Current Mass Spectrometric Tools for the Bioanalyses of Therapeutic Monoclonal Antibodies and Antibody-Drug Conjugates

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The increase in the use of therapeutic monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) has made the detailed bioanalysis of these drugs essential not only for planning optimal therapeutic programs for clinical practice, but also for evaluating the biological equivalencies in the development of other biosimilars. The ligand binding assays that are widely in use now are being replaced rapidly by the highly accurate, sensitive, and selective analytical method using a mass spectrometer. This review will discuss the progress in and challenges observed during the development of a mass spectrometry-based bioanalytical method for therapeutic mAbs and ADCs.

Keywords Therapeutic monoclonal antibody, mass spectrometry, bioanalysis, liquid chromatography, antibody-drug conjugate

(Received September 15, 2017; Accepted November 29, 2017; Published April 10, 2018)

1 Introduction

Therapeutic monoclonal antibodies (mAbs) are medicines containing monoclonal antibodies (mAb), which constitute the main part of the biological immune system, and bind specifically with antigens serving as markers in biological fluids or on the surface of cancerous or therapeutic target cells. More than 50 items of the therapeutic mAbs are currently approved in the United States and Europe, and are used for the treatment of...
cancer, rheumatoid arthritis, infectious diseases, etc. Currently, therapeutic mAbs represent five out of the top ten pharmaceutical products in terms of sales worldwide, and by 2020 it is predicted that more than 70 products will be in the market and their total sales would reach $125 billion. Therapeutic mAbs have the advantages of high in vivo stability and effectiveness sustained over a long time; their half-lives in the blood are commonly 10 to 25 days, and they can be administered just once a week or once in several weeks. In contrast to that seen in the administration of low molecular weight drugs, the pharmacokinetics (PK) of most therapeutic mAbs show non-linear profiles without any concentration dependence. This could be due to the effect of the fetal Fc receptors that catabolize the IgG proteins before the blood transfer and bind to its target antigen. Therapeutic mAbs are administered at high concentrations, usually 1 - 10 mg/kg. For example, the anticancer drug bevacizumab is administered at 5 – 7 mg/kg per dosage, its maximum drug concentration reaches the μg/mL level. Therefore, bioanalyses of the therapeutic mAbs in blood samples seem easier than other bioanalyses targeting trace amounts of a peptide, or disease related proteins. However, there are inherent difficulties underlying the present analytical methods that involve selective analysis of certain therapeutic mAbs, differing only in the sequence of the variable region of IgG proteins, present in a large amount in the blood. In recent years, next-generation antibody drugs such as POTENTIAL antibodies,3,4 recycling antibodies,5,6 or bispecific antibodies7,8 have been developed in order to reduce the dosage and cost of the drug. Since these drugs can be administered at the current dose of 1/10000, a trace analysis of these drugs in the blood would be necessary at the sub-ng/mL level in the future. In the clinical trials and PK studies at pharmaceutical companies, a ligand binding assay (LBA) such as enzyme-linked immunosorbent assay (ELISA) is generally performed, and antibody drugs, which specifically recognize the complementarity-determining regions (CDRs) of each antibody, are used as the capture antibodies. There have been increasing reports that compare the existing ELISAs with the newly developed LC/MS/MS methods for the same analytes, and it is becoming increasingly evident that the concentration results from both techniques may not necessarily be superimposable.9,10 Although the LBA enables a relatively easy and high-throughput analysis, it takes a long time to establish and optimize the method, and the analysis result depends on the quality of the capture antibody. Additionally, the comparison of PK analysis results obtained through ELISA and LC/MS/MS methods reveal that the results of the LC/MS/MS method for maximum blood concentration (C_max) and the area under the blood concentration-time curve (AUC) were different.11,12 For analyzing therapeutic mAbs, global pharmaceutical companies are vigorously developing bioanalytical methods based on the tryptic digestion- LC/MS/MS approach in collaboration with analytical instrument manufacturers.11,13 For the bioanalysis of therapeutic mAbs using LC/MS/MS, high accuracy and robustness along with low-molecular weight drugs are becoming required. This method would also comply with the US Food and Drug Administration (FDA) bioanalysis validation14 in the near future. While the free drug (unliganded form) concentration can be measured by the ELISA method, both the total (liganded and unliganded forms) and free drug concentrations can be quantified by the LC/MS/MS method.

Figure 1 shows a flow diagram of the bioanalysis of the therapeutic mAbs by the tryptic digestion-LC/MS/MS method. The blood samples are diluted with the buffer to attain pH 7.5 – 8.5, followed by the denaturation of the mAbs by the addition of chaotropic reagents such as urea or guanidine hydrochloride. After reducing the disulfide bonds with dithiothreitol (DTT), the free thiol groups are alkylated with iodoacetamide (IAA). Trypsin is added to the reaction at this point, and the digestion is carried out at 37°C for a duration of >12 h. The resultant signature peptides that are specific to the therapeutic mAbs are desalted and purified using a solid-phase extraction cartridge and then analyzed by the reverse-phase LC-MS/MS in the selected reaction monitoring (SRM) mode. As therapeutic mAbs are mostly identical in their sequence to the sequence of human IgG proteins, the signature peptides derived from the CDRs present in the variable regions of each drug are utilized for the analysis. It may be noted here that since the trypsin digestion efficiency might differ with the cleavage site, the MS/MS quantification with two or more signature peptides is recommended.

In this review, we outline the current bioanalytical methods using LC/MS for therapeutic mAbs with our main focus on sample pretreatment, enzymatic digestion process, internal standard compound (IS) selection for high precision analysis, LC separation, and mass spectrometry. We also discuss the intact bioanalytical methods for therapeutic mAbs and antibody-drug conjugates (ADCs) that have recently been reported.

2 Pretreatment of the Biological Samples

2-1 Purification of therapeutic mAbs

According to the report by Heidi et al. of Novartis, the total recovery rate of mAbs from plasma by tryptic digestion-LC/MS/MS method was only 14%; 72% of the loss was attributed...
to the pretreatment process while 32% of the loss was attributed to tryptic digestion. The blood contains many proteins with concentrations ranging over 10 orders of magnitude; 55% of the plasma protein is albumin potentially interfering with quantification of targets. Therefore, the key to improve the recovery rate of IgGs or target drug would be to extract them from the blood. To purify the therapeutic mAbs from the blood samples, affinity purification methods, using either magnetic beads or solid-phase cartridge as the carrier, have been used. For the affinity ligand, either an anti-idiotype antibody that recognizes the CDR of the target drug or the Protein A/G that specifically binds to the constant region of the human IgG, is used. The purification that uses anti-idiotypic antibodies can collect only the free form of the drug, whereas the one using Protein A/G can collect both the free as well as the bound forms from the blood samples. Measurements of the free drug concentration can be used for the evaluation of bioavailability and efficacy of the drugs, whereas that of the total drug concentration allows for the evaluation of the therapeutic index and PK. Therefore, appropriate purification methods can be chosen according to the purpose.

Zhang et al. had successfully automated the immunoaffinity purification process using a liquid handling robot. Magnetic beads bound to the anti-human IgG antibody were placed in a 96-well plate and combined with a robotic dispensing operation to automate the purification of human IgG protein from the plasma. Upon comparing these analytical results with those given by the ELISA method, considerable differences were observed due to the difference in the capture antibody used in each analysis. Taken together, we see that in the affinity purification, the quality of capture antibody used is very important for obtaining accurate analytical results.

2-2 Efficiency technology, speed up, and automation of tryptic digestion

Trypsin is quite frequently used as a digestion enzyme to obtain signature peptides, but other endoproteinases that cleave at different sites such as Lys-C, Glu-C, Arg-C, or Asp-N are also commercially available in sequencing grade, and can be used for obtaining the appropriate peptide fragments. For the tryptic digestion, reaction at 37°C for a duration of >12 h is recommended and this step determines the analysis time. Depending on the location of the target amino acid sequences either on the surface or in the interior of the protein, the accessibility of trypsin and hence its digestion efficiency might differ. To improve this efficiency, a surfactant-aided precipitation/on-pellet-digestion (SOD) using a surfactant, sodium dodecyl sulfate (SDS), has been reported. In the SOD method, after adding the surfactant (SDS) to the sample such as plasma, a reduction by DTT and alkylation of the SH group by IAA are performed as mentioned in the earlier section. Thereafter, it is repeatedly washed with acetone and aqueous acetone solution and the obtained pellet sample is subjected to tryptic digestion. The addition of SDS is expected to (1) improve the denaturation, reduction, and alkylation efficiency, (2) remove the matrix components, and (3) inactivate the endogenous protease inhibitor. By this method, appreciable digestion efficiency and good quantitativeness of the target mAbs could be achieved within a digestion time of 45 min.

Pressure cycling technology (PCT) has been reported to be another method to speed up the tryptic digestion process. In PCT, a digestion reaction is in more than 60 alternating cycles of ultra-high pressure (20000 psi; 1360 atm) for 50 s and atmospheric pressure for 10 s. Under ultra-high pressure, the mAbs undergo a conformational change, hence allowing trypsin to access the interior of the structure. It has been shown that PCT has particularly high digestion efficiency for the hydrophobic proteins and IgGs, which explains how PCT enhances the solubility of the hydrophobic sites of the proteins in the buffer. Since this approach can shorten the trypsin digestion time to about 1 h, PCT-dedicated machines are already in the market. To the best of our knowledge, there is no application of this method to the analysis of therapeutic mAbs yet, but applications are sure to come up in the future due to its high potential. As an improved method to achieve both rapid speed and automated tryptic digestion, the use of a trypsin-immobilized enzyme reactor (IMER) as a pretreatment column, placed in front of an LC system, has been reported. Not only IMERs, but on-line tryptic digestion-LC system combined with the IMER, dedicated buffers, and column-switching systems are also commercially available now.

As a pretreatment column in IMER, Monospin Trypsin column™...
which is a trypsin-immobilized monolithic silica spin column, has been used.28 Tryptic digestion is completed by adding the DTT- and IAA-treated; denatured sample to this column and repeating the 5-min centrifugation steps twice while passing the buffer solution.

The SMART Digest kit20,30 achieves faster and easier workflow than the traditional trypsinic digestion by its unique design of co-immobilizing the immunoaffinity reagents (either streptavidin, protein A, or protein G) and the heat-activated, thermally stable trypsin onto a single bead. Following the binding of a capture reagent to the bead, and enrichment of the target, the enzyme is activated at elevated temperatures for an accelerated digestion under protein-denaturing conditions.

Meanwhile, Iwamoto and colleagues have reported an nSMOL (nano-surface and molecular-orientation limited proteolysis) method, which can selectively obtain the trypsin-digested (nano-surface and molecular-orientation limited proteolysis) signature peptide are optimized by an analysis. They have established these analytical methods in just one week.

### 3 Selection of ISs for a High Precision Analysis

For a high-precision and an accurate quantification of the therapeutic mAbs in real samples, an isotopic dilution method that uses a stable isotope labeled (SIL)-signature peptide (SIL-peptide), such as those labeled with $^{13}$C or $^{15}$N, as an IS is common.42 However, to maintain the efficiency of trypsic digestion, either an extended peptide (flanking peptide, winged peptide), to which a digestion site sequence is attached, may be used, at both ends of the SIL-peptide,43 or a combination with SIL-peptide$^44$ may be used. In pharmaceutical companies and others, it is becoming a common practice to use SIL-protein, prepared from cells grown in a medium containing SIL-labeled amino acids, as IS. Li et al. examined, in detail, the influence of the three types of IS on analytical accuracy and precision using three kinds of mAbs ($\alpha$D-G2, $\alpha$K-G2, $\alpha$DA-G1) (Fig. 5).45 When the three concentrations (low, medium, and high) of the quality control (QC) samples were analyzed, it was noticed that both the accuracy (upper row) and the precision (lower row) fulfilled the regulation values (20% or less) when SIL-protein was used as the IS, hence emphasizing that the SIL-protein was an excellent candidate for IS. On the other hand, when SIL-peptide or SIL-extended peptide was used as the IS, the accuracy and precision could vary significantly depending on the type of antibody, site of signature peptide, and the measured concentration. Although the methods using the SIL-protein as the IS showed excellent accuracy, it is quite difficult to prepare an exactly equivalent SIL-protein because even a slight difference in the culture conditions or amino acid modifications. In order to overcome these problems, a hybrid calibration method that employs non-labeled mAb as calibrator and SIL-peptide or SIL-extended peptide as IS has been reported44 (Fig. 6). The usefulness of this method has been evaluated by experiments using an anti-hepatitis C virus mAb (anti-HCV
mAb) as a model antibody. By applying the hybrid calibration method to the SIL-peptide and SIL-extended peptide, respectively, an accuracy close to 20% could be achieved even without using the SIL-protein.

4 Selection of Mass Spectrometer

A triple quadrupole (QqQ) mass spectrometer, with excellent sensitivity and yield, has been most commonly used for the bioanalysis of therapeutic mAbs. However, the mass spectrometer that combines QqQ and linear ion trap (QqQ-LIT), TOF-MS, and Orbitrap, which are high-resolution mass spectrometers (HRMS) with more than 30000 resolutions, are now being used increasingly, instead of the QqQ mass spectrometer. The QqQ-LIT mass spectrometer is effective for the analysis of a limited number of peptides, and the MS^n detection can improve the identification ability of the signature peptide and ensure high sensitivity due to background reduction. On the other hand, although the use of the HRMS provides a lower dynamic range and detection sensitivity than that of the QqQ mass spectrometer with the SRM mode,
it is possible to achieve an appreciable quantification with the selected ion monitoring (SIM) mode due to its high resolution. As will be discussed later, with the improvement of the sensitivity of the HRMS in recent years, bioanalysis involving the direct quantification of the therapeutic mAbs or their fragments, rather than the trypsin-digested fragments, has become common.

Fig. 5 Accuracy (%Bias) and precision (%CV) of the QCs from 3 mAbs (αDA-G2, αK-G2, and αDA-G1) obtained with three different SIL-ISs. Accuracy of the whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively (top left, middle, right) Precision of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively (bottom left, middle, right). Adopted with permission from Ref. 44. Copyright 2017 American Chemical Society.

Fig. 6 Two-dimensional representations of the quantitative biases by (A) peptide-, extended-peptide-, and protein-level calibration approaches (left-right), and (B) the two “hybrid” calibration approaches (left-right). Adopted with permission from Ref. 43. Copyright 2017 American Chemical Society.
Antibody-drug conjugates (ADCs) are the next generation antibody drugs in which the targeted mAbs and a low-molecular-weight drug are linked by a linker, and are expected to have high therapeutic effects, low side effects, and low effective dosages. Brentuximab vedotin (marketed as Adcetris) is prescribed for the treatment of Hodgkin’s lymphoma, while trastuzumab emtansine (T-DM1, marketed as Kadcyla) has been licensed for the treatment of patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer. In addition, gemtuzumab ozogamicin has been approved in Japan. At present, more than 40 ADCs are at the stage of clinical trials, and by the year 2018, the ADC market is expected to reach $28 billion.

Depending on the conjugation chemistry used, a variety of drug molecules can be attached to a single mAb. However, the differences in the drug/antibody ratio (DAR) can affect the ADC distribution and pharmacokinetics (PK). Similar to the therapeutic mAbs, the tryptic digestion-LC/MS/MS method has been used in the bioanalysis of ADCs as well. Utilizing a highly sensitive QqQ mass spectrometer could enable the simultaneous bioanalysis of the low-molecular-weight drugs released from ADCs.

Bioanalysis of Therapeutic mAbs and ADCs as Use of Intact Protein

Unlike the tryptic digestion-LC/MS/MS, the use of intact LC/MS for the quantitative bioanalysis of the therapeutic mAbs is difficult due to the in vivo modification and elimination in the mAbs such as pyroglutamylation, deamidation, methionine oxidation, etc. For the separation of the mAbs and ADCs by intact analysis, a reversed phase mode using propyl- or butyl-silica gel columns, and mixed modes of hydrophilic interaction chromatography (HILIC) and size-exclusion chromatography (SEC) using polyhydroxyethyl aspartamide (marketed as PolyLC) columns are used. In order to obtain appreciable sensitivity, the microcapillary columns of their stationary phases are used.

As an example of LC/MS analysis of an ADC without the tryptic digestion, a bioanalysis of the ADC in the plasma and serum by immunonoaffinity-capillary LC/MS has been reported. In this report, the ADC was purified from real samples using the magnetic beads with an immobilized antibody against monomethyl auristatin E (MMAE), which is a low-molecular-weight drug moiety of the ADC, and directly separated by reverse-phase LC using a polymer capillary column (0.3 × 50 mm), followed by the final quantification by ESI-q-TOFMS. The ADC is quantified by its original molecular weight, which was calculated by the deconvolution of the multiple charged ions generated by the ESI. This method was shown to be applicable to the control of drug-antibody ratios (DARs) during their manufacture, and to the transition of DAR during the toxicokinetic analysis in vivo (Fig. 7). He et al. explored the biotransformations, such as the deacetylation and glycation, of ADCs by a high-resolution and accurate-mass spectrometry (HR/AM) approach using the orbitrap mass spectrometer, and demonstrated its superiority over the TOF-MS analysis.

Although not purely a mass spectrometric detection, the bioanalysis of trastuzumab by an LC-native fluorescence detection method has been reported as an intact LC-based bioanalysis of therapeutic mAbs. In this method, trastuzumab is purified from the serum sample by an affinity purification, and then separated by a high-temperature reverse phase LC (HT-RPLC), which achieves an excellent separation of IgG and the quantification is performed by the detection of the native fluorescence. Our group improved this method and reported the bioanalytical methods for bevacizumab and infliximab. Our methods improved especially the pretreatment process of blood samples compared with the previous report, and by using immunoaffinity magnetic beads, it was possible to achieve both an improvement in accuracy and an increase in sensitivity due to increased recovery rates. The proposed methods could be successfully applied to the clinical samples obtained from patients suffering from cancer and rheumatoid arthritis, after being treated with each drug. In the HT-RPLC reported first by Dillon et al., a wide-pore octyl
column with a mobile phase containing the solvents of high eluotropic strength, such as isopropanol and acetonitrile, at high temperatures (>70°C), were utilized. Thus, the adsorption of IgGs onto the stationary phase, refolding of the denatured IgGs, and aggregation of the IgGs could be greatly reduced. This leads to a good separation of the IgGs with corresponding sharp peaks. By such a combination of the LC mode and intact MS approach, analyses of the Lys variant,66 IgG2 antibodies isoforms,67 glycosylation,68 or mismatch of the disulfide bonds in the IgG2 antibodies68 have been performed.

In recent years, a middle-down LC/MS method has been reported in which the target drugs are fragmented by an enzymatic digestion or a chemical reduction, and HRMS analysis of the resulting fragments, with more uniform structure, is performed.69–71 Treatment of the IgGs with IdeS, a protease derived from *Streptococcus pyogenes*, rapidly and specifically cleaves the IgG into F(ab′)2 and Fc fragments.72 The generated fragments are analyzed by HRMS such as TOF-MS or Orbitrap Fourier transform mass spectrometer.70 Despite being a recently reported methodology, this approach is being applied to the characterization of the therapeutic mAbs and related products such as glycosylation, oxidation, charge variants, pyroglutamination, aggregation, deamidation, or change of DARs.69

Applications of this approach in the bioanalysis of therapeutic mAbs and ADCs have also been reported,71 and similar methods based on the middle-down LC/MS methods might be developed soon.

Mills et al. of Mayo Clinic performed the quantification of rituximab and examined its application to the individualized diagnosis by the monoclonal immunoglobulin rapid accurate mass measurement (miRAMM) method.73,74 In this method, rituximab and vedolizumab (IS) were first chemically reduced, and the resultant light chains were analyzed by the microflow-LC-ESI-Q-TOF MS to identify their 11+-charged ions. After the purification of the serum samples (collected from the patient) by Melon Gel, the samples were reduced by dithiothreitol and then analyzed by LC-Q TOF MS. In the clinical samples obtained from the patients who had been administrated rituximab for over a year, not only the administered rituximab, but also the endogenous clones, which have similar recognition ability as rituximab, could be detected by this method (Fig. 8). In the near future, simultaneous analysis of the target mAbs with such endogenous clones generated after prolonged drug administration can be a useful method for evaluating the therapeutic effects of the drugs.

7 Conclusions
In this review, we have outlined the current bioanalytical methods for therapeutic mAbs using mass spectrometers. There are still many points to consider in the sample preparation, IS/uni00A0 substance selection, precision control, mass spectrometer selection, etc. Since the patent for infliximab expired in 2013, many kinds of biosimilars have been scheduled for launch by many pharmaceutical companies. Therapeutic mAbs are high-molecular weight drugs, and their structures are heterogeneous and complicated, hence rendering it difficult to demonstrate that the active ingredients of the biosimilars are the same as their original drugs. The methods introduced in this review would be useful for bioequivalence assessments of biosimilars in preclinical and clinical trials. In addition, as treatments using therapeutic mAbs and ADCs become more familiar, that should lead to more opportunities for bioanalyses of these drugs, for the determination of their treatment efficacies and formulation of an administration plan.
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