High Sensitivity and Precision High-Temperature Reversed-Phase LC Analysis of Bevacizumab for Intact Bioanalysis of Therapeutic Monoclonal Antibodies

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Abstract
We optimized several analytical conditions for more sensitive and precise HT-RPLC analysis of the therapeutic monoclonal antibody (mAb), bevacizumab. Specifically, we (1) optimized the sample preparation process to reduce adsorption and aggregation of bevacizumab, (2) introduced a sample concentration process using a centrifugal ultrafiltration unit to increase detection sensitivity, and (3) used another therapeutic mAb as an internal standard to improve analytical precision. The optimized method for bevacizumab analysis was shown to have low detection and quantification limits of 0.010 and 0.032 µg/mL, respectively, good correlation coefficients ($r^2 > 0.9997$), and good intra- and inter-day precisions within < 12.0 %. This study provides an important methodology for the intact bioanalysis of therapeutic mAbs, not merely their LC measurement.

Keywords: High-temperature reversed-phase LC; Bevacizumab; Therapeutic monoclonal antibody; Bioanalysis

1. Introduction
In recent years, therapeutic monoclonal antibodies (mAbs) and related drugs have been widely used in the treatment of various ailments such as cancer, rheumatoid arthritis, and autoimmune and infectious disease. As of December 2016, over 60 therapeutic mAbs have been approved in Japan, the United States, and Europe. Among the ten top-selling pharmaceuticals in the world, five are therapeutic mAbs and their use is expected to continue to expand in the future [1-3].

The pharmacokinetics (PK) and pharmacodynamics (PD) of therapeutic mAbs are very complicated compared with those of low-molecular-weight drugs [4,5]. These profiles are concentration-dependent for many mAbs that exhibit non-linear PK due to the presence of fetal Fc receptors, which function in mAbs for catabolism before blood transition, binding to the target antigens, and transportation [5-8]. To date, PK and PD analyses of therapeutic mAbs that have performed mainly by ligand-binding assays (LBAs), such as enzyme-linked immunosorbent assay (ELISA) [9,10]. Although LBAs permit high-sensitivity and high-throughput analysis, some potential exists for cross-reactivity of capture antibodies and low accuracy [11]. In contrast, tryptic digestion-liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been applied to the analysis of therapeutic mAbs in serum or plasma samples [12-18]. These methods enable sensitive bioanalysis of therapeutic mAbs, but also present several limitations, such as time-consuming trypsin digestion and manual purification for tryptic peptides using solid-phase extraction cartridges. It is difficult to control the accuracy of the pretreatment process and peptide analysis [12,14]. We have recently developed simple and rapid quantification methods for the therapeutic mAbs bevacizumab and infliximab in the plasma of cancer and rheumatoid arthritis (RA) patients, using a combination of immunoaffinity...
magnetic purification and high-temperature reversed-phase LC (HT-RPLC) with fluorescence detection [19]. In this method, target drugs in plasma samples are purified using immunoaffinity magnetic beads immobilized with anti-idiotype mAbs. The purified drugs are separated further using HT-RPLC, which enables excellent separation of mAbs with good peak shape, using a large pore-size octyl column [20,21]. The separated drugs are detected with high sensitivity by their own fluorescence. This method requires no tryptic digestion or expensive LC-MS/MS instruments. Although this method was successfully applied to clinical analyses, several problems remain, such as decreased quantitativeness at low concentrations due to adsorption and/or aggregation of mAbs, insufficient sensitivity for trough concentration analysis using native fluorescence detection, and the necessity for rigorous sample preparation when not using an internal standard.

In this study, to overcome these problems, we optimized several analytical conditions to allow more sensitive and precise HT-RPLC analysis for anti-cancer drug, bevacizumab. Specifically, we (1) optimized the sample preparation process to reduce adsorption and aggregation of mAbs, (2) introduced a sample concentration process using a centrifugal ultrafiltration unit to increase detection sensitivity, and (3) used of another therapeutic mAb as an internal standard to maintain accuracy. This study provides an important methodology for the intact bioanalysis of therapeutic mAbs, not merely their LC measurement.

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, isopropanol and methanol were purchased from Kanto Chemicals (Tokyo, Japan). Bevacizumab (Avastin® 400 mg/16 mL Intravenous Infusion), tocilizumab (ACTEMRA® 80 mg for Intravenous Infusion), and trastuzumab (HERCEPTIN® Intravenous Infusion 150, 150 mg/7.2 mL) were produced by Chugai Pharmaceutical (Tokyo, Japan). Infliximab (REMICADE for Intravenous Infusion 100) was produced by Mitsubishi Tanabe Pharma (Osaka, Japan). Trevalese dehydrate, polysorbate 20, and trifluoroacetic acid for amino acid sequence analysis were purchased from Wako (Osaka, Japan), respectively. All other chemicals were of the highest purity available and were used as received.

2.2. Preparation of therapeutic mAb solutions

The Avastin preparation (100 mg/4 mL) contained 23.2 mg sodium dihydrogen phosphate, 4.8 mg disodium hydrogen phosphate, 240 mg trehalose dihydrate, and 20 mg polysorbate 20 as additives [22]. Therefore, an aqueous solution having the same composition was used for dilution of therapeutic mAbs. Bevacizumab (0.1-10 µg/mL) and other mAb (10 µg/mL) solutions were prepared. To 450 µL of each solution of bevacizumab, 50 µL of 10 µg/mL trastuzumab (internal standard: IS) solution was added, and the solution then underwent the concentration procedure described in Section 2.3. The resulting solution was analyzed by HT-RPLC, as described in Section 2.1.

2.3. Concentration procedure for bevacizumab solution

Centrifugal ultrafiltration, an Amicon Ultra Centrifugal Filter device (MWCO 100 kDa, 0.5 mL, Merck, Darmstadt, Germany) was used in accordance with the manufacturer's operating instructions. The device consisted of housing, membrane, and collection tube, composed of styrene/butadiene co-polymer, low-adsorption regenerated cellulose membrane, and polypropylene, respectively. Prior to using the device, filter units were washed with 10 % methanol and water. An aliquot of bevacizumab solution (500 µL), prepared by dilution with the sample preparation solution, was loaded onto the centrifugal ultrafiltration device, and the resulting concentrated solution (ca 25 µL) was analyzed by HT-RPLC. Using centrifugal ultrafiltration, 500 µL of sample solution could be concentrated 20-fold, to approximately 25 µL.

2.4. HT-RPLC system and conditions

We used the Prominence UFLC liquid chromatograph system (Shimadzu, Kyoto, Japan), which consisted of a CBM-20A system controller, an SIL-20AC auto sampler, 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 excitation and emission wavelengths of 278 and 343 nm, respectively. The collected data were analyzed using a LabSolutions LC (v. 1.21; Shimadzu); the peak areas and heights were estimated using the baseline-to-baseline method. 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 water containing 0.1 % trifluoroacetic acid (TFA); while solvent B was 70% isopropanol, 20% acetonitrile, 9.9% water, and 0.1% TFA. The gradient elution conditions used for individual analysis of bevacizumab were as follows: 0-1 min, 90% A; 1-2 min, linear gradient from 90% A to 75% A; 2-15 min, linear gradient from 75% A to 50% A; 15-20 min, 100% B; 20-28 min, 90% A. Gradient elution conditions used for simultaneous analysis of bevacizumab and other mAbs were as follows: 0-1 min, 90% A; 1-2 min, linear gradient from 90% A to 75% A; 2-25 min, linear
gradient from 75% A to 60% A; 25-30 min, 100% B; 30-35 min, 90% A. The flow rate of the mobile phase and the column temperature were set at 0.2 mL/min and 75°C, respectively.

2.5. Method validation
The proposed analytical method was evaluated partially based on the FDA bioanalytical method validation [23]. To obtain the validation parameters (intra- and inter-day precisions, accuracy, linearity, limit of detection (LOD) and limit of quantification (LOQ)), peak areas were integrated by LabSolutions LC, and the baseline-to-baseline method was used for quantification.

2.5.1. Precision
The precision of the assays was determined by the repeated evaluation of five (0.1, 0.5, 1, 5, 10 μg/mL; n = 5) bevacizumab samples. For intra-day precision, these levels were analyzed three times daily, whereas for inter-day precision, samples at the same concentrations were analyzed three times daily for three days (n = 9).

2.5.2. Accuracy
The accuracy was determined by the repeated evaluation of three concentrations (0.1, 1, and 10 μg/mL; n = 5) of QC samples. The minimum acceptable biases were < 20 % at 0.1 μg/mL and 15 % at other concentrations.

2.5.3. Calibration curve, limit of detection, and limit of quantification
For the quantitative analysis, calibration standard solutions (n = 5) with concentrations ranging from 0.1 to 10 μg/mL (0.1, 0.5, 1, 5, 10 μg/mL) were prepared by diluting the stock solutions. The calibration curve equations were determined using least squares linear prediction. The limit of detection (LOD) and the lower limit of quantification (LOQ) were determined from signal-to-noise ratios of 3 and 10, respectively.

3. Results and discussion
3.1. Effect of additives in bevacizumab solution
Aqueous solutions of bevacizumab were prepared by diluting with sample preparation solution having the same composition as the Avastin preparation. For comparison, aqueous solutions of bevacizumab at the same concentrations were also analyzed. The chromatogram for bevacizumab solution prepared with either sample preparation solution or water is shown in Fig. 1a. Each calibration curve is shown in Fig. 1b. The difference in retention times of the two peaks were considered to be due to the influence of additives in the sample solutions. In the sample solution diluted with water, a drastic decrease in peak intensity was observed for concentrations less than 10 μg/mL, and the calibration curve showed strong linearity, with $r^2 = 0.9949$. These results indicate that bevacizumab was adsorbed onto the sample vial and pipette tip by hydrophobic interaction. In contrast, when diluted with the sample preparation solution, adsorption and aggregation

![Fig. 1. (a) HT-RPLC chromatograms of bevacizumab at 5 concentrations prepared with different solvents, and (b) their obtained calibration curves. Peaks: 1, bevacizumab diluted with sample preparation solution; 2, bevacizumab diluted with water.](image-url)
were suppressed, and increases in peak intensities and improved linearity of the calibration curve \((r^2 =0.9998)\) were observed. The addition of phosphate salts stabilized pH, and the addition of trehalose suppressed the denaturation, aggregation, and adsorption of mAbs through its strong hydration force [24]. Polysorbate 20 contributed to suppressing the adsorption of mAbs onto containers and the aggregation of mAbs by solubilizing as a surfactant [25].

### 3.2. Effect of concentration procedure on detection sensitivity

By centrifugal ultrafiltration, 500 μL of sample solution could be concentrated 20-fold, to approximately 25 μL. Figure 2 shows the chromatograms of bevacizumab solutions at each concentration before and after the concentration procedure. Table 1 shows the results of comparisons between the fluorescence intensities at each concentration before and after concentration. Through the concentration procedure, peak intensities increased 5.2- to 17.7-fold at each concentration, and the calibration range could be lowered from 1 μg/mL to 0.1 μg/mL. However, the concentration ratio for each sample was not constant, which caused a decrease in the linearity of the calibration curve. This may be the result of hydrophobic adsorption of bevacizumab onto the filter unit and/or the housing of the concentrating device. However, we addressed this decreased linearity by introducing an internal standard method.

### Table 1. Relative peak intensities of bevacizumab solution with and without the concentration procedure and concentration ratios.

| Bevacizumab (μg/mL) | Fluorescence peak area \((\times 10^5 \mu V \cdot \text{min})\) Before concentration | After concentration | Concentration ratio (fold) |
|---------------------|---------------------------------|--------------------|--------------------------|
| 0.1                 | –                               | 0.1                | –                        |
| 0.5                 | –                               | 0.4                | –                        |
| 1                   | 0.2                             | 1.3                | 5.2                      |
| 5                   | 1.4                             | 18.3               | 12.5                     |
| 10                  | 3.5                             | 63.3               | 17.7                     |
| 50                  | 22.1                            | –                  | –                        |
| 100                 | 44.9                            | –                  | –                        |

### 3.3. Improvement of analytical accuracy by internal standard compound

In choosing the internal standard compound, we considered its separation from bevacizumab and appropriate retention time. As shown in Fig. 3, four commercially available therapeutic mAbs (trastuzumab, infliximab, tocilizumab, bevacizumab) could be well separated by HT-RPLC, with trastuzumab being separated farthest from bevacizumab with \(R_s >1.5\). From these results, trastuzumab was selected as the internal standard compound.

![Fig. 3. HT-RPLC chromatograms of four mAb preparations. 1, trastuzumab; 2, infliximab; 3, tocilizumab; and 4, bevacizumab. Each peak corresponds to 100 μg/mL.](image)

Figure 4 shows HT-RPLC chromatograms of bevacizumab and I.S. undergoing the optimized preparation and concentration procedure. The calibration curve for bevacizumab calculated from the peak height ratio between bevacizumab and IS showed good correlation coefficients \((r^2 > 0.9997)\). The LOD and LOQ of bevacizumab were 0.010 and 0.032 μg/mL, respectively. In contrast, these values, when analyzed by the same LC system without the concentration procedure were 0.209 and 0.696 μg/mL, respectively. Thus, the concentration process achieved 21-fold higher sensitivity. The intra- and inter-day assay

![Fig. 2. HT-RPLC chromatograms of bevacizumab solution. Peaks: 1, treated with ultrafiltration concentration procedure; 2, not treated with ultrafiltration concentration procedure.](image)
precisions obtained by six-replicate analysis of bevacizumab ranged from 3.26-10.7%, 3.25-12.0%, respectively.

4. Conclusion
In this paper, we proved that the adsorption of therapeutic mAbs onto the container during sample preparation and/or dilution greatly affects detection sensitivity and quantitativity in HT-RPLC analysis. Furthermore, using the same solution composition as that of the diluting solution used for sample preparation, the quantitativity of the mAb could be greatly improved. This method achieved both sufficient sensitivity and excellent quantitativity in HT-RPLC analysis of bevacizumab. In the future, the proposed method could be successfully applied to the intact bioanalyses of various therapeutic mAbs and antibody-drug conjugates (ADC) by combining immune-affinity purification. Recently, LC-TOF MS bioanalytical methods which analyze intact mAb itself or light chains of mAbs have been reported [26, 27], and the findings of this study may also be useful in refining these analyses.

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