Fluorescence Bioanalysis of Bevacizumab Using Pre-Column and Post-Column Derivatization – Liquid Chromatography After Immunoaffinity Magnetic Purification

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Abstract
This report presents two fluorescence labeling methods for therapeutic monoclonal antibody, bevacizumab, to increase its detection sensitivity for fluorescence detection. One method is high-temperature reversed-phase LC (HT-RPLC) following post-column fluorogenic derivatization using o-phthalaldehyde with thiol. Another method is pre-column derivatization using Zenon Alexa Fluor 488 protein-tag following size-exclusion chromatography (SEC). The calibration curves of bevacizumab were 1–50 μg/mL (post-column method) and 0.1–10 μg/mL (pre-column method). Both methods showed good correlation coefficients ($r^2 > 0.991$). The LOD and the LOQ of bevacizumab were, respectively, 0.13 and 0.43 μg/mL (post-column method) and 0.03 and 0.1 μg/mL (pre-column method). The sensitivities were about 2 and 10 times higher than that of native fluorescence detection. The proposed methods were applied to bevacizumab spiked human plasma samples. The bevacizumab in plasma samples was purified selectively with immunoaffinity beads and detected as a single peak using HT-RPLC or SEC with fluorescence detection.

Keywords: Bevacizumab; Fluorescence derivatization; High-temperature reversed-phase liquid chromatography; Immunoaffinity purification; Bioanalysis

1. Introduction
Therapeutic monoclonal antibodies (mAbs) typically possess long drug efficacy and few side effects. However, their pharmacokinetics (PK) and pharmacodynamics (PD) are much more complicated than those of low-molecular-weight pharmaceuticals [1,2]. Recently, PK and PD analyses of bevacizumab have attracted attention for planning optimal therapeutic programs in combination dose therapy such as FORFIRI plus bevacizumab [3,4], and for the evaluation of biological equivalencies in biosimilar development. To date, PK and PD analyses of bevacizumab have primarily used the ligand binding assay (LBA) [5-7]. While LBA allows for high sensitivity and high throughput analysis, there are several potential cross-reactivity and low accuracy [8]. In contrast, various liquid chromatography – tandem mass spectrometry (LC-MS/MS) methods have been applied to analysis of therapeutic mAbs in serum or plasma samples [9-15]. These methods enable sensitive bioanalysis of therapeutic mAbs, but these methods present several limitations such as time-consuming trypsin digestion and manual purification process for tryptic peptides using solid-phase extraction cartridges. Against this background, we recently developed simple, sensitive, accurate and rapid quantification of therapeutic mAbs, bevacizumab and infliximab, in cancer and rheumatoid arthritis (RA) patient plasma using a combination of immunoaffinity magnetic purification and high-temperature reversed-phase LC (HT-RPLC) with fluorescence detection.
[16]. In this method, target drugs in blood samples are purified using immunoaffinity magnetic beads immobilized with anti-idiotypic mAbs. The purified drugs are separated further using HT-RPLC [17-20], which is suitable for excellent separation among IgGs, using a large pore-size octyl column. The separated drugs are detected sensitively with their own fluorescence. This method does not require tryptic digestion or expensive LC-MS/MS instruments. We applied both methods to the bioanalyses of plasma samples obtained from cancer and RA patients who had been administered each drug. The recommended dosages of most therapeutic mAbs are of milligram per kilogram of body mass. Their blood concentrations are also of microgram per milliliter order. Therefore, similar methodology is expected to be applicable for the bioanalysis of many commercial therapeutic mAbs. However, applying this method to the bioanalysis of next-generation therapeutic mAbs such as antibody-drug-conjugate [21,22], recycling antibodies [23,24], and potelligent antibodies [25,26], which would be marketed as low-dose mAbs in the near future, is expected to be difficult because of its low detection sensitivity. Consequently, more sensitive detection methods than native fluorescence detection are expected to be necessary. Fluorescence labeling methods of proteins in LC or capillary electrophoresis analysis are roughly classifiable into two groups: chemical and biochemical modifications. Chemical modification targets functional groups such as amines and thiols of amino acid residues in proteins. For this purpose, low-molecular fluorescent probes represented by fluorescein, tetramethyl rhodamine, Alexa Fluor dyes, and CyDyes have generally been used [27]. However, for fluorescence derivatization targeting amines in proteins, control of the quantities of labeled fluorophores in a molecule is difficult. Furthermore, multiple introduction of fluorophores can induce a decrease of solubility and aggregation of analytes. Therefore, for high-sensitivity and high-resolution analysis of proteins, post-column fluorescence derivatization methods are expected to be preferred. Biochemical modification using a protein-tag such as Protein G [28] and Zenon labeling technology [29-31] has been used widely in fluorescence imaging and immunostaining.

For this study, we developed two fluorescence labeling methods to enhance therapeutic mAbs bevacizumab detection sensitivity for fluorescence detection. The one is HT-RPLC following post-column fluorogenic derivatization using o-phthalaldehyde with thiol. These reagents react with ε-amino groups of lysine residue in bevacizumab under alkaline conditions to form highly fluorescent isoindole derivatives (Fig. 1a). The other is pre-column derivatization using Zenon Alexa Fluor 488 protein-tag followed by size-exclusion chromatography (SEC) (Fig. 1b). The post-column derivatization enables automatic fluorescence labeling in the LC system, and the pre-column derivatization allows IgG-specific fluorescence labeling. Both are expected to detect fluorescence in longer wavelength region than native fluorescence and increase detection sensitivity. The developed methods were validated in terms of sensitivity, linearity, and precision. Both methods were applied to bevacizumab containing human plasma samples. The bevacizumab in plasma samples was purified selectively using immunoaffinity beads immobilized with anti-idiotypic mAbs. Our literature review indicates that this report is the first to describe sensitive LC analysis of commercial therapeutic mAb by pre-column and post-column fluorescence derivatization.

2. Experimental

2.1. Reagents and solutions

Deionized and distilled water that had been purified using purelab flex system (ELGA, Marlow, UK), was used to prepare all aqueous solutions. LC grade acetonitrile was purchased from Honeywell (Morris Plains, NJ, USA). Isopropanol was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). OPA and 2-mercaptoethanol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bevacizumab (Avastin® 400 mg/16 mL Intravenous Infusion) was produced by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Zenon Alexa Fluor 488 Human IgG Labeling kit, Dynabeads M-280 Tosyl Activated (2.8 μm particle size) and DynaMag Spin were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Anti-bevacizumab idiotype antibody was ELISA grade, obtained from Abnova Corp. (Taipei, Taiwan). Control human plasma was obtained from healthy volunteers. These volunteers understood the purpose and significance of this experiment and donated blood after providing written consent. All other chemicals were of the highest purity available and were used as received.
2.2. HT-RPLC post-column fluorescence derivatization

The liquid chromatograph system (Prominence; Shimadzu Corp., Kyoto, Japan) consisted of a system controller (CBM-20A), an auto sampler (SIL-20AC), SPD-20A, four LC-20AD pumps, an online degasser (DGU-20A), a column oven (CTO-20A), and a fluorescence spectrometer (RF-10AXL) equipped with a 12-μL flow cell. The analytical column (Aeris Widepore XB-C8) was a core–shell type analytical column packed with 3.6-μm core-shell particles (150 × 2.1 mm I.D.; Phenomenex Inc., Torrance, CA, USA). Mobile phase A was water containing 0.1% trifluoroacetic acid (TFA), whereas solvent B was 70% isopropanol : 20% acetonitrile : 9.9% water : 0.1% TFA. Gradient profiling involved isocratic elution with A/B (90 : 10) for 1 min, linear gradient elution from A/B (90 : 10) to A/B (75 : 25) for 1 min, linear gradient elution from A/B (75 : 25) to A/B (50 : 50) for 13 min, isocratic elution with A/B (0 : 100) for 5 min, and isocratic elution with A/B (90 : 10) for 8 min. The flow rates of the mobile phase and the column temperature were set respectively at 0.25 mL/min and 75°C. The effluent from the analytical column was then mixed with a stream of reagent solutions. The reagent solutions were 30 mM OPA in methanol and 100 mM borate buffer (pH 9.1) containing 30 mM 2-mercaptoethanol; those were delivered by two pumps (LC-20AD; Shimadzu Corp.) at flow rates of 0.1 mL/min. The fluorescence derivatization reaction was performed in a reaction (stainless tubing, 1 m × 0.13 mm I.D.) immersed in a column oven set at 75°C. The resulting fluorescence was monitored, respectively, at excitation and emission wavelengths of 278 and 343 nm for native fluorescence and 350 and 450 nm for OPA derivatives. The collected data were analyzed (Lab Solutions LC v. 1.21; Shimadzu Corp.). The peak areas and heights were estimated using the baseline-to-baseline method.

2.3. Pre-column fluorescence derivatization-SEC

A mixture of bevacizumab solution (37 μL) and 2 M aqueous sodium hydroxide (2 μL) was added to 200 μg/mL Zenon Alexa Fluor 488 Humin IgG Labeling Kit (1 μL) and was then let to stand at ambient conditions for 10 min. The resulting solution (2 μL) was injected into the SEC system. The Nexera an ultra-high-performance liquid chromatography system (Shimadzu Corp.), consisted of a system controller (CBM-20A), an AC autosampler (SIL-30), a pump (LC-30AD), an online degasser (DGU-20A), a column oven (CTO-30A), and a fluorescence spectrometer (RF-20Ax) equipped with 12-μL flow cell. After the data were processed (Lab Solutions LC ver. 1.21), peak areas were estimated using the baseline-to-baseline method for quantification. An SEC column was used (300 × 4.6 mm I.D., 3 μm, KW402.5-4F; Shodex / Showa Denko K.K., Tokyo, Japan) for isocratic elution using 100 mM phosphate buffer (pH 7.0). The flow rate of the mobile phase and the column temperature were set, respectively, at 0.35 mL/min and ambient. The UV detection was monitored at 210 nm. The fluorescence was monitored, respectively, at excitation and emission wavelengths of 278 and 343 nm (native fluorescence) and 490 and 525 nm (Alexa Fluor 488 fluorescence). The collected data were analyzed (Lab Solutions LC v. 1.21). Peak areas and heights were estimated using the baseline-to-baseline method.

2.4. Fluorescence spectroscopic analysis

Fluorescence spectra were measured using a spectrophotometer (FP-8300; Jasco Corp. Tokyo, Japan) in 10 mm × 10 mm quartz cells. Spectral bandwidth of 5 nm was used for both excitation and emission monochrometers. OPA in methanol (100 μL, 5 mM) and 5 mM 2-mercaptoethanol in 0.1 M borate buffer (pH 9.1) were added to an aliquot of aqueous bevacizumab solution (100 μL, 100 μg/mL). After vortex mixing, the mixture was left to stand at room temperature for 10 min, then it was mixed with 300 μL of isopropanol-acetonitrile-0.1% aqueous TFA (7:2:1, v/v). The resulting solution was subjected to fluorescence spectroscopic analysis. Fluorescence emission spectrum of OPA-bevacizumab derivative was recorded for 350–600 nm with excitation wavelength of 340 nm.

2.5. Preparation of immunoaffinity magnetic beads

Anti-bevacizumab idiotype antibody was coupled to tosyl-activated magnetic beads as described in our previous report [16]. A suspension of the dynabeads (33 μL, 1 mg) was placed in a 1.5-mL polypropylene tube. After removal of the solvent, the idiotype antibody (30 μg) dissolved in 100 μL of 100 mM sodium phosphate buffer (pH 7.4) containing 1.5 M ammonium sulfate was added. This mixture was vortex-mixed at room temperature for 20 hr using a microtube mixer MT-360 (Tomy Seiko Co. Ltd., Tokyo, Japan). After addition of 50 μL of 1 M tris-HCl buffer (pH 7.4) containing 1% peptone, the mixture was further vortex-mixed at room temperature for 20 hr. After removal of the solution, the remaining beads were washed with 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% Tween 20 (washing buffer). After washing, beads were dispersed in 100 μL of 100 mM sodium phosphate buffer (pH 7.4) and stored as a suspension at 4°C.

2.6. Immunoaffinity purification of bevacizumab from human plasma samples

Affinity purification was done using 1 mg immunoaffinity magnetic beads per sample. After removal of the solvent, the immunoaffinity beads were added to 100 μL of bevacizumab-spiked plasma sample diluted 10 times with 100 mM phosphate buffer (pH 7.4). The mixture was incubated with vortex-mixing at room temperature for 1 hr.
After incubation, the beads were washed twice with 100 μL of the washing buffer. Bevacizumab was then eluted once with 100 μL of 100 mM citrate buffer (pH 3.1). For HT-RPLC – post-column derivatization system, volumes of 2 μL aliquots were subjected. For pre-column derivatization – SEC analysis, volumes of 37 μL aliquots were subjected to the derivatization procedure described in Section 2.3. Following elution, the remaining beads were reused after equilibration with 100 μL of 100 mM sodium phosphate buffer (pH 7.4).

2.7. Method validation

To ascertain the validation parameters, peak areas were estimated using LabSolution LC. The baseline-to-baseline method was used for quantification. The assay precision was determined from repeated measurements of six concentrations of 1–50 μg/mL (1, 2, 5, 10, 20 and 50 μg/mL) for HT-RPLC – post-column fluorescence derivatization, and from 0.1–10 μg/mL (0.1, 0.5, 1, 2, 5, and 10 μg/mL) for pre-column fluorescence derivatization—SEC analyses, respectively. To ascertain the intra-day precision, these levels were analyzed five times daily. For inter-day precision, these levels were analyzed three times per day for five days (n = 15). These samples were subjected to the respective analytical procedures. For the quantitative analysis, calibration standard solutions (n = 5) were prepared by diluting the stock solutions with six concentration ranges of 1–50 μg/mL (1, 2, 5, 10, 20, and 50 μg/mL) for HT-RPLC – post-column fluorescence derivatization, and from 0.1 to 10 μg/mL (0.1, 0.5, 1, 2, 5, and 10 μg/mL) for pre-column fluorescence derivatization—SEC analyses, respectively. The equations of the calibration curves were determined using least squares linear prediction. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined, respectively, from signal-to-noise ratios of 3 and 10. Concentrations of bevacizumab were calculated using the standard addition method.

3. Results and discussion

3.1. HT-RPLC – post-column fluorescence derivatization method

For HT-RPLC analysis, a widepore Zorbax-300SB C8 column is used at high temperatures (>70°C) with a mobile phase containing solvents of high eluotropic strength, such as isopropanol and acetonitrile. Our previous study [16] revealed that HT-RPLC can achieve good separation with sharper peaks for four therapeutic mAbs (trastuzumab, infliximab, tocilizumab, and bevacizumab), which have very similar molecular weights, within 20 min. This result implies that the identification and quantification of target mAbs is possible even though immunoaffinity purification is insufficient. To optimize the LC-fluorescence detector conditions; fluorescence emission spectra of OPA derivative were measured (data not shown). As shown in the experimental section, reaction conditions for bevacizumab were set to be same as those of post-column derivatization. The emission maximum was approximately 450 nm, as reported for other amine-OPA derivatives [32,33]. The post-column derivatization reaction for bevacizumab proceeded successively in the presence of OPA and thiol in alkaline buffer with heating at 75°C. We optimized several factors related to the post-column fluorescence derivatization of bevacizumab (Fig. 2).

![Fig. 2. Effects of (a) kind of thiol, (b) pH of borate buffer, (c) OPA concentration, and (d) reaction coil length on the post-column fluorescence derivatization of bevacizumab.](image-url)
Among three thiols we examined (2-mercaptoethanol, \(N\)-acetylcysteine, sodium 2-mercaptopropanesulfonate), 2-mercaptoethanol gave the greatest peak area (Fig. 2a). By varying the borate buffer pH in the range of 8.2–9.7, we found the maximum peak area to be 9.1 (Fig. 2b). We examined the effect of OPA concentration on fluorescence development for 5–50 mM (Fig. 2c). The fluorescence development was maximized at 30 mM. We also examined the reaction coil length of 25–200 cm on fluorescence development (Fig. 2d). Product formation increased to a maximum at 50 cm, after which constant peak area of the derivative was observed. We selected 100 cm as the optimum coil length. The total volume inside the coil was about 133 μL, and the reaction time calculated from the flow rate of the mixed reaction solution was about 0.30 minutes. It was shown that the post-column derivatization reaction using OPA proceeds rapidly even in such a short time. Figure 3 portrays a chromatogram of 50 μg/mL of bevacizumab analyzed using HT-RPLC – post-column fluorescence derivatization (red line). For comparison, the chromatogram of bevacizumab analyzed by HT-RPLC with native fluorescence detection is also shown as a black line. By post-column derivatization, the fluorescence peak intensity of bevacizumab was increased about twice despite having twice the volume of column elution into the fluorescence detector. As the chromatogram shows, immunoaffinity-purification was able to collect bevacizumab selectively from drug-spiked human plasma samples, and subsequent HT-RPLC post-column fluorescence derivatization helped to analyze bevacizumab with high sensitivity (Fig. 4).

3.2. Pre-column fluorescence derivatization – SEC method

For pre-column fluorescence derivatization of bevacizumab, we adopted Zenon Human IgG Labeling technology. In this technology, Zenon protein fragment binds specifically to Fc regions of IgGs with a ratio of 2:1 [29]. This technology is widely used in fluorescence and enzyme labeling of antibodies [30,31]. Because pre-column fluorescence derivatization using Zenon technology uses protein–protein interaction for labeling, HT-RPLC separation of the resulting derivatives performed with high eluotropic strength organic solvents, which are isopropanol and acetonitrile, and acidic solution as mobile phases, was impossible. Consequently, these resulting derivatives were separated by SEC mode.

For pre-column fluorescence derivatization, bevacizumab was reacted with a Zenon labeling kit according to the manufacturer’s instructions. Figure 5 shows SEC chromatograms of 100 μg/mL of bevacizumab or its fluorescence derivative with native fluorescence detection. The pH value represents the pH at which bevacizumab reacts with the Zenon labeling kit. The chromatograms respectively show (a) Zenon Alexa Fluor-bevacizumab derivative (pH 7.5), (b) Zenon Alexa Fluor-bevacizumab derivative (pH 3.5), (c) only Zenon reagent, and (d) only bevacizumab. From Fig. 5a, a peak corresponding to the complex of bevacizumab and Zenon reagent was detected around 6 min and was clearly discriminated with peaks of the Zenon reagent (Fig. 5c) and bevacizumab itself (Fig. 5d). Furthermore, formation of the complex produced a significant decrease of peak of the Zenon reagent itself at around 7.5 min by complexation. Without pH adjustment of reaction solution, insufficient formation of the complex was confirmed (Fig. 5b). These results suggest that reaction pH is an important factor affecting the complex formation. Therefore, pH adjustment of the eluate should be neutralized with a strong base such as sodium hydroxide.
Fig. 5. SEC chromatograms of 100 μg/mL of bevacizumab or its fluorescence derivative with native fluorescence detection: (a) Zenon Alexa Fluor-bevacizumab derivative (pH 7.5), (b) Zenon Alexa Fluor-bevacizumab derivative (pH 3.5), (c) Zenon reagent only, and (d) bevacizumab only.

Figure 6 shows SEC chromatograms of 5 μg/mL of bevacizumab or its fluorescence derivatives with different detection: (a) bevacizumab derivative with Alexa Fluor488 detection (ex. 490 nm, em. 525 nm), (b) bevacizumab with native fluorescence detection, and (c) bevacizumab with UV 210 nm. By comparing Fig. 6a and 6b, the pre-column fluorescence derivatization enhanced its fluorescence peak intensity by about 10 times.

Fig. 6. SEC chromatograms of 5 μg/mL of bevacizumab or its fluorescence derivatives with different detection: (a) bevacizumab derivative with Alexa Fluor488 detection (ex. 490 nm, em. 525 nm), (b) bevacizumab with native fluorescence detection, and (c) bevacizumab with UV 210 nm.

Figure 7 shows a chromatogram of 5 μg/mL of bevacizumab spiked in plasma sample treated with immunoaffinity purification analyzed using pre-column derivatization – SEC.

Fig. 7. Chromatogram of 5 μg/mL of bevacizumab spiked in plasma sample treated with immunoaffinity purification analyzed using pre-column derivatization – SEC.

3.3. Method validation
3.3.1. HT-RPLC – post-column fluorescence derivatization

Relations between the peak area and the bevacizumab concentration were all linear (correlation coefficients; 0.998, n = 5) over the concentration range of 1–50 μg/mL per 2-μL injection. The LOD and LOQ of the bevacizumab by post-column derivatization were, respectively, 0.13 μg/mL and 0.43 μg/mL. The intra-day and inter-day precisions were, respectively, 2.3%–8.8% and 4.6%–13.8%.

3.3.2. Pre-column fluorescence derivatization – SEC method

The relations between the peak area and the bevacizumab concentration were all linear (correlation coefficients; 0.991, n = 5) over the concentrations of 0.1–10 μg/mL per 2-μL injection. The LOD and LOQ of the bevacizumab by pre-column derivatization were, respectively, 0.03 μg/mL and 0.1 μg/mL. These values were almost 2 times lower than those given by HT-RPLC with native fluorescence detection. The intra-day and inter-day precisions were, respectively, 1.3%–6.2% and 3.0%–10.9%.

4. Conclusion

For this study, we developed two fluorescence derivatization – LC methods for bevacizumab to increase its detection sensitivity for fluorescence detection. Bevacizumab was purified selectively from plasma using magnetic beads immobilized with commercial anti-idiotype antibody. In both methods, a peak corresponding to bevacizumab was detected within 10 min and detected as a single peak without interfering peaks. By pre-column and post-column fluorescence derivatization, detection sensitivities increased respectively about 10 and 2 times compared to native fluorescence detection. The intra-day and inter-day precisions showed good results for use in bioanalysis. Proposed methods are highly diverse. They are expected to be theoretically applicable to various therapeutic mAbs. Our methods enabled selective bioanalysis of bevacizumab without LC-MS/MS instruments. Therefore, these methods are expected to
become general-purpose analytical methods that are capable of complementing results obtained using conventional LBA or LC-MS/MS.

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