the retention of native IgG glycopeptides on 2 completely different liquid chromatography-mass spectrometry systems.

INTRODUCTION

Glycosylation is one of the most common co- or post-translational modifications, as >50% of eukaryotic proteins are glycosylated.1–3 This modification can affect the structure, function, interaction, and folding of proteins and is linked to numerous diseases, including rheumatoid arthritis, various types of cancer, Crohn’s disease, tuberculosis, among >50 others.4–12 N-Linked glycosylation involves the linking of a carbohydrate through a nitrogen atom onto an asparagine residue that follows the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid residue except proline.13 This modification adds a substantial carbohydrate to the modified protein, increasing the polarity and mass of the protein to a high degree. The analysis and characterization of glycans moieties are essential to understand their function.

Several notable examples of glycosylated proteins in humans include IgGs, which comprise 75% of the antibodies circulating in human blood serum.14 IgGs are essential in the biotherapeutics realm, as many engineered mAb are used to treat diseases. Therefore, the characterization of IgG glycosylation is imperative.8, 10, 14 Examples of biotherapeutic IgGs include anti-TNF treatments for rheumatoid arthritis and Crohn’s disease, as well as trastuzumab (a human IgG mAb) to inhibit human epidermal growth factor receptor 2-dependent tumors in breast carcinomas.15–17 There are 4 subclasses of human IgGs (IgG1, -2, -3, and -4) that have minimal differences in their constant region (>90% homology) but have a glycosylation site at the N297 position, allowing for selective binding to Fcγ receptors. The majority of the glycans at this position have a complex biantennary structure that is core fucosylated, with some having bisecting structures or varying degrees of sialylation. Glycan structures vary based on a human’s physiologic conditions.1, 8, 10, 18 One such example is age, as the level of galactosylation changes with age, in addition to a decrease in sialylation the older that one gets.14, 18 Another is pregnancy, which leads to an increase in both sialylation and galactosylation.8, 10, 14 These examples highlight the importance of the knowledge of the identities of glycans present on the IgGs.

The microheterogeneity and diversity of glycans make identification challenging, especially between structural or linkage isomers.6 Tandem mass spectrometry (MS/MS) has emerged as a vital tool for glycan analysis, as it can provide structural information that can help in identification. However, isomeric identification can be challenging without use of a method of separation before MS analysis. Since glycosylation is a highly hydrophilic addition, hydrophilic interaction liquid chromatography (HILIC) has been shown to help in this regard and to provide a consistent, predictable retention.20–22 The monitoring of retention can aid in the identification of relevant sialic acid linkage isomers in IgGs that contribute to anti-inflammatory responses. For instance, α2- to -6-linked sialylation increases anti-inflammatory activity, whereas α2- to -3-linked sialylation does not. This difference is identifiable with methods of separation that use the change in hydrophilicity based on linkage.3, 7, 10, 14, 23–25

*ADDRESS CORRESPONDENCE TO: Ron Orlando, Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Rd., Athens, GA 30602, USA (Phone: 706-542-4412; Fax: 706-542-4412; E-mail: rorlando@ccrc.uga.edu). doi: 10.7171/jbt.18-2904-002

Journal of Biomolecular Techniques 29:98–104 © 2018 ABRF
A model was previously created that predicts HILIC peptide retention from amino acid composition, and another model predicts glycan retention on columns with the same HILIC stationary phase. Ideally, those models can be paired together to predict the retention of glycopeptides. If successful, the integrated model would help to facilitate glycopeptide identification and characterization, as well as to suggest the identity of structural or linkage isomers. The glycan and peptide model was combined by merely replacing the retention coefficient attributed to the procainamide, with the retention coefficient calculated for the peptide. The main portion of the work presented here details the analysis of the glycopeptide retention from human and rabbit IgGs on a Halo Penta-HILIC column and provides a comparison with predicted retention from the peptide and glycan models previously created. The predicted retention values of the human IgG glycopeptides were in reasonable agreement with those determined experimentally, deviating by an average of 0.13 glucose units (GU) or 15 s in the 80 min liquid chromatography (LC) gradient used to analyze these glycopeptides. The analysis of the human glycopeptides allowed us to derive a coefficient for the positional isomers of the A2G1 structures and for bisected N-acetylglucosamine (GlcNAc) moieties, which were not included in our glycan HILIC model. The combined model with the new coefficients was evaluated using LC-MS data from tryptic digests of rabbit IgGs. Good agreement was found with the rabbit glycopeptide retention data, which had an average deviation of 0.17 GU or 19 s between the predicted and the actual experimental retention values. The close agreement between the predicted and experimental retention times of these glycopeptides suggests that this is a useful tool for glycoprotein characterization and suggests that the expansion of this model to other glycopeptides is a worthwhile endeavor.

**MATERIALS AND METHODS**

**Materials**

Acetonitrile (ACN), dextran ladder, DTT, human serum (human male, AB plasma), iodoacetamide (IDA), and N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin were purchased from MilliporeSigma (Burlington, MA, USA). Sequencing-grade trypsin and chymotrypsin were purchased from Promega (San Luis Obispo, CA, USA). Ammonium bicarbonate (AMBIC), ammonium formate, and formic acid (FA) were purchased from Fluka (Mexico City, Mexico). Rabbit serum was obtained from Glyco-scientific LLC (Athens, GA, USA).

**Glycoprotein separation and digestion**

Human IgGs were separated from human serum (MilliporeSigma) using a HiTrap Protein G column (General Electric Company, Fairfield, CT, USA). Proteins were reduced using 10 mM DTT and then alkylated using 55 mM IDA. Sequencing-grade trypsin or chymotrypsin was added at 50:1 (w/w, protein/trypsin) for incubation overnight in 50 mM AMBIC (pH 7.0) at 37°C.

Proteins from rabbit IgGs were reduced using 200 mM DTT and alkylated using 1 M IDA. An aliquot of 200 mM DTT was then added to each sample. N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin was added at 50:1 (w/w, protein/trypsin) for an 18 h incubation in 50 mM AMBIC (pH 7.0) at 37°C. Glycopeptides were then purified from the digest using J.T. Baker (Center Valley, PA, USA) Octadecyl disposable extraction columns.

**LC-MS/MS settings and instrumentation**

The human IgG samples were analyzed on a 4000 QTRAP (Applied Biosystems/MDS Sciex, Foster City, CA, USA). Samples were suspended in 25% H2O, 75% ACN, and 0.1% FA for direct injection into the LC system. Peptides were separated by a 2.1 mm × 15 cm Halo Penta-HILIC column packed with 2.7 μm diameter superficially porous particles that have a 90 Å pore diameter (Advanced Materials Technology, Wilmington, DE, USA) using a Nexera ultra-fast LC (Shimadzu, Kyoto, Japan). The temperature of the column was 60°C. The gradient used for each sample was 22–52% H2O over 80 min at a 0.4 ml/min flow rate. The aqueous solvent contained 0.1% FA and 50 mM ammonium formate, and the organic solvent was pure ACN. Spectra were obtained using an electrospray ionization source. A selected reaction monitoring method was used to select precursor and fragment masses for both peptides and glycopeptides of interest.

Rabbit IgG samples were analyzed on the same LC-MS system as human IgG samples. The only differences were the following: the samples were suspended in 30% H2O, 70% ACN, and 0.1% FA; the gradient used was 30–40% H2O at a 0.6 ml/min flow rate; and the column temperature was 70°C.

Data were also acquired using a Finnigan LTQ (Thermo Fisher Scientific, Waltham, MA, USA) in an 1100 Series Capillary LC system (Agilent Technologies, Palo Alto, CA, USA) with an electrospray ionization source that used spray tips made in-house. Peptides/glycopeptides were separated using a 200 μm × 150 mm Halo Penta-HILIC column packed with 2.7 μm diameter superficially porous particles at room temperature. The gradient elution conditions used a linear increase in the aqueous solvent from 5 to 70%, >90 min, at a 2 μl/min flow rate. The aqueous solution contained 0.1% FA with 50 mM ammonium formate, and the organic solvent was ACN with 0.1% FA. The settings for the mass spectrometer included the procurement of the 5 most intense ions from each full mass spectrum for fragmentation using collision-induced dissociation, and the resulting MS/MS spectra were recorded.
Glycopeptide retention analysis

Glycopeptide retention times were determined manually using the apex of the peaks displayed in Analyst software (Applied Biosystems/MDS Scien). The dextran ladder reference was labeled with procainamide and analyzed using identical experimental conditions both before and after the samples. The retention times for each GU analyte were determined using the Analyst software, and these values were then graphed, and a logarithmic line was fit that was subsequently used to convert retention in minutes to GU for sample analytes. This was done so the model may be used to predict retention times on different LC-MS systems with different chromatographic conditions. Peptide retention times in minutes were converted to GU using the dextran standard data, and experimental retention times were compared with predicted retention times using the peptide and glycan models created in-house.

RESULTS AND DISCUSSION

Human IgG1 to -4 yield glycopeptides with 3 different amino acid sequences at the glycosylation site of interest (N297) after trypsin digestion, as IgG2 and IgG3 have the same sequence. The nonglycosylated forms of these peptides were identified in the IgG samples after digestion, and their actual retention values were compared with predicted retention values from a previously made model (Table 1).

The peptide model is based on amino acid composition and is able to sum amino acid coefficients related to their hydrophilicities with an intercept to predict retention.20 The retention values are expressed in GU from procainamide-labeled dextran samples that were used as retention time calibrants. The GU retention index enables the comparison of retention on different LC-MS systems with various chromatographic conditions (size of the column, temperature of the column, gradient slope, buffer composition, and flow rate). The deviations from experimental times and predicted times in Table 1 are extremely low, suggesting that the prediction is reasonably accurate for these species. The IgG peptides containing N297 only differ by the substitution of phenylalanine for tyrosine, which have coefficients in the previously made peptide retention prediction model of −0.967 and −0.430 GU, respectively.20 Their negative values indicate that they are hydrophobic, and peptides with these residues will elute earlier on the HILIC column. The substitution of 1 phenylalanine residue for a tyrosine residue (IgG1–IgG4) would result in a predicted difference of 0.537 GU from the coefficients, and the actual difference was 0.550 GU (a 0.013 GU difference). The substitution of 2 F residues for 2 Y residues (IgG1–IgG2/3) would result in a predicted difference of 1.074 GU, and the actual difference was 1.202 GU (a 0.128 GU difference). Both of these comparisons demonstrate that the peptide model is fully capable of accurately predicting the retention times of native peptides that are very similar in composition.

Glycopeptide retention prediction

Glycosylated forms of the peptides in Table 1 were identified in the IgG samples, and their structures and retention times were analyzed (Table 2). The number of glycopeptide identifications for each subclass is in direct correlation with their abundances in human serum, as IgG1 has an ~66% abundance, IgG2 and -3 have a combined ~30% abundance, and IgG4 has an ~4% abundance.26 The N-linked glycans studied herein are comprised of several retention-affecting elements, namely GlcNAc, mannose (Man), galactose (Gal), and core fucose. These chromatographically influencing elements, in combination with the individual influences of the peptide amino acids, affect retention reproducibly, allowing for the creation of a predictive model. The retention of glycopeptides is driven by the interaction of hydrophilic functional groups of the N-linked glycan and the peptide with the HILIC stationary phase and water-rich layer, and changes in glycopeptide structure or composition will result in greater or lesser retention on the HILIC column. The resolution of isomeric glycoforms, such as α2,3- or α2,6-linked sialic acid species, is possible because of these differences in the degree of interaction between the glycans and the HILIC column, allowing for separate analysis of not only individual glycan species but also of their structural isomers.27

A variety of glycopeptides with different glycan structures were analyzed (see Fig. 2 for cartoon diagrams of the glycan structures). The A2 structure, which has 2 GlcNAc moieties, has the shortest retention, and the retention

| Table 1 |
|---|
| Experimental retention times of human IgG native peptides compared with predicted retention values |
| Source | Peptide | Mass | Exp RT, min | Exp RT, GU | Predicted RT, GU | Deviation, GU |
| IgG1 | EEQYNSTYR | 1189.5 | 54.06 | 5.888 | 5.946 | 0.058 |
| IgG2/3 | EEQFNSTFR | 1173.5 | 48.82 | 4.687 | 4.872 | 0.185 |
| IgG4 | EEQFNSTYR | 1157.5 | 51.81 | 5.339 | 5.409 | 0.070 |

Exp, Experimental; RT, retention time.
increased as the glycan chain is extended with additional hydrophilic monosaccharides. Glycopeptides that included a G1 structure had doublets corresponding to the linkage of Gal. These isomeric glycans differ by having the terminal Gal on either the antennae, originating with the 3- or 6-linked Man residue. The addition of the Gal to the 6-Man antennae is more hydrophilic, as this antenna is more extended than the alternative branch, and thus, these 2 species can be chromatographically resolved with HILIC—in this case, by an average of 0.211 GU. The difference in retention of the 3-/6-branch Gal attachment was not included in the glycan retention model, as there were not a sufficient number of glycans differing by this moiety to permit an accurate coefficient for these 2 species.22

The ability to combine the HILIC glycan and peptide retention model was evaluated by the calculation of the values for the procainamide-tagged glycans and the native peptides and then the substitution of the predicted values for the peptides with the coefficient for the procainamide. This process mimics what happens structurally, as both the peptide the procainamide moiety are on the reducing terminus of the glycan. Comparison of the predicted and experimental retention of the IgG glycopeptides (Fig. 1 and Table 2) shows good conformity, deviating by an average of 0.13 GU or 16 s in the 80 min LC gradient used to analyze these glycopeptides. The excellent agreement demonstrates that this unified model can be used to give accurate retention values for both of these classes of biopolymers.

Comparison of the calculated and experimental retention values for glycopeptides containing a bisected GlcNAc residue, such as that found on the A3G1 glycans (Fig. 2), was not performed, as bisected GlcNAc residues were not included in the glycan HILIC model.22 Comparisons of the experimental retention to the values obtained for these glycopeptides when the bisected GlcNAc is treated as nonbisected GlcNAcs (Table 3) revealed that these

| Glycan structure | Peptide | Predicted RT, GU | Exp RT, GU | Difference, GU | Difference, s |
|------------------|---------|-----------------|------------|----------------|---------------|
| A2               | IgG₁    | 13.271          | 13.686     | 0.415          | 47            |
| F1A2             | IgG₁    | 14.367          | 14.378     | 0.011          | 1             |
| A2G1             | IgG₁    | 14.458          | 14.545     | 0.087          | 10            |
| A2G1             | IgG₁    | 14.458          | 14.799     | 0.341          | 38            |
| F1A2G1           | IgG₁    | 15.186          | 15.265     | 0.079          | 10            |
| F1A2G1           | IgG₁    | 15.186          | 15.475     | −0.124         | −14           |
| A2G2             | IgG₁    | 15.645          | 15.615     | −0.030         | −3            |
| F1A2G2           | IgG₁    | 16.005          | 16.334     | −0.039         | −4            |
| F1A2             | IgG₂    | 13.293          | 13.183     | −0.110         | −12           |
| F1A2G1           | IgG₂    | 14.112          | 14.020     | −0.092         | −10           |
| F1A2G1           | IgG₂    | 14.112          | 14.229     | 0.117          | 13            |
| F1A2G2           | IgG₂    | 14.931          | 15.068     | 0.137          | 16            |
| F1A2             | IgG₃    | 13.830          | 13.760     | −0.070         | −8            |
| F1A2G2           | IgG₃    | 15.468          | 15.683     | −0.153         | −17           |
| Average absolute deviation | | | | 0.129 | 14 |

FIGURE 1
Plot of the retention times in GU that were predicted by model vs. those obtained experimentally for the peptides and glycopeptides identified by LC-MS analysis of trypsin-digested human serum IgGs.
glycopeptides eluted, on average, 0.9 GU or nearly 2 min earlier than predicted. It is anticipated that the bisecting GlcNAc moiety could be shielded from stationary-phase interaction by the other monosaccharide subunits, thus reducing the interaction of the bisected GlcNAc and causing these glycopeptides to elute earlier than predicted. The use of a coefficient of 0.932 GU for bisecting GlcNAc residues increases the agreement between the predicted and experimental results.

To assess the accuracy of the combined glycopeptide model with the addition of the coefficients for the G1 positional isomers and bisected GlcNAc residues, LC-MS data from a sample that was not used during the creation of either the glycan model or the peptide model were analyzed; specifically, rabbit IgG glycopeptides were analyzed. The amino acid sequence of the glycopeptide is EQQFNSTIR. There is only 1 rabbit IgG Fc glycopeptide, as this species has only 1 subclass of IgGs. The rabbit IgG sample was analyzed in duplicate, and 14 glycopeptides were identified, and the retention times in minutes for each analyte were recorded. With the use of a dextran ladder reference that was run before and after each sample, a graph was constructed, and the resulting logarithmic fit equation was used to convert the experimental retention times in minutes into GU for comparison with the model prediction values. The experimental retention and the predicted using the combined model are listed in Table 4. The comparison of these values reveals an average difference of 0.13 GU (15 s) between actual and predicted values. This level of deviation is comparable with that seen with in the LC-MS analysis of human IgGs and further suggests that the glycopeptide prediction model is reasonably accurate.

**FIGURE 2**
Glycan structures analyzed in the glycopeptide retention prediction model. Each structure with a “G1” can have 2 possible linkages of Gal, and both isoforms are shown. Blue square, GlcNAc; yellow circle, Gal; green circle, Man; red triangle, fucose.

**TABLE 3**
Predicted and actual retention times of glycopeptides containing bisected glycans that were identified in human IgG1

| Glycan structure | Peptide | Predicted RT, GU | Exp RT, GU | Difference, GU | Difference, s |
|-----------------|---------|-----------------|------------|----------------|--------------|
| F1A3            | IgG1    | 15.399          | 14.906     | -0.493         | -56          |
| A3G1            | IgG1    | 15.031          | 13.955     | -1.076         | -122         |
| A3G1            | IgG1    | 15.031          | 14.123     | -0.908         | -103         |
| F1A3            | IgG2    | 14.325          | 13.647     | -0.678         | -77          |
| A3G1            | IgG2    | 13.957          | 12.748     | -1.209         | -137         |
| A3G1            | IgG2    | 13.957          | 12.933     | -1.024         | -116         |
| A3G1            | IgG1    | 14.494          | 13.350     | -1.144         | -130         |
| A3G1            | IgG1    | 14.494          | 13.573     | -0.921         | -104         |
| **Average absolute deviation** | | | 0.932 | 105 |
especially because the rabbit samples were run at a different gradient, column temperature, and flow rate than the human samples.

**CONCLUSIONS**

The ability to sum the predictions from the peptide model and the glycan model demonstrates the ease of predicting N-linked glycopeptide retention. Even though this study was only done on glycopeptides from IgG samples, it suggests that a generic model for the analysis of glycopeptides can be created. We anticipate that combining retention prediction will assist in the identification of isomeric glycans. For instance, the 0.662 GU difference in retention between a pair of glycans, differing only by a 2→3- or a 2→6-linked N-acetylneuraminic acid is well outside of the error in the glycopeptide model demonstrated here. These isomeric glycans would be challenging to differentiate solely by MS/MS analysis. As shown here, our model is capable of distinguishing bisected GlcNAc from other GlcNAc residues. Additional approaches, such as the addition of an internal standard to mark the retention times and the calculation of the relative shifts in glycopeptide retention, instead of the absolute retention of individual components, should improve the accuracy, which in turn, will provide more confident structural assignments. Lastly, there appears to be a synergistic combination of obtaining structural information from both LC retention and MS/MS, and this combination is more powerful than either approach by itself.

**REFERENCES**

1. Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta Gen Subj.* 1999;1473:4–8.

2. Khoury GA, Baliban RC, Floudas CA. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Sci Rep.* 2011;1:pii: srep00090.

3. Zhang P, Woen S, Wang T, et al. Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs. *Drug Discov Today.* 2016;21:740–765.

4. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol.* 2007;25:21–50.

5. Bianco GA, Toscano MA, Ilarregui JM, Rabinovich GA. Impact of protein-glycan interactions in the regulation of autoimmunity and chronic inflammation. *Autoimmun Rev.* 2006;5:349–356.

6. Devakumar A, Mechref Y, Kang P, Novotny MV, Reilly JP. Identification of isomeric N-glycan structures by mass spectrometry with 157 nm laser-induced photofragmentation. *J Am Soc Mass Spectrom.* 2008;19:1027–1040.

7. Abès R, Teillaud JL. Impact of glycosylation on effector functions of therapeutic IgG. *Pharmaceuticals (Basel).* 2010;3:146–157.

8. Huhn C, Selman MH, Ruhaak LR, Deelder AM, Wuhrer M. IgG glycosylation analysis. *Proteomics.* 2009;9:882–913.

9. Henner T. Diseases of glycosylation beyond classical congenital disorders of glycosylation. *Biochim Biophys Acta.* 2012;1820:1306–1317.

10. Lin CW, Tsai MH, Li ST, et al. A common glycan structure on immunoglobulin G for enhancement of effector functions. *Proc Natl Acad Sci USA.* 2015;112:10611–10616.

11. Parekh RB, Dwek RA, Sutton BJ, et al. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature.* 1985;316:452–457.

12. Varki A, Cummings R, Esko J, et al. *Essentials of Glycobiology,* 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2009.
13. Drickamer K, Taylor ME. Evolving views of protein glycosylation. *Trends Biochem Sci*. 1998;23:321–324.
14. Shade K-TC, Anthony RM. Antibody glycosylation and inflammation. *Antibodies*. 2013;2:392–414.
15. Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. *N Engl J Med*. 2007;357:39–51.
16. Kempeni J. Preliminary results of early clinical trials with the fully human anti-TNFalpha monoclonal antibody D2E7. *Ann Rheum Dis*. 1999;58(Suppl 1):I70–I72.
17. Targan SR, Hanauer SB, van Deventer SJ, et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn’s disease. Crohn’s Disease cA2 Study Group. *N Engl J Med*. 1997;337:1029–1035.
18. Wuhrer M, Stam JC, van de Geijn FE, et al. Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. *Proteomics*. 2007;7:4070–4081.
19. Endo T, Kochibe N, Kobata A. Structural study of the carbohydrate moieties of two human immunoglobulin subclasses (IgG2 and IgG4). *Glycoconj J*. 1989;6:57–66.
20. Badgett MJ, Boyes B, Orlando R. Prediction of peptide retention times in hydrophilic interaction liquid chromatography (HILIC) based on amino acid composition. *Chromatography Today*. 2015;8:39–42.
21. Badgett MJ, Boyes B, Orlando R. The separation and quantitation of peptides with and without oxidation of methionine and deamidation of asparagine using hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-MS). *J Am Soc Mass Spectrom*. 2017;28:818–826.
22. Betchy E, Boyes B, Orlando R. Development of a hydrophilic interaction liquid chromatography retention model for procaipamide tagged N-linked glycans. *Chromatography Today*. 2017;10:8–12.
23. Anthony RM, Nimmerjahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science*. 2008;320:373–376.
24. Mancera-Arteu M, Giménez E, Barbosa J, Sanz-Nebot V. Identification and characterization of isomeric N-glycans of human alfa-acid-glycoprotein by stable isotope labelling and ZIC-HILIC-MS in combination with exoglycosidase digestion. *Anal Chim Acta*. 2016;940:92–103.
25. Toegel S, Pabst M, Wu SQ, et al. Phenotype-related differential alpha-2,6- or alpha-2,3-sialylation of glycoprotein N-glycans in human chondrocytes. *Osteoarthritis Cartilage*. 2010;18:240–248.
26. Hashira S, Okitsu-Negishi S, Yoshino K. Placental transfer of IgG subclasses in a Japanese population. *Pediatr Int*. 2000;42:337–342.
27. Tao S, Huang Y, Boyes BE, Orlando R. Liquid chromatography-selected reaction monitoring (LC-SRM) approach for the separation and quantitation of sialylated N-glycans linkage isomers. *Anal Chem*. 2014;86:10584–10590.