High-Temperature Reversed-Phase LC Separation of Heavy and Light Chain Fragments of Therapeutic Monoclonal Antibodies and Antibody-Drug Conjugate Produced by Chemical Reduction

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Abstract
To construct liquid chromatography (LC)-based bioanalytical method for therapeutic monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), twelve commercially available therapeutic mAbs and one ADC were chemically reduced, and the generated fragments were analyzed by high-temperature reversed-phase LC. For most therapeutic mAbs, single peaks of light and heavy chains were detected, indicating a possibility of homogeneous LC analysis using light chains. However, characteristic fragmentations were observed in infliximab, pembrolizumab, ramucirumab, and trastuzumab emtansine. We also performed a simple validation using the fragmented light chains for the bioanalysis of bevacizumab. The limit of detection (LOD) and limit of quantification (LOQ) of bevacizumab were 0.63 and 2.10 µg/mL, respectively, with dithiothreitol reduction, and 0.74 and 2.48 µg/mL, respectively, with tris (2-carboxyethyl) phosphine reduction. These results indicate that both the reductants confer sufficient linearity, LOQ, and LOD for the light chain analysis of bevacizumab. Thus, this method, combined with affinity purification, can be used for the bioanalysis of bevacizumab.

Keywords: High-temperature reversed-phase LC; Therapeutic monoclonal antibody; Antibody-drug conjugate; Chemical reduction; Bioanalysis

1. Introduction
With the increasing use of therapeutic monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), there is an increasing demand for the bioanalysis of these drugs, in order to evaluate their efficacies and establish optimal treatment plans. For the bioanalysis of therapeutic mAbs and ADCs in complex biological samples such as plasma and serum, tryptic digestion–liquid chromatography-tandem mass spectrometry (LC-MS-MS) and ligand-binding assays such as enzyme-linked immunosorbent assay are the two most commonly used strategies, primarily because of their high sensitivities and specificities [1,2]. These methods require complex and time-consuming pretreatment, precision control, and homogeneity of the reagents used [2]. Therefore, a new trend has emerged for the direct analysis of the target drugs or their light chain fragments by LC, in conjugation with high-resolution mass spectrometry [3,4]. We have also reported bioanalytical methods for the direct analysis of therapeutic mAbs in human blood samples. The method was a combination of high-temperature reversed-phase LC (HT-RPLC) [5] and immunoaffinity [6] or DNA aptamer affinity [7,8] purification.

Mills et al. of MAYO Clinic used rituximab administered to rheumatoid arthritis patients; the resulting anti-drug antibodies were used to evaluate the therapeutic efficacies by quantifying them as light chain fragments derived from chemical reduction [9]. The quantification of light chain fragments is a useful approach leading to highly selective, accurate, and sensitive bioanalysis of therapeutic mAbs,
because they can be detected by LC and MS, without considering the heterogeneity arising from the difference in the number of added sugar chains, charged variants, and in vivo modification and degradation.

In this study, the light chain fragments generated by chemical reduction were analyzed by HT-RPLC to investigate whether the differences in the generation of light chain fragments were caused by the differences in the target drugs and/or reduction methods. This, in turn, led to the development of a bioanalytical method for the detection of therapeutic mAbs and ADCs. Specifically, 12 commercially available therapeutic mAbs and 1 ADC were chemically reduced using water-soluble reducing agents, dithiothreitol (DTT) or tris (2-carboxyethyl) phosphine (TCEP), and the generated fragments were subjected to HT-RPLC. Figure 1 shows the structure of the reducing agents used in this study and the chemical reduction scheme of fragmentation of the mAb drugs.

Table 1 summarizes the trade name, drug name, and the characteristics of the therapeutic mAbs and ADCs investigated. HT-RPLC, developed by Dillon et al., is a suitable method for the separation of immunoglobulin G (IgG) proteins [10] and is capable of separating and recognizing the number of sugar chains attached [11], the different isoforms [11], and the mislinkage of disulfide bonds [12]. We also found that sufficient retention and separation could be achieved not only for intact therapeutic mAbs, but also their F(ab')2 and Fab' fragments upon digestion by papain and pepsin [13].

The analysis on fragmentation of each therapeutic mAb by chemical reduction gives useful findings not only in the HT-RPLC but also in other chromatographic modes (reversed-phase, size exclusion, and ion exchange). Furthermore, we established a simple validation using fragmented light chains for the bioanalysis of bevacizumab.

2. Experimental
2.1. Reagents and solutions
Deionized and distilled water purified using the ELGA Purelab Flex system (ELGA, Marlow, UK) was used to prepare all aqueous solutions. LC-grade acetonitrile and isopropanol were purchased from Kanto Chemicals (Tokyo, Japan). The specifications and sources of the therapeutic mAb formulations used in this study are as follows: bevacizumab (AVASTIN 400 mg/16 mL intravenous infusion), pertuzumab (PERJETA 420 mg/14 mL for intravenous infusion), tocilizumab (ACTEMRA 80 mg for intravenous infusion), and trastuzumab (HERCEPTIN intravenous infusion 150, 150 mg/7.2 mL). Trastuzumab emtansine (KADCYLA intravenous infusion 100 mg) was procured from Chugai Pharmaceutical (Tokyo, Japan). Nivolumab (OPDIVO 20 mg/2 mL for injection) and infliximab (REMIKAD, 100 mg for intravenous infusion) were procured by Ono Pharmaceutical (Osaka, Japan) and Mitsubishi Tanabe Pharma (Osaka, Japan), respectively. Cetuximab (ERBITUX Injection 100 mg) was purchased from Sigma-Aldrich (St. Louis, MO, USA), respectively. All other chemicals were of the highest purity available and were used as received. Each therapeutic mAb formulation was from Merck KGaA (Darmstadt, Germany), while Pembrolizumab (KEYTRUDA Injection 20 mg) was purchased from MSD K.K. (Kenilworth, NJ, USA). Ramucirumab (CYRAMZA intravenous infusion 100 mg) was obtained from Eli Lilly and Company (Indianapolis, IN, USA) and Durvalumab (IMFINZI injection 120 mg) was obtained from AstraZeneca, Cambridge, England). Rituximab (RITUXAN injection 10 mg/mL (10 mL)) was obtained from Zenyaku Kogyo Company, Tokyo, Japan) and panitumumab (VECTIBIX injection for intravenous infusion 100 mg) was
obtained from Takeda Pharmaceutical Company (Osaka, Japan). Trehalose dehydrate was purchased from Wako (Osaka, Japan). Polysorbate 20 diluted to the desired concentration using a dilution buffer for mAbs (175 mM trehalose, 42 mM sodium dihydrogen phosphate, 8 mM disodium hydrogen phosphate, 0.4% Tween-20, pH 7.4) [5].

2.2. Chemical reduction of therapeutic mAbs

DTT reduction [9]: To 20 µL sample solution, 10 µL 200 mM aqueous DTT and 20 µL 50 mM aqueous ammonium bicarbonate were added and then incubated at 55 °C for 15 min. An aliquot (2 µL) of the resulting solution was subjected to HT-RPLC.

TCEP reduction [14]: To 20 µL sample solution, 10 µL 8 mM aqueous TCEP and 10 µL 8 mM aqueous EDTA-2Na were added and then incubated at 40 °C for 30 min. After the addition of 10 µL of aqueous 1% (v/v) trifluoroacetic acid (TFA), an aliquot (2 µL) of the resulting solution was subjected to HT-RPLC. Sample solution at a concentration of 100 µg/mL was used to evaluate the separation of mAbs and ADC.

2.3. HT-RPLC system and conditions

Most of the experimental parameters for HT-RPLC were the same as in previous studies [5-7]. We used the Prominence UFLC liquid chromatography system (Shimadzu), which consisted of a CBM-20A system controller, a SIL-20AC autosampler, two LC-20AD pumps, a DGU-20A online degasser, a CTO-20AC column oven, an SPD-M20A PDA detector, and an RF10AXL fluorescence spectrometer equipped with a 12 µL flow cell. The fluorescence intensity was monitored at an emission wavelength of 343 nm, upon excitation at 278 nm. The collected data were analyzed using LabSolutions LC (v. 1.21; Shimadzu). The Aeris Widepore XB-C8 column, which is a core-shell type analytical column packed with 3.6 µm core-shell particles (150 × 2.1 mm I.D., Phenomenex, Torrance, CA, USA) was used. Mobile phase A was 98% water, 2% isopropanol, and 0.1% TFA; while mobile phase B was 70% isopropanol, 20% acetonitrile, 10% mobile phase A. The gradient elution carried out as follows: 0–0.5 min, 5% B; 0.5–1.0 min, linear gradient from 5% B to 20% B; 1.0–7.5 min, linear gradient from 20% B to 50% B; 7.5–10.0 min, linear gradient from 50% B to 100% B; 10.0–14.0 min, linear gradient from 100% B to 5% B. Flow rate of mobile phase, column temperature, autosampler temperature, and injection volume were set at 0.4 mL/min, 75 °C, 9 °C, and 2 µL, respectively.

2.4. Calibration curve, limit of detection, and limit of quantification

For the quantitative analysis of bevacizumab, calibration standard solutions (n = 3) with concentrations ranging from 5 to 50 µg/mL (5, 7.5, 10, 15, 20, 25, and 50 µg/mL) were prepared by diluting the stock solution. Peak heights were used for constructing the calibration curve. The equation of the calibration curve was determined using least-squares linear prediction. The limit of detection (LOD) and lower limit of quantification (LOQ) were determined from the signal-to-noise ratios of 3 and 10, respectively.

3. Results and discussion

3.1. HT-RPLC separation of antibody fragments produced by chemical reduction

In the chemical reduction schemes shown in Fig. 1, not only the reducing agents, but also the heating temperature and reaction time were different. Nonetheless, both the reactions resulted in the fragmentation of proteins [9,14], and sufficient amount of the reducing agent remained for the therapeutic mAbs. Of the 12 mAbs examined, 9 mAbs generated two peaks upon DTT reduction (the exceptions were infliximab, pembrolizumab, and ramucirumab). These peaks corresponded to light and heavy chains, with light chains eluting first. As a representative example, the HT-RPLC chromatograms of trastuzumab, rituximab, and bevacizumab after chemical reduction are shown in Fig. 2. In several cases, the retention times of both heavy chain and its intact form were close to each other, which could be attributed to the strong retention of the crystallizable region of the hydrophobic fragments in the heavy chain. We then evaluated the differences in fragmentation owing to the differences in chemical reduction. In the TCEP reduction of trastuzumab and rituximab, two peaks derived from light and heavy chains were detected at almost the same retention time as that obtained by the DTT reduction.

Fig. 2. HT-RPLC chromatograms of trastuzumab, rituximab, and bevacizumab (left to right) after DTT or TCEP reduction. Each peak corresponds to 100 µg/mL of sample solution. Blue and black lines in (a) indicate intact mAbs and their fragments, respectively. Black (1) and blue (2) lines in (b) indicate fragment peaks obtained by DTT and TCEP reduction, respectively.
The injection amounts of reduced mAbs were the same in both reduction conditions, however, the difference in the peak intensities of the heavy chains, such as trastuzumab, and the similar peak intensities of the light and heavy chains, such as rituximab, suggested to be caused by the difference in the reactivity of the reductants to mAbs. For bevacizumab, TCEP reduction resulted in two overlapping peaks derived from the heavy chains.

Figure 3 shows the chromatograms of the fragments of chemically reduced infliximab, pembrolizumab, and ramucirumab. Interestingly, the chemical reduction of infliximab and pembrolizumab resulted in single peaks whose retention times differed from that of the intact forms. Although pembrolizumab and nivolumab are both anti-programmed cell death 1 (anti-PD-1) antibodies belonging to the same subclass of IgG4, there were no common fragments upon reduction. In the chromatogram of ramucirumab, a total of three peaks, two of which corresponded to heavy chains, were detected upon DTT reduction, while two peaks were detected upon TCEP reduction, indicating a difference in fragmentation by the two reducing agents.

Table 2. Number of antibody fragment peaks detected by chemical reduction.

| Drug name    | DTT reduction | TCEP reduction |
|--------------|---------------|----------------|
| cetuximab    | 2             | 2              |
| rituximab    | 2             | 2              |
| infliximab   | 1             | 1              |
| trastuzumab  | 2             | 2              |
| pertuzumab   | 2             | 2              |
| pembrolizumab| 1             | 2*             |
| tocilizumab  | 2             | 2              |
| bevacizumab  | 2             | 3*             |
| ramucirumab  | 3             | 2              |
| durvalumab   | 2             | 3*             |
| panitumab    | 2             | 2              |
| nivolumab    | 2             | 2              |
| trastuzumab emtansine | 3* | 3* |

Three peaks, two of which corresponded to heavy chains were detected on the chromatogram. However, the two peaks could not be separated by changing the HT-RPLC conditions. Since a single peak derived from the light chains was observed at approximately the same retention time as trastuzumab, it was expected that the fragmented light chain could also be used for the quantification of trastuzumab emtansine.

Figure 4 shows the chromatogram of the ADC trastuzumab emtansine under various conditions. Unlike trastuzumab (Fig. 2a, left), the intact form of trastuzumab emtansine gave a broad peak, reflecting the multiple binding of emtansine and linkers, and was eluted later than trastuzumab. In both DTT and TCEP reduction, a total of three peaks, two of which corresponded to heavy chains were detected on the chromatogram. Since a single peak derived from the light chains was observed at approximately the same retention time as trastuzumab, it was expected that the fragmented light chain could also be used for the quantification of trastuzumab emtansine.

In the bioanalysis of ADCs, measurement of in vivo drug-antibody ratio is an important index for toxicokinetics; thus, bioanalysis using light chain fragments may be useful for the measurement of total drug concentration for this purpose. The results of the fragmentation of each antibody drug and ADC produced upon chemical reduction are summarized in Table 2.
3.2. HT-RPLC analysis of bevacizumab, which targets light chain fragment produced by chemical reduction

A calibration curve was constructed using light chain fragment of bevacizumab, and the quantification range was 5–50 µg/mL as sample solution of bevacizumab. With either reductant, an increase in the peak intensities of the light and heavy chain was observed, depending on the concentration of bevacizumab (Fig. 5a). The linearity of the calibration curves were good, with $R^2$ values of 0.998 (DTT reduction) and 0.999 (TCEP reduction) (Fig. 5b). The LOD and LOQ of bevacizumab were 0.63 and 2.10 µg/mL, respectively, upon DTT reduction and 0.74 and 2.48 µg/mL, respectively, upon TCEP reduction. According to the drug package inserts, the effective blood concentrations of bevacizumab ranged from 50 to 500 µg/mL [15]. The present method proved to cover this concentration range with simple sample dilution. These results indicate that both the reductants provide sufficient linearity, LOQ, and LOD for the light chain analysis of bevacizumab, and this method combined with affinity purification can be used for the bioanalysis of bevacizumab. Quantification using light chain fragments having a peak intensity of about one-third that of the parent mAbs causes a decrease in detection sensitivity in native fluorescence detection. In contrast, this approach leads to highly sensitive quantification, because it allows detection in a smaller $m/z$ range and can be detected as a single component in MS measurements.

4. Conclusion

In this paper, to construct LC-based bioanalytical method for the detection of therapeutic mAbs and ADCs, twelve commercially available therapeutic mAbs and one ADC were chemically reduced, and the generated fragments were analyzed by HT-RPLC. For most of the therapeutic mAbs, single peaks derived from light and heavy chains were detected, indicating the possibility of homogeneous LC analysis using light chains. However, characteristic fragmentations were observed in some antibody drugs and ADC. Next, we performed a simple validation using the fragmented light chains for the bioanalysis of bevacizumab. The results indicated that the proposed method conferred sufficient linearity, LOQ, and LOD for the bioanalysis of bevacizumab. This HT-RPLC method, which does not involve nonvolatile salts, can be easily combined with high-resolution MS and can be expected to be used for further highly sensitive and precise bioanalysis. The findings obtained in this study can be applied to semi-intact bioanalysis of many therapeutic mAbs and nanobodies [16], which are regarded as post-therapeutic mAbs.

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