Sensitive Method for LC Analysis of Therapeutic Monoclonal Antibodies Using a Centrifugal Filtration Device with Adsorption Suppression Treatment

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Abstract
Herein, we report an intact LC–native fluorescence analysis method for therapeutic monoclonal antibodies (mAbs) based on a centrifugal filtration device with adsorption suppression treatment. Coating the centrifugal filtration device with MPC monomer suppressed the non-specific adsorption of mAbs, especially in the low concentration range; trastuzumab could be quantitatively and sensitively analyzed in the 0.2–10 μg/mL range. In this analysis, the average concentration factor over the entire concentration range was approximately 25 times. The other mAbs (bevacizumab, rituximab, nivolumab) also showed good linearity with $R^2 \geq 0.996$, and the average concentration factors were similar to that obtained for trastuzumab. This method can potentially be used in combination with affinity purification for simple and sensitive bioanalysis.

Keywords: Therapeutic monoclonal antibody; Adsorption Suppression Treatment; MPC monomer; Bioanalysis

1. Introduction
In recent years, therapeutic monoclonal antibodies (mAbs) have been widely applied for the treatment of various diseases such as cancer, rheumatoid arthritis, autoimmune diseases, and infections [1,2]. In addition, next-generation therapeutic mAbs that are effective at lower concentrations, such as antibody-drug conjugates, bispecific antibodies and recycling antibodies, have been developed. Thus, the importance of simple and highly sensitive bioanalytical methods for these drugs in preclinical/clinical studies and therapy evaluation is increasing. Trypsin digestion-liquid-chromatography mass-spectrometry (LC-MS/MS) is commonly used for bioanalysis of therapeutic mAbs [3-7]. However, not only is the pretreatment to obtain the signature peptide for quantification time-consuming, but it also requires much attention in order to maintain precision and trueness. For this reason, with the progress of high-resolution mass spectrometry (HR-MS), bioanalytical methods in which therapeutic mAbs are affinity-purified and then subjected to HR-MS, in either intact or fragmented form, have been reported [7-9].

We have recently reported a bioanalysis method for therapeutic mAbs that combined immunoaffinity purification or aptamer affinity purification with LC-fluorescence detection [10,11]. In addition, we reported a high-sensitivity LC analysis method in which bevacizumab was concentrated using a centrifugal filtration device, and direct LC analysis was performed [12]. By using the centrifugal filtration device, the bevacizumab solution was concentrated about 20 times, and the concentration could be adjusted to allow microanalysis by HR-MS. However, in this method, non-specific adsorption of bevacizumab on the housing of the filter device was observed, and the quantitativity in the low concentration range decreased.

Herein, we first applied 2-methacryloyloxyethyl phosphorylcholine (MPC) monomer as a coating agent for the centrifugal filtration device; an LC-native fluorescence analysis method for therapeutic mAbs based on this device showed superior quantitativity and sensitivity. MPC monomer contains the biocompatible phosphorylcholine group and suppresses protein adsorption [13]; consequently, it is widely used in nanobioscience, biocompatible materials, and biosensing [14].

By the proposed method, low-concentration therapeutic mAbs can be concentrated while maintaining quantitativity, and the lack of sensitivity of the conventional methods can
be easily improved. This study goes beyond mere measurement ingenuity and provides an important methodology for bioanalysis of therapeutic mAbs in LC-HRMS and LC-UV analysis.

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). Trastuzumab (HERCEPTIN intravenous infusion 150, 150 mg/7.2 mL) and bevacizumab (AVASTIN 400 mg/16 mL intravenous infusion) were procured from Chugai Pharmaceutical (Tokyo, Japan). Trastuzumab (OPDIVO 20 mg/2 mL for injection) and rituximab (REMIACA, 100 mg for intravenous infusion) were obtained from Ono Pharmaceutical (Osaka, Japan) and Mitsubishi Tanabe Pharma (Osaka, Japan), respectively. Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate-12 water, trehalose dihydrate, and formic acid (FA) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Trifluoroacetic acid (TFA) and Tween 20 were purchased from Kanto Chemicals. All other chemicals were of the highest purity available and used as received. 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. Aqueous solutions of this composition were used for all the preparation and dilution of the therapeutic mAbs that were examined.

2.2. Adsorption suppression processing for centrifugal filtration device

The Amicon Ultra centrifugal filter device (MWCO 100 kDa, 0.5 mL, Merck, Darmstadt, Germany) and 1.5 mL polypropylene tube that serves as a saucer were coated. MPC monomer-containing coating reagent 500 µL (FastGene™ NovyCoat, Nippon Genetics, Tokyo, Japan) was added to the filter device and centrifuged for 10 s. The device was then inverted and centrifuged for another 10 s. The saucer tube was vortexed twice with the remaining reagent for 5 s each time. Both the device and the tube were air dried overnight.

2.3. Concentration procedure using prepared centrifugal filtration device

The sample solution (500 µL) was placed in the coated device prepared in the previous section and centrifuged at 14,000 g for 10 min at 25 °C. Then, the device was turned over and centrifuged at 14,000 g for 10 min, and the concentrated sample solution was collected in the saucer tube.

2.4. LC system and conditions

The Prominence UFLC liquid chromatography system (Shimadzu, Kyoto, Japan), 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 a RF10AXL fluorescence spectrometer equipped with a 12 µL flow cell was used. 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 heights were estimated using the baseline-to-baseline method.

The MonoSelect RP-mAb (20 × 2.1 mm I.D., GL Sciences, Tokyo, Japan), a silica monolith column specialized for mAb analysis, was used for separation. The mobile phase A was water containing 0.075% FA and 0.025% TFA, while the mobile phase B was acetonitrile containing 0.075% FA and 0.025% TFA. The gradient elution was carried out as follows: 0–5 min, linear gradient from 20% B to 60% B; 5–7 min, linear gradient from 60% B to 20% B; 7–10 min, 20% B. The flow rate of the mobile phase, column temperature and injection volume were set at 0.3 mL/min, 75 °C, and 2 µL, respectively.

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

For the quantitative analysis of trastuzumab, calibration standard solutions (n = 6) with concentrations ranging from 0.2 to 10 µg/mL (0.2, 0.5, 1, 5, and 10 µg/mL) were prepared by diluting the stock solution. For the quantitative analysis of bevacizumab and rituximab, calibration standard solutions (n = 2) with concentrations ranging from 0.1 to 10 µg/mL (0.1, 0.2, 0.5, 1, 5, and 10 µg/mL) were prepared. For the quantitative analysis of nivolumab, calibration standard solutions (n = 2) with concentrations ranging from 0.1 to 10 µg/mL (0.1, 0.2, 0.5, 1, 5, and 10 µg/mL) were prepared. Peak heights were used for constructing the calibration curve. The calibration curve equation was determined using the least-squares linear prediction. The detection limit was determined from the signal-to-noise (S/N) ratio of 3.

3. Results and discussion

3.1. Effect of adsorption suppression by MPC polymer coating

In order to verify the effect of MPC monomer coating on the adsorption of mAbs, calibration curves for trastuzumab were generated before and after centrifugal concentration in the range 1–50 µg/mL. In this experiment, centrifugation was carried out for 5 min each for concentration and recovery. In the absence of coating (Fig. 1a), non-specific
adsorption to the device occurred in the low concentration range and a significant decrease in linearity ($R^2 = 0.987$) was observed. This was attributed to the non-specific adsorption of the hydrophobic Fc region of mAbs to the housing composed of styrene/butadiene copolymer. It was evident that the MPC monomer coating (Fig. 1b) suppressed this nonspecific adsorption in the low concentration range and showed good linearity ($R^2 = 0.994$), including in the low concentration range.

Then, we optimized the centrifugation time for a 0.5 μg/mL trastuzumab solution (Fig. 2). The maximum concentration effect was observed when the centrifugation treatment was performed for 20 min; thus, the centrifugation time was set to 20 min in subsequent experiments.

3.2. Improved sensitivity and quantitativity of trastuzumab by centrifugal concentration

Figure 3 shows the chromatograms of trastuzumab at 0.5 - 5 μg/mL with and without centrifugal concentration.
To quickly evaluate the effect of concentration by centrifugal filtration, MonoSelect RP-mAb, a phenyl-bonded monolithic column, was used for LC separation. Trastuzumab could be analyzed as a single sharp peak around 3 min. However, the column was too short to separate therapeutic mAbs or human endogenous IgG. High-temperature reversed-phase LC [10,11,15,16], which we have already reported, can be applied here as it allows simultaneous analysis of multiple antibody drugs and bioanalysis.

Table 1 shows the peak heights and concentration factors before and after centrifugal concentration of the trastuzumab solutions. The average factor was 26.1, and peaks were detected after concentration even from 0.2 and 0.5 μg/mL trastuzumab solutions that were not detected otherwise. The detection limit of trastuzumab calculated at S/N=3 was 0.055 μg/mL, which was 16.4 times higher than that obtained without concentration (0.898 μg/mL).

Table 1. Peak intensities of trastuzumab (n = 6) without and with centrifugal concentration, and concentration factors.

| Trastuzumab (μg/mL) | Without concentration | With concentration | Concentration factor |
|---------------------|-----------------------|--------------------|---------------------|
| 0.2                 | -                     | 8,008              | -                   |
| 0.5                 | -                     | 28,168             | -                   |
| 1                   | 2,635                 | 63,197             | 24.0                |
| 5                   | 13,653                | 365,256            | 26.8                |
| 10                  | 27,033                | 743,486            | 27.5                |

3.3. Application to other therapeutic mAbs

To show the versatility of this method, calibration curves for three mAbs (bevacizumab, rituximab, and nivolumab) were generated before and after concentration (Figure 4). Upon concentration, bevacizumab and rituximab were detected in the range 0.2–10 μg/mL, while nivolumab peaks were obtained in the range 0.1–10 μg/mL, with good linearity (R² ≥ 0.996).

Table 2 summarizes the peak heights and concentration factors before and after concentration of the three drug solutions at each concentration. The average factors were 24.7, 24.9, 26.4, and the peaks could be detected from 0.1 or 0.2 μg/mL drug solutions by concentration. The concentration factor by ultrafiltration was similar, regardless of the therapeutic mAb type; thus, quantitation of one drug can be corrected for variability during enrichment by adding another drug as an internal standard.

The detection limits of bevacizumab, rituximab, and nivolumab calculated at S/N=3 were 0.013, 0.032, and 0.026 μg/mL, which were 14.5, 21.9, and 19.6 times higher than that obtained without concentration (0.190, 0.693, and 0.509 μg/mL), respectively.

Fig. 4. Calibration curves of (a) bevacizumab, (b) rituximab, and (c) nivolumab before and after centrifugal concentration in the range of 1-10 μg/mL. Plots: 1, after concentration; 2, before concentration.
Recently, Vasicek et al. reported a bioanalytical method in which immunoaffinity-purified therapeutic mAbs were deglycosylated and subjected to intact mass spectrometric analysis with HR-MS [17]. In that report, the detection limit was 0.1 μg/mL, even with large injections (30 μL) of the sample. On the other hand, in the bioanalysis of antibody-drug-conjugate: trastuzumab emtansine by LC–HRMS after immunoaffinity purification, the linear dynamic range was 5–100 μg/mL [18]. These were less sensitive than the trypsin digested LC-MS/MS method [7]. Our enrichment approach can also contribute to the high sensitivity of intact bioanalysis of therapeutic mAbs by such a middle-down or top-down approach.

### 4. Conclusion

Herein, we reported an LC-based analytical method for therapeutic mAbs by concentrating the sample solution on a centrifuge filter coated with MPC monomer and quantifying the mAb by native fluorescence detection. The method showed sufficient sensitivity and good quantitativity, and it could be applied to at least four therapeutic mAbs. Since a sufficient amount of mAbs can be concentrated and introduced into the detector, we expect that it can be used for bioanalysis such as intact mass spectrometry using HR-MS and LC-UV analysis, which lack detection sensitivity. The results presented here could greatly contribute to simple, sensitive, and precise bioanalysis of therapeutic mAbs.

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### Table 2. Peak intensities of bevacizumab, rituximab, and nivolumab (n = 2) without and with centrifugal concentration, and concentration factors.

| Drug (μg/mL) | Bevacizumab | Rituximab | Nivolumab |
|-------------|-------------|-----------|-----------|
|             | Without concentration | With concentration | Without concentration | With concentration | Without concentration | With concentration |
| 0.1         | N.D.        | N.D.      | N.D.      | N.D.        | N.D.        | -         | 9,027      |
| 0.2         | N.D.        | N.D.      | -         | -           | -           | N.D.      | N.D.  |
| 0.5         | -           | 11,296    | -         | 29,749      | -           | 44,104    | -         |
| 1           | -           | 28,970    | -         | 87,427      | -           | 88,839    | 22.8      |
| 2           | 2,608       | 64,405    | 24.8      | 8,202       | 166,399     | 20.3      | 7,128     | 175,566   |
| 5           | 5,523       | 135,233   | 24.5      | 17,010      | 440,096     | 25.9      | 17,323    | 516,898   |
| 10          | 14,933      | 368,851   | 24.7      | 30,059      | 838,351     | 27.8      | 33,551    | 950,697   |

N.D. means “not determined”, “-” means “not detected or not calculated”.

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