Analysis of therapeutic monoclonal antibody glycoforms by mass spectrometry for pharmacokinetics study

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ABSTRACT

Monoclonal antibodies (mAbs), are one of the most important protein drugs have attracted increasing attention. However, the pharmacokinetics of mAbs has not been fully investigated due to the complexity of protein drugs. Traditional immuno-based approaches can not recognize the proteoforms of mAbs because of the long development cycles, prohibitive cost, and interactions between different proteins. Therefore, reliable qualitative and quantitative analysis of the proteoforms of mAbs in biological samples is of crucial importance. Herein, a novel method was developed for absolute quantitation of mAbs and their glycoforms in complex biological samples such as serum and tissues. With the combination of HILIC enrichment and parallel reaction monitoring by high resolution mass spectrometry, most of the glycoforms can be accurately quantified at the fmol level through the use of the model mAb of bevacizumab. More importantly, the structural confirmation can be achieved simultaneously without the need for additional experiments. This strategy can be readily applied to the pharmacokinetic study of glycosylation modification and biomarker discovery for clinical applications.

1. Introduction

Since the first generation of therapeutic monoclonal antibodies (mAbs) has been developed and approved for clinical use, mAbs have provided valuable new platforms and strategies for the treatment of a wide range of diseases such as cancer, inflammation, and neurological diseases [1–5]. In 2012, the United States Food and Drug Administration (FDA) issued guidelines on biosimilar drugs development that emphasizing the importance of evaluating the formulation or minor structural differences that can significantly affect the effectiveness and/or safety of biosimilar drugs, with special reference to certain changes in glycosylation patterns [6]. As glycoproteins with two heavy and light chains, mAbs typically have an N-linked glycosylation site at asparagine in the Fc region of each heavy chain [7] and some specific glycoforms have been shown to have an impact on their effectiveness and safety. For example, firstly, the absence of core fucosylation will enhance antibody-dependent cellular cytotoxicity (ADCC) due to the increasing affinities for the FcγRIIIa receptor [8]. On the other hand, a high level of Fc sialylation has the opposite ADCC effects by decreasing affinities for the FcγRIIIa receptor [9]. Secondly, antibody molecules bearing terminal sialic acid are more sensitive to protease degradation [10]. Thirdly, high mannose glycoforms can decrease serum half-lives, increase immunogenicity and also enhance ADCC effects [11]. These findings indicated the importance of the establishment of precise method to characterize the glycoforms of mAbs.

With the development of mass spectrometry, multiple reaction monitoring (MRM) methods have been widely used in targeted quantitative proteomic analysis via triple quadrupole mass spectrometry [12]. For example, Toyama et al. combined MRM and energy-resolved structural analysis to quantitatively analyze the microheterogeneity of N-glycans in therapeutic antibodies [13]. Hong et al. quantified immunoglobulin G (IgG) and its glycoforms from serum by MRM without glycoprotein enrichment [14]. More recently, the introduction of high resolution and accurate mass spectrometer-the quadrupole orbitrap instruments, which can be operated in parallel reaction monitoring (PRM) mode, offer an alternative to targeted quantitative analysis [15,16]. MRM differs from PRM in that the third quadrupole of a triple quadrupole is replaced with a high resolution and accurate mass analyzer that allows the parallel detection of all target product ions in a single analysis [17]. And consecutive full MS/
MS spectra of a precursor ion can be obtained by PRM analysis in the entire chromatographic elution profile. Therefore, PRM provides a more precise characterization result. In this report, we developed a novel method for qualitative and quantitative analysis of the glycoforms of therapeutic monoclonal antibodies by high resolution mass spectrometry operated in the PRM mode. The method allows the direct determination of the tryptic digest of mAbs in pooled mice serum or liver tissue using PRM analysis at the glycopeptides level without glycopeptide enrichment, which enabled the simultaneous absolute quantitation of the mAbs. Meanwhile, a lower detection limit of glycopeptides can be obtained by this method after hydrophilic interaction chromatography (HILIC) enrichment is performed at the glycopeptides level. This strategy is applicable to the characterization of glycoforms of all glycoproteins and relative/absolute quantitative analysis of various biological samples. It is anticipated that PRM will have potential value for the characterization of constitutions of unknown complex glycopeptides. We anticipate that this method will become widely used for pharmacokinetic investigations and for the discovery and characterization of biomakers.

2. Experimental section

2.1. Materials and reagents

Bevacizumab was purchased from a local drug store. Stable isotope labeled peptide PTFSIDTSDK^{(13C6, 15N2)} was synthesized by Beijing Scilight Biotechnology, LLC. PNGase F was purchased from New England Biolabs (Ipswich, MA, USA). Urea was purchased from Bio Basic Inc (Ontario, Canada). ACN (acetonitrile, HPLC grade) was purchased from Waters (Milford, MA, USA). GELoader tips (20 µL) were purchased from Eppendorf (Hamburg, Germany). Click maltose-HILIC beads (4 µm, 100 Å) were kindly provided by Prof. Xinniao Liang (Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian, China). Dionized water was used in all of the experiments and was purified with a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Sample preparation

100 µg mAbs (25 µg/µL) standard was dissolved in 94 µL denaturing buffer (50 mM HEPES and 8 M urea, pH=8.0). 2 µL DTT (1 M) was added to reach the final concentration of 20 mM and the solution was then incubated in a 60 °C water bath for 1 h. Then 0.74 mg IAA was added to reach the final concentration of 40 mM, followed by incubation in the dark at room temperature for 40 min. 700 µL buffer (50 mM HEPES, pH=8.0) was added to make the concentration of urea be 1 M, then 6 µg trypsin was added and the solution was incubated in a 37 °C water bath for 16 h. 5 µL FA was added to quench the digestion. 10 µL stable isotope labeled internal standard (SIL-IS) peptide solution (25 ng/µL) was added and then the solution was desalted by Oasis HLB cartridge. The elution was lyophilized and then redissolved by 1000 µL 0.1% FA for analysis by nanoLC-HRMS.

The N-glycans were released by adding 1 µL (500 units) of PNGase F in 200 µL of 10 mM NH₄HCO₃ and incubating at 37 °C overnight with gentle shaking after the digestion and lyophilization.

2.3. Preparation of calibration standards, quality control samples and matrix effect samples

The mAbs standard (25 µg/µL) was diluted with water to prepare a series of standard solutions (0.10, 0.20, 0.50, 1.00, 5.00 and 10.00 µg/µL) and quality control (QC) solutions (0.20, 1.00 and 8.00 µg/µL). A similar stock solution of SIL-IS was diluted to give a 25 ng/µL IS working solution. For serum calibration standards and QC samples, 2 µL standard solutions were spiked into 2 µL pooled mice serum. Then the mixtures were subsequently digested with trypsin and lyophilized by the method described above. For liver tissue calibration standards and QC samples, 2 µL standard solutions were spiked into 6 µL pooled mice liver tissue extract. Then the mixtures were subsequently digested with trypsin and lyophilized by the method described above. For matrix effect (ME) samples, 2 µL QC solutions were directly digested with trypsin and lyophilized by the method described above. The BAL B/C mice purchased from Dalian Medical University (Dalian, China). All samples were redissolved with 100 µL 0.1% FA for analysis by nanoLC-HRMS operated in PRM mode.

2.4. Enrichment of N-glycopeptides by HILIC

HILIC enrichment were performed by HILIC-SPE tips which were prepared as described in a previous report [18]. The GELoader tip was first packed with a little cotton wool which served as the sieve. Then 5 mg Click maltose-HILIC beads suspended in 50 µL 80% ACN/1% TFA (v/v) were spiked into the tip by centrifugation at 4000 g and then the beads were washed by 50 µL 80% ACN/1% TFA (v/v) again. Lyophilized tryptic digest of 0.4 µg mAbs with serum or liver tissue extract dissolved by 50 µL 80% ACN/1% TFA (v/v) was spiked into the tip by centrifugation at 4000 g for 20 min. Then, the tip was washed by 40 µL 80% ACN/1% TFA (v/v) to remove the nonspecific adsorption for 3 times. Finally, the tip was eluted by 40 µL 10% ACN for 2 times. The elution was lyophilized and stored at −20 °C until analysis. All samples were redissolved with 100 µL 0.1% FA for analysis by nanoLC-HRMS operated in PRM mode.

2.5. Mass spectrometry analysis

All analyses were performed by a Q-Exactive (Quadrupole-orbitrap) mass spectrometer equipped with a nanoelectrospray ionization source (Thermo, USA). Samples were loaded by a home-made capillary C18 trap column (5 cm×200 µm) and then separated by a homemade C18 column (15 cm×75 µm) on an Ultimate 3000 series UHPLC (Thermo, USA). The flow rate of NC_pump was 300 nL/min and linear gradient was 60 min (from 5–45% B over 30 min; mobile phase A=0.1% FA/H₂O and B=80% ACN/0.1% FA).

For the analysis of glycopeptides from mAbs, full MS scans were acquired in the mass range of 400–2500 m/z with mass resolution of 70000. The AGC target value was set at 1000000 and the maximum injection time was 100 ms. Twelve of the most intense peaks were fragmented with higher-energy collisional dissociation (HCD) with collision energy of 22. MS/MS was obtained with a resolution of 17500. The AGC target was 500000 and the maximum injection time was 60 ms. Dynamic exclusion was set for 30.0 s. The system control and data collection were performed in Xcalibur software version 2.1.

For the quantitative analysis of mAb and/or its glycopeptides in PRM mode, one Full-MS SIM and ten Targeted-MS2 was set as a detection cycle. Full MS scans were acquired in the mass range of 400–2500 m/z with a mass resolution of 70000. The AGC target value was set at 3000000 and the maximum injection time was 100 ms. The MS1 selection window was set at ± 1 Da. MS/MS was obtained with a resolution of 35000. The AGC target was 1000000 and maximum injection time was 120 ms. The system control and data collection were performed in Xcalibur software version 2.1.

2.6. Database search

For unique peptides analysis, the obtained *.raw files were first converted to *.mgf files by Proteome Discoverer 1.3 (Thermo, USA) with default parameters and then searched by Mascot Daemon (version
2.3.0. Matrix Science, London, UK) against a bevacizumab database, a Uniprot human database and Uniprot mouse database respectively. Preference settings are shown below: the mass tolerance was 10 ppm and MS/MS tolerance was 0.05 Da. Enzyme was set as Trypsin with an allowance for two missed cleavage sites. The fixed modification was carboxyamidomethylation (C, 57.0215 Da) and the variable modification was oxidized methionine (15.9949 Da). The significance threshold was set as \( p < 0.01 \) and the ion score cut-off was 20.

For N-glycopeptide analysis, *.csv files of deglycosylated peptides were obtained first by the search method described above. Deamidated asparagine (0.9840 Da) was set as a variable modification and other preference settings were just the same as above. And the *.csv files were converted to *.ppl files by ArMone (http://www.bioanalysis.dicp.ac.cn/proteomics/software/ArMone2.html) [19]. Then, *.raw files of N-glycopeptides analysis were converted to *.mzXML files by MS convert (http://proteowizard.sourceforge.net/). Finally, the *.ppl files and *.mzXML files were loaded into ArMone to obtain the N-glycopeptide information by automatical matching.

3. Results and discussion

In this study, we developed a method for qualitative and quantitative analysis of the glycoforms of a therapeutic monoclonal antibody using parallel reaction monitoring by high resolution mass spectrometry. Specifically, we systematically studied the unique peptides and glycopeptides of bevacizumab. Bevacizumab has one glycosylation site (R.EEQYN*STYR.V) which was identical to that of the IgG1 [20], therefore we also studied the glycoforms of human IgG1. Based on these measurement we found that the glycoforms only belonged to bevacizumab. Calibration curve samples were directly analyzed by Q-Exactive mass spectrometry using the PRM mode without performing glycopeptide enrichment. Using this technique we were able to simultaneously determine the relative quantitation of glycopeptides and the absolute quantitation of mAbs. Moreover, a lower detection limit of glycopeptides could be obtained by PRM analysis after HILIC enrichment was performed at the glycopeptide level.

3.1. Quantitation of Bevacizumab

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that contains 667 amino acid residues and has a molecular weight of approximately 149 kDa with one N-glycosylation site that is identical to IgG1 (R.EEQYN*STYR.V). The structure of bevacizumab is similar to that of IgG's, but the various regions of the light and heavy chains especially the complementarity-determining region (CDR) can differ greatly between antibodies. In silico tryptic digestion of variable regions of bevacizumab revealed eight theoretical trypsin peptides that were not present in IgG. The sequences of bevacizumab are shown in Fig. 1. It was determined that six of the eight peptides could not be used for quantification purposes due to their molecular mass and size, as well as the existence of post-translational modifications and unstable amino acids (such as methionine or cysteine) in their sequences. Moreover, based on additional LC-MS/MS analysis of tryptic digest, it was found that one of the other two peptides could not be used for quantitation because of its poorly resolved chromatographic peak. Finally, only one peptide (peptide 1, R.FTFSLDTSK.S) was chosen for quantitative purpose that was not present in any other known proteins. The stable isotope labeled peptide FTFSLDTSK(13C6, 15N2) was synthesized as internal standard for quantitation.

As orbitrap analyzer permits the parallel detection of all target product ions in one scan, only the m/z value of the precursor ion need to be determined. We set \([\text{M}+2\text{H}]^{2+}\) m/z 523.26 and 526.88 as theoretical values for peptide 1 and its SIL-IS respectively. The sequence and retention time of the two peptides are listed in Table 1. The MS/MS spectra of peptide 1 and its SIL-IS are shown in Fig. 2(a) and (b). We extracted the chromatographic peaks of the three top intensities of the product ions and established their respective calibration curves. We then chose one that had the best linear regression coefficient as extracted ion to establish calibration curves. Details regarding to the extracted ions for the peptide1 and SIL-IS are summarized in Table 1.

Equal volumes of calibration curve samples, QC samples and ME samples were directly detected using the PRM method by nanoLC-HRMS. The linearity of calibration curves were evaluated by linear least-squares regression with a 1/X2 weighting using internal standard with the Xcalibur software version 2.1. This PRM method demonstrated good linearity of serum and liver tissue calibration curves using the chosen unique peptide combined SIL-IS in the range from 2.00 µg/mL to 200.00 µg/mL. The regression coefficient (\( r^2 \)) of serum and liver tissue calibration curves were 0.9912 and 0.9976 respectively. The calibration curves are shown in Fig. S1(a) and (b). As shown in Table 2, accurate (as relative error, %RE) and precision (as relative standard deviation, %RSD) of serum and liver tissue QC samples were estimated by analysis of three replicate QC samples and were all within ± 15%. The matrix effect of serum samples were 59.37 ± 2.03, 44.84 ± 1.49, 32.40 ± 1.88% at low, medium and high QC concentrations respectively and 28.48 ± 1.48% for the IS. The matrix effect of liver tissue samples were 190.01 ± 16.49, 104.61 ± 7.31, 67.73 ± 0.93% at low, medium and high QC concentrations respectively and 64.20 ± 7.23% for the IS.

3.2. Characterization of Glycopeptides

In contrast with traditional small molecule drugs, mAbs have very complicated pharmacokinetic or pharmacodynamic behavior due to the complexity of protein drugs. In particular, many studies have shown that glycosylation has a great impact on the effectiveness and safety of mAbs. Therefore, it is essential to comprehensively characterize the glycoforms of mAbs and quantitatively monitor their specific glycoforms. However, the identification of intact glycopeptides has been a challenge in MS-based proteomics. In this study, the characterization of glycopeptides was performed by searching a database in ArMone that was developed in our previous report for automatic and high throughput characterization of intact N-glycopeptides [19]. Both tryptic digest of bevacizumab standard and deglycosylation samples were submitted to LC-MS/MS for glycopeptide analysis. The Y1 ion (i.e. the peptide with a GlcNAc) and the structure of glycan were determined by the spectra of intact glycopeptides and searching a glycan database, respectively. By combining the information regarding the N-glycosites provided by deglycosylation samples with the molecular weight calculated from Y1 ion, the sequences of intact glycopeptides were identified. All of these processes were automatically performed using ArMone software platform. Moreover, this strategy can also be applied to large scale and high throughput characterization of intact glycopeptides from complex biological samples.

We characterized 21 glycoforms of bevacizumab including those common glycoforms in mAbs such as H3N4F1 (Hex3HexNAc4Fuc1, also called G0F), H4N4F1(Hex4HexNAc4Fuc1, also called G1F), H5N4F1 (Hex5HexNAc4Fuc1, also called G2F), H4N3F1 (Hex4HexNAc3Fuc1, also called G1F-GlcNAc) and those that can enhance ADC likeH4N4 (Hex4HexNAc4, also called A-Fuc) and so on. Because Q-Exctive instrument can trap low molecular weight fragment ions, either the Y1 ion or the oxonium ions such as those found at m/z 204.08 (HexNAc), 274.09 (NeuAc-H2O), 366.14(Hex1HexNAc) can be clearly observed in the MS/MS spectra. The MS/MS spectrum of glycopeptide II is shown in Fig. 2(c). Accurate molecular weight combined with oxonium ions and Y1 ion can improve the accuracy of glycopeptides identification. Since the glycosylation site (R.EEQYN*STYR.V) of bevacizumab was the same as that of IgG1’s, we excluded the glycopeptides that present in both bevacizumab and IgG to improve the accuracy of the quantitation of complex biological samples. Through a combination of a data base search and a compr-
Fig. 1. The sequences and the disulfide bonds information of bevacizumab. The sequences with yellow background are the variable regions of the light and heavy chains of bevacizumab. The red asparagine is the N-glycosylation site.

Table 1

<table>
<thead>
<tr>
<th>number</th>
<th>name</th>
<th>glycan structure</th>
<th>precursor ion (m/z)</th>
<th>extract ion (m/z)</th>
<th>retention time (min)</th>
<th>collision energy (%)</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>H3N3</td>
<td></td>
<td>1142.96 (+2)</td>
<td>1392.59</td>
<td>29.77</td>
<td>22</td>
</tr>
<tr>
<td>II</td>
<td>H5N2</td>
<td></td>
<td>1203.47 (+2)</td>
<td>1392.59</td>
<td>29.58</td>
<td>22</td>
</tr>
<tr>
<td>III</td>
<td>H6N2</td>
<td></td>
<td>1284.50 (+2)</td>
<td>1392.59</td>
<td>29.52</td>
<td>22</td>
</tr>
<tr>
<td>IV</td>
<td>H4N3F1S1</td>
<td></td>
<td>962.04 (+3)</td>
<td>1392.59</td>
<td>30.53</td>
<td>22</td>
</tr>
<tr>
<td>V</td>
<td>H8N2</td>
<td></td>
<td>964.70 (+3)</td>
<td>1392.59</td>
<td>29.29</td>
<td>22</td>
</tr>
<tr>
<td>VI</td>
<td>H6N3F1</td>
<td></td>
<td>973.05 (+3)</td>
<td>1392.59</td>
<td>29.17</td>
<td>22</td>
</tr>
</tbody>
</table>

Notes: H (circle), hexose; N (square), HexNAc; F (triangle), fucose; S (rhombus), N-acetylneuraminic acid.
A comprehensive review of the literature regarding IgG glycopeptide, [14,20–24] we finally identified six glycopeptides that were only present in bevacizumab as listed in Table 1.

3.3. Quantitation of glycopeptides

The six glycopeptides were determined using PRM by nanoLC-HRMS. The oxonium ions were chosen as extract ions in a previous study. [13,14] For complex samples, however, other glycopeptides may be detected at an overlapping retention time and cannot be distinguished using oxonium ions. In this case, the Y1 ion has prominent advantage because that the peptide sequences of glycopeptides are different. Therefore we chose Y1 ion as a product ion to extract chromatographic peak for quantitation analysis. Information regarding the glycopeptides are listed in Table 1, while the chromatograms are shown in Fig. 3(b) and (d) and the MS/MS spectra of the other five glycopeptides are shown in Fig. S2 in the Supporting Information.

Quantitation of glycopeptides and bevacizumab protein could be performed in a single run without the need for enrichment. When we analyzed calibration standard samples, the glycopeptides were quantified simultaneously. First, to eliminate the presence of endogenous interference, blank matrix samples were determined by the PRM method. The extracted ion chromatograms (XIC) for the blank matrix samples are shown in Fig. 3(a) and (c). For serum calibration standards samples, except one glycopeptide found in low abundance (H4N3F1S1), the other five glycopeptides were determined when the

Table 2

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Serum</th>
<th>Liver Tissue</th>
</tr>
</thead>
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<tr>
<td>Accuracy (RE%)</td>
<td>−12.50</td>
<td>−6.50</td>
</tr>
<tr>
<td>Precision (RSD%)</td>
<td>0.57</td>
<td>9.64</td>
</tr>
<tr>
<td>Matrix effect (%)</td>
<td>59.37 ± 2.03</td>
<td>190.01 ± 16.49</td>
</tr>
</tbody>
</table>

Fig. 2. The representative MS/MS spectra of (a) peptide 1 (R.FTFSLDTSK.S), (b) SIL-IS peptide (R.FTFSLDTSK* .S) and (c) glycopeptide (H5N2) (R.EEQYN*STYR.V) analyzed by Orbitrap mass spectrometry.
concentration of bevacizumab was 20 µg/mL. The two high abundance glycopeptides (H3N3, H5N2) were determined when the concentration of bevacizumab was 4 µg/mL. For liver tissue calibration standards samples, except one low abundance glycopeptide (H4N3F1S1), the other five glycopeptides were determined when the concentration of bevacizumab was 10 µg/mL. The two high abundance glycopeptides (H3N3, H5N2) were determined when the concentration of bevacizumab was 2 µg/mL. The results showed that a varying degree of signal suppression was caused by the different kinds of matrices, and this variation may be related to the degree of complexity of the matrix proteins. The LOD peaks for the glycopeptides are shown in Fig. S3.

Moreover, in order to evaluate the reliability of PRM method of glycopeptide quantitation, we investigated the linearity of the glycopeptides chromatography peak areas and protein concentrations and achieved highly linear fits. In the case of glycopeptide H5N2, the calibration curves of serum and tissue calibration standards are shown in Fig. S1(c) and (d). These results indicated that PRM methods for glycopeptides quantitation analysis provide a reliable approach for glycopeptides quantitation analysis. Furthermore, we can determine the site-specific glycopeptides abundance and the concentration of the protein. Therefore we can evaluate the changing degree of glycosylation in different biological samples.

In complex biological samples, the concentration of mAbs is usually thousands of folds lower than that of the endogenous IgGs. Moreover, non-glycopeptides and some background ions are usually more easily ionized than glycopeptides, and thus the signal of glycopeptides will be suppressed when they are coeluted. Therefore, enrichment is necessary to ensure the detection of glycopeptides. In this study, the enrichment of the glycopeptides was performed by HILIC-SPE tips strategy which can effectively reduce the sample loss and the experimental error [18]. We determined the same spiked concentration of bevacizumab (4.00 µg/mL) with and without HILIC enrichment as shown in Fig. 4. For serum sample, only two highly abundant glycopeptides (H3N3, H5N2) were determined without enrichment, while all of the six glycopeptides were determined with HILIC enrichment. For liver tissue sample, two highly abundant glycopeptides (H3N3, H5N2) were determined without enrichment, while five of the six glycopeptides (except H6N3F1) could be determined after HILIC enrichment. The sensitivity for all glycopeptides was increased by HILIC enrichment. These results indicated that a lower detection limit could be obtained by HILIC enrichment using the HILIC-SPE tips strategy. This enrichment strategy provides an alternative way to improve the sensitivity of glycopeptide analysis especially for the complex biological samples. We have done the preliminary test of pharmacokinetics study of mAb using our developed approach as shown Fig. 5. We anticipate that this strategy will find widespread use for pharmacokinetic investigations of mAbs.

4. Conclusions

A simple and reliable approach was established for quantitative analysis of the glycopeptides of a therapeutic monoclonal antibody by high resolution mass spectrometry operated in the parallel reaction monitoring mode, and the absolute quantitation of mAbs protein was performed simultaneously. The glycopeptides could be detected and quantitatively analyzed at the fmol level in serum and tissue samples and a lower detection limit could be reached by HILIC enrichment. This method demonstrated the advantages of qualitative and quantitative characterization of site-specific glycopeptides and we expected the broad application for pharmacokinetics study for mAbs with the approach developed. Moreover, it provided an alternative method for further study in protein-specific glycoforms characterization of complex biological samples.

Acknowledgement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.01.023.
References


Fig. 4. The chromatograms of six glycopeptides of (a) serum sample without HILIC enrichment, (b) serum sample with HILIC enrichment, (c) liver tissue sample without HILIC enrichment and (d) liver tissue sample with HILIC enrichment.

Fig. 5. Relative blood concentrate-time curve of glycopeptide H5N2 of bevacizumab with HILIC enrichment after administration of a single intravenous 50 mg/kg and 10 mg/kg dose with rats.