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RESEARCH PAPER

Development of an LC-MS/MS method with protein G purification strategy for quantifying bevacizumab in human plasma

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Abstract Biopharmaceutical products such as protein drugs and monoclonal antibodies (mAb) are currently of great interest with monoclonal antibody drugs being one of the fastest growing categories of biopharmaceutical products. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has gained high interest for measuring mAb drugs in biological samples in recent years due to its high selectivity. Bevacizumab is a humanized immunoglobulin G (IgG) mAb drug against human vascular endothelial cell growth factor A (VEGF-A). It is used for treating many types of cancers. Recent studies have indicated that clinical outcomes vary among patients treated with bevacizumab and produce various side effects, such as vascular disorders. In this study, we developed an LC-MS/MS method to quantify bevacizumab concentration. We selected readily available and economic materials for sample preparation to facilitate its wider use in clinical fields.—Protein G was used to trap bevacizumab from human plasma. In place of an extended stable isotope-labeled internal standard (SIL-IS), the IgG-based drug-IS tocilizumab was used because of its better calibration performance. The method was validated in terms of its precision, accuracy, linearity, and sensitivity. The accuracies which were expressed as percentage recoveries for three concentration levels were within 92.8 ± 3.2 to 112.7 ± 4.5%. Repeatability and intermediate precision in terms of peak area ratios were lower than 5.2 and 12.9% RSD, respectively. The application to patients' sample measurements revealed a wide individual variability of drug concentrations, and the proposed simple and general method may facilitate personalized medicine for improving therapeutic efficacy and safety.

Keywords Bevacizumab · Protein G purification · Monoclonal antibody (mAb) · Plasma · In-solution digestion · LC-MS/MS

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Introduction

Biopharmaceutical products such as protein drugs and monoclonal antibodies are currently of great interest. These types of drugs have the potential to improve therapeutic effects and reduce side effects. Monoclonal antibody (mAb) drugs are one of the rapidly growing categories of biopharmaceutical products. From 2010 to 2014, greater than 30% of the approved biopharmaceuticals in the USA and Europe belong to mAb-based products [1]. Bevacizumab is a humanized immunoglobulin G (IgG) monoclonal antibody (mAb) drug

against human vascular endothelial cell growth factor A (VEGF-A), and it is also the first anti-angiogenesis agent to be approved by the FDA for metastatic colorectal cancer treatment in 2004 [2]. Subsequently, bevacizumab has been approved for treating many types of cancers, such as non-small cell lung cancer, metastatic renal cell carcinoma, and glioblastoma.

Personalized medicine is increasingly important in the clinic, and therapeutic drug monitoring (TDM) is one important strategy to achieve personalized medicine. TDM of monoclonal antibody concentrations has also been conducted to improve therapeutic efficacy, reduce side effects, and improve the control of disease activity [3]. A previous study found that pharmacokinetic (PK) behaviors are usually more complex with biopharmaceutical products than with conventional small molecule drugs [4]. Although mAbs can provide a better safety profile, a recent study indicated that clinical outcomes vary among patients treated with bevacizumab, and the extent of the side effects such as vascular disorders also varied [5]. As a result, simple and accurate quantification methods for these biopharmaceutical drugs in biological fluids are vital and allow dose adjustment depending on the pharmacokinetic profiles of different patients.

Currently, ligand binding assay (LBA), for example, enzyme-linked immunosorbent assay (ELISA) is the most widely used methods for the measurement of mAb concentrations in the plasma/serum [6–8]. However, these LBA methods have several limitations. First, the customized of specific reagents such as antigens or antibodies are usually required for LBA assays, and the reagent's quality and consistency between different batches would seriously affecting the method performances including specificity, robustness, and also sensitivity [9, 35]. Second, the generation of the specific reagent for LBA is commonly time and cost consuming [11]. Third, the interference in the sample matrix may also lead to inaccurate quantification and insufficient dynamic range due to the potential cross reaction [12, 13]. A recent study compared the accuracy between LBA and LC-MS for infliximab quantification in human plasma. A significant bias was found with commercial ELISA [13]. Therefore, to develop an alternative method with the advantages of simple and cost-effective for wider applications of TDM in clinical fields is still required. Due to its high sensitivity and selectivity, LC-MS is being used increasingly for quantifying monoclonal antibodies in human plasma [14–16]. However, serious matrix effects may lead to inaccurate quantification. Therefore, many sample cleanup methods have been proposed for purifying mAbs from biological matrixes [17–20]. Iwamoto et al. developed a nano-surface and molecular-orientation limited (nSMOL) proteolysis method for quantification of bevacizumab in human plasma; however, it required a special device. Also, clinical validation was not performed in their study for evaluation the utility of their method in clinical measurement [17]. Todoroki recently used an anti-idiotype antibody to purify bevacizumab from human plasma followed by LC with fluorimetric detection analysis. Since idiotype antibodies are generated by a customized service, the sample preparation cost would be relatively higher [21]. Therefore, a simple and cost-effective method is still required to enable a wider application of TDM in clinical fields.

In this study, we proposed a general purification method coupled to LC-MS/MS to determine bevacizumab concentrations in human plasma. Protein G magnetic beads were chosen for sample purification due to their wide availability and low cost. Protein G magnetic beads selectively trap IgG class antibodies in human plasma. This procedure can help to reduce the sample matrix complexity and the large amount of endogenous proteins such as albumin and other proteins, including immunoglobulin A and immunoglobulin M, can be removed. To calibrate the potential loss by trapping, another IgG-based pharmaceutical product, tocilizumab, was used as an internal standard. The tandem MS displayed the great selectivity for the surrogate peptide quantification in human plasma. This LC-MS/MS method was validated in term of its precision, accuracy, linearity, and sensitivity. Finally, the validated method was used to analyze plasma samples obtained from breast cancer patients with brain metastasis to demonstrate its usefulness for TDM in clinical fields.

Materials and methods

Reagents and materials

Bevacizumab was purchased from Roche Applied Science (Indianapolis, IN, USA). Trypsin was purchased from Promega (Madison, WI, USA). Protein G Mag Sepharose Xtra beads were purchased from GE (Piscataway, NJ, USA). MS-grade methanol was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Acetonitrile (ACN) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium bicarbonate, formic acid (FA) solution (99%), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO, USA). Sodium chloride and sodium phosphate dibasic anhydrous were obtained from J.T. Baker (Phillipsburg, NJ, USA). Potassium chloride was purchased from Fluka (Buchs, St. Gallen, Switzerland). Potassium phosphate monobasic was obtained from Sigma (St. Louis, MO, USA). The extend SIL-IS was synthesized by GenScript (Piscataway, NJ, USA). Tocilizumab was purchased from Genentech (South San Francisco, CA, USA).

LC-MS/MS system

An Agilent 1290 UHPLC system equipped with an Agilent 6460 triple quadrupole system (Agilent Technologies, Waldbronn, Germany) was used for the analysis. An Aeris™

PEPTIDE XB-C18 100 × 2.1 mm (1.7 μm) column (Phenomenex, Torrance, USA) was selected for the separation. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in ACN (solvent B) at a flow rate of 0.3 mL min^{-1}. The gradient profile was as follows: 0–1.5 min, 5% B; 1.5–5 min, 5–50% B; 5–5.5 min, 50–100% B; 5.5–6.5 min, 100% B; and column re-equilibration with 5% B for 2 min. The sample reservoir was maintained at 4 °C, and the column oven was set at 40 °C. The injection volume was 20 μL. A positive electrospray ionization mode was utilized with the following parameters: a 325 °C dry gas temperature, a 7 L min^{-1} dry gas flow rate, a 45 psi nebulizer pressure, a 325 °C sheath gas temperature, an 11 L min^{-1} sheath gas flow rate, a 3500 V capillary voltage, and a 500 V nozzle voltage. MS acquisition was executed in multiple reaction monitoring (MRM) mode. The transitions for surrogate peptides were *m*/*z* 588.3 → 602.3 and 523.3 → 797.4, and the transition for extended stable isotope-labeled peptide was 590.3 → 602.3. The transition for tocilizumab was 514.8 → 526.25.

Trap and digestion of bevacizumab from human plasma

In this study, we used protein G beads to trap bevacizumab from human plasma. The protein G bead solution was first conditioned with 200 μL of phosphate-buffered saline (PBS) buffer twice. Next, 5 μL of plasma and 20 μL of tocilizumab (50 μg mL^{-1}) were added to the bead solution and incubated for 1 h at 4 °C. After the incubation, 200 μL of PBS buffer and 200 μL of deionized water were used as washing solutions to remove unbound proteins. To elute the bevacizumab from the beads, 200 μL of 100 mM formic acid solution was added to the sample twice. The eluent was dried under N_2. The dried eluent was reconstituted with 150 μL of 100 mM ammonium bicarbonate buffer and 150 μL of deionized water, and the solution was heated at 90 °C for 25 min. Ten microliters of 100 mM DTT was added to the solution and then heated at 60 °C for 60 min. After cooling, 10 μL of 100 mM IAA was added for alkylation at 30 °C for 30 min in the dark. For trypsin digestion, 18 μL of trypsin (20 μg/200 μL) and 20 μL of 100 mM ammonium bicarbonate were added to the solution and heated at 37 °C for 12 h. To terminate the digestion, 20 μL of 10% FA was added to the trypsin digestion solution. The digested sample was subjected to LC-MS/MS analysis. To evaluate the calibration performance, 10 μL of extended stable isotope-labeled internal standard (SIL-IS) (10 μg/mL) was added before trypsin added.

Method validation

Selectivity

Six plasma blank samples (without administration of bevacizumab and tocilizumab) were applied to evaluate the selectivity of this method. We compared the chromatograms of plasma blank and bevacizumab and tocilizumab spiked plasma to confirm whether there were any interference exist at the same retention time.

Linearity, limits of detection, and limits of quantification

A series dilution of the stock solution (25,000 μg mL^{-1}) was conducted to produce solution concentrations ranging from 1000 to 30 μg mL^{-1} of bevacizumab in human plasma samples. Aliquots of bevacizumab solution were added to plasma blank to obtain 30, 50, 100, 300, 500, and 1000 μg mL^{-1} spiked samples to generate the calibration curve, and each concentration was analyzed for three replicates. The peak area of the analyte was integrated using the Agilent software. The calibration curve was obtained with a weighting factor of $1/X$ and by linear regression analysis. The limit of detection (LOD) was defined as a signal to noise (S/N) ratio of three. The limit of quantification (LOQ) was defined as a signal to noise (S/N) ratio of ten.

Accuracy and precision

To evaluate accuracy, three different concentrations (30, 300, and 1000 μg mL^{-1} in the plasma) of bevacizumab were spiked into the different plasma samples from healthy controls and patients with infection, and the back calculated concentrations were compared to the spiked concentrations. Five determinations per concentration were applied to evaluate the accuracies according the FDA guidance for bioanalytical method validation. For repeatability evaluations, the samples were analyzed by five determinations per concentration within the same day. For intermediate precision, three samples per concentration were prepared individually and analyzed at three different days.

Stability, matrix effect, and extraction recovery

Processed sample stability was evaluated by measuring the sample concentration after 24 h kept in 4 °C autosampler. To measure the matrix effect, three concentrations of digested bevacizumab standards were individually spiked into six plasma blank samples and one reagent blank sample. The matrix effects at three concentrations were calculated using surrogate peptide intensity in plasma blank divided by surrogate peptide intensity in reagent blank. The reagent blank was composed of same component as the bevacizumab digestion buffer.

To evaluate the extraction recovery, bevacizumab was spiked into the plasma samples at three different concentrations (30, 300, and 1000 μg mL^{-1} in the plasma) before incubation with protein G beads as the prespiked samples. The post-spiked samples were prepared by adding bevacizumab into the plasma blank samples after the protein G trapping

step. The extraction recoveries were calculated by dividing the peak area for the prespiked sample by the peak area for the post-spiked sample and multiplying by 100%.

Collection of clinical samples

Plasma samples were collected at the National Taiwan University Hospital. The study was approved by the institutional review board of the National Taiwan University Hospital. Avastin® (bevacizumab) is administered intravenously at a dose of 15 mg kg^{-1}. The blood samples were centrifuged at 10,000×*g* for 15 min, and the resultant plasma samples were stored at − 80 °C until use.

Results and discussion

Method development

Selection of surrogate peptides of bevacizumab

This study used surrogate peptides for bevacizumab quantification. Plasma blank and bevacizumab-spiked samples were profiled by time of flight mass spectrometry to identify unique peptides that were contributed by bevacizumab. The amino acid sequences of surrogate peptides were investigated using the website PeptideMass (http://web.expasy.org/peptide_mass/). Two surrogate peptides, VLIYFTSSLHSGVPSR and FTFSLDTSK, were identified, and they were further investigated for their mass transition for bevacizumab quantification. The surrogate peptide (VLIYFTSSLHSGVPSR) with higher abundance was used for quantification, and the other surrogate peptide (FTFSLDTSK) was used for confirmation. The amino acid sequences, mass transitions, and the corresponding mass parameters are listed in Table 1. These surrogate peptides sequences of bevacizumab were the same as the previous studies [17, 22]. Figure 1 shows that the selected surrogate peptides were selective and that no interference could be detected.

Trapping of immunoglobulin G monoclonal antibodies by using protein G beads

Protein G beads were used to selectively extract IgG class antibodies including bevacizumab. A schematic diagram for protein G trapping procedure is shown in Scheme 1. The entire immunoglobulin G trapping process can be divided into three steps: incubation, wash, and elution. Bevacizumab in human plasma was first trapped using protein G beads with an end-to-end mixer to obtain sufficient interactions between the protein G beads and bevacizumab. Phosphate-buffered saline (PBS buffer) was then used to remove unbound interfering substances. Finally, an acid solution (100 mM formic acid) was added to disrupt the binding between the protein G beads and the target analyte. This immune-affinity trapping workflow provides a convenient method for reducing the complexity of the sample matrix.

Optimization of the protein G trapping procedure

To effectively trap bevacizumab from plasma samples, we evaluated different protein G bead volumes for extraction of bevacizumab in 5 μL of plasma sample. Theoretically, the capacity of 40 μL of protein G beads is sufficient to bind all of the endogenous IgG and bevacizumab in plasma samples (3.5 μg of human IgG per μL of 10% medium slurry, according to the manufacturer). Five different plasma bead volume ratios of 1:2, 1:4, 1:8, 1:12, and 1:16 were investigated to determine the optimal volume of protein G beads required to provide sufficient capacity for the bevacizumab in the samples. The samples were incubated at 4 °C with end-to-end rotation. The results are shown in Fig. 2a. When the ratio was lower than the theoretical capacity (1:8), the signal intensity of the surrogate peptide was relatively low with a high standard deviation. The peak area of the surrogate peptide reached a plateau when the ratio was above 1:8 and was without improvement when the ratio was increased to 1:12 and 1:16. As a result, we selected 1:8 as the optimal plasma: bead reaction ratio to trap bevacizumab from the plasma samples.

The incubation time for protein G beads and plasma samples was further evaluated. To evaluate the time effect on the trapping efficiency, we tested four incubation times from 30 to 180 min. As shown in Fig. 2b, the abundance of surrogate peptide clearly increased from 30 to 60 min; however, as the incubation time increased, the amount of surrogate peptide did not increase noticeably. To provide an efficient method, we selected 60 min as the incubation time.

Other parameters, such as buffer volume and manner of incubation, were also optimized to effectively trap bevacizumab from human plasma samples. A large volume of buffer (420 μL) with end-to-end rotation was found to provide better trapping performance due to the more thorough contact between the protein G beads and bevacizumab.

Optimization of the trypsin digestion procedure

To acquire the optimal trypsin digestion results, we investigated the digestion time and the trypsin/protein ratio. For the evaluation of the digestion time, we tested five digestion times from 1 to 16 h. We observed that from 1 to 12 h, the abundances of surrogate peptide clearly increased; however, as the incubation time increased, there was no enhancement of the abundance of surrogate peptide (Fig. 3a). Therefore, 12 h was selected as the digestion time.

The trypsin/protein ratio is also a critical parameter that should be considered in order to provide better digestion

Table 1 MRM ion transitions and mass parameters for the surrogate peptides, SIL-IS, and tocilizumab

Amino acid sequence	MRM transition	Collision energy (eV)	Fragmentor (V)	Cell accelerator voltage (V)
VLIYFTSSLHSGVPSR[a]	588.3 → 602.3	35	150	7
FTFSLDTSK[b]	523.3 → 797.4	20	150	7
V*LIYFTSSLHSGVPSR	590.3 → 602.3	30	150	7
LLIYYTSR (tocilizumab)	514.8 → 526.3	25	150	7

[a] Used for quantification

[b] Used for confirmation

efficiency. A trypsin/protein ratio from 1:20 to 1:100 (*w*/*w*) is frequently recommended for these digestions. The protein amount is calculated using an average value of IgG among general adult populations [23]. This study evaluated trypsin/protein ratios from 1:10 to 1:100, and the results are shown in Fig. 3b. The results indicate that the surrogate peptide abundances for trypsin/protein ratios of 1:10 and 1: 20 were significantly lower than for a trypsin/protein ratio of 1:50. However, when the ratio was increased to 1:100, the trypsin amount was insufficient to digest the target analyte in plasma samples. To provide a sensitive and robust quantification method, a 1:50 ratio of trypsin/protein was selected.

Selection of internal standards for improving quantification accuracy

LC-MS/MS protein quantification studies commonly use extended SIL peptides as internal standards (IS) [19, 24]. However, extended SIL peptides could not be trapped by protein G beads and a potential trapping loss therefore could not be calibrated. Hongyan et al. suggested the use of the entire molecule as the internal standard to calibrate fluctuations in the sample processing steps [20]. Consequently, we compared two types of internal standards for their correction efficiency: extended SIL peptide-IS and an IgG-based drug-IS. Three concentrations of quality control (QC) samples were used to evaluate the calibration performance. For IgG-based drug-IS, we adapted another IgG-based drug, tocilizumab, as the internal standard to correct for a possible preparation error from the bead trapping step to trypsin digestion. The sequence and the corresponding mass parameters of two types of internal standards are listed in Table 1. Both MRM chromatograms for these internal standards in plasma blank samples and plasma spiked samples are shown in Fig. 1c, d. There was no interference with these transitions for the two internal standard peptides. A comparison of the correction efficiency is shown

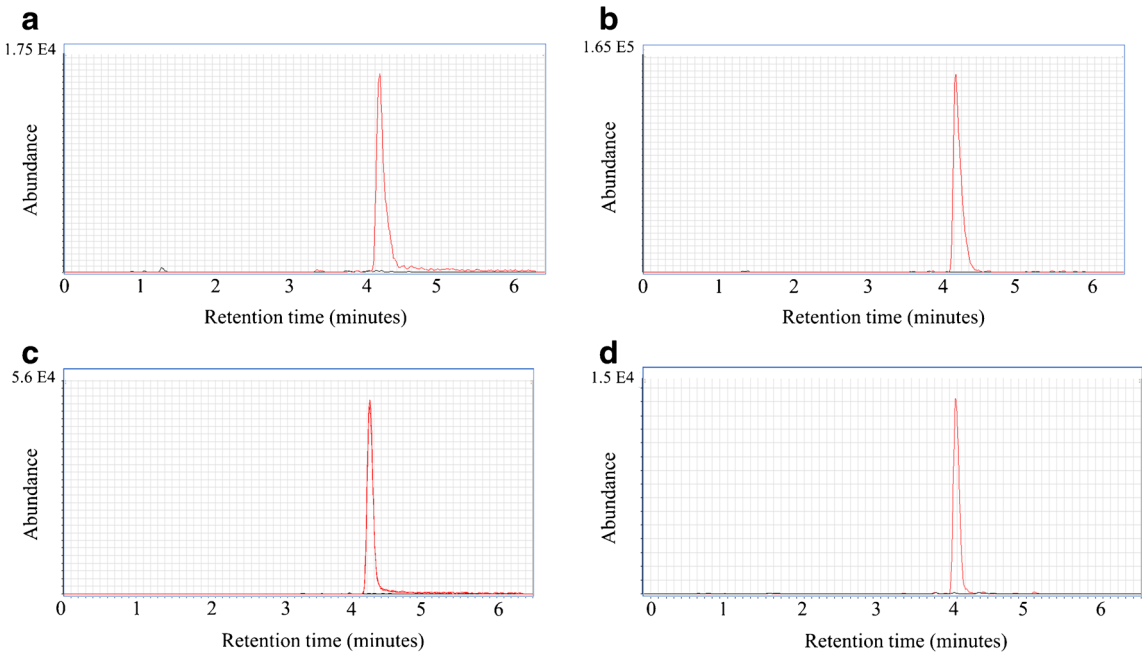
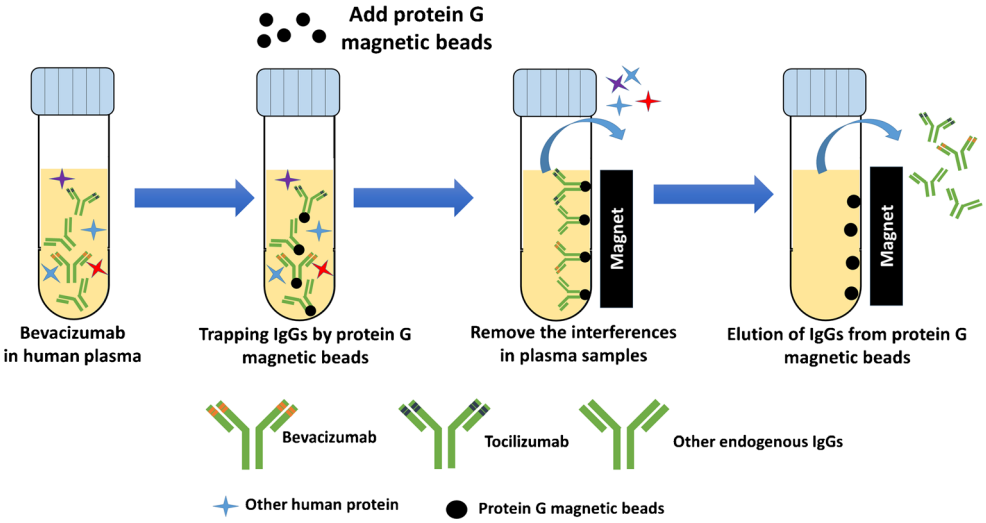


Fig. 1 Overlay of MRM chromatograms of surrogate peptides from spiked and blank plasma samples. **a**, **b** Two surrogate peptides of bevacizumab: VLIYFTSSLHSGVPSR, 588.3 → 602.3 and FTFSLDTSK, 523.3 → 797.4. **c** A surrogate peptide of SIL-IS, V*LIYFTSSLHSGVPSR, 590.3 → 602.3. **d** A surrogate peptide of tocilizumab, LLIYYTSR, 514.8 → 526.3. The red color indicates a spiked plasma sample, and the black color indicates a plasma blank



Scheme 1 Schematic diagram for protein G trapping of bevacizumab from human plasma

in Fig. 4. Both quantification accuracies and precision were used to evaluate the correction efficiency of different internal standards. As shown in Fig. 4a, without applying any internal standard correction, the biases were higher than 40% at low and high concentrations. When using the extended SIL peptide as the internal standard, the accuracy was not noticeably improved. On the other hand, when using the tocilizumab as the internal standard, the bias of all concentration levels were decreased to less than 15%. We additionally compared the precision of peak areas obtained from three replicate samples. As shown in Fig. 4b, the results indicated that without applying any internal standard correction, the RSD is approximately 15–30% for different concentrations. When using the extended SIL peptide as the internal standard, the RSD was not noticeably decreased; in the low and high concentration groups, the RSDs were even greater. On the other hand, when using tocilizumab as the internal standard, the RSD significantly decreased to less than 12%. The good correction efficiency of tocilizumab could be attributed to both tocilizumab and bevacizumab belong to the IgG class and also present in intact form, and as a result, the preparation including protein G trapping and digestion efficiency may be mimicked. According to accuracy and precision comparison results, we could conclude that tocilizumab represents as an effective internal standard for bevacizumab quantification.

Method validation

Selectivity

Selectivity was evaluated using six plasma samples obtained from healthy controls and patients. Chromatograms obtained from bevacizumab-spiked plasma and plasma blank were compared. There was no any interference in six tested plasma blank.

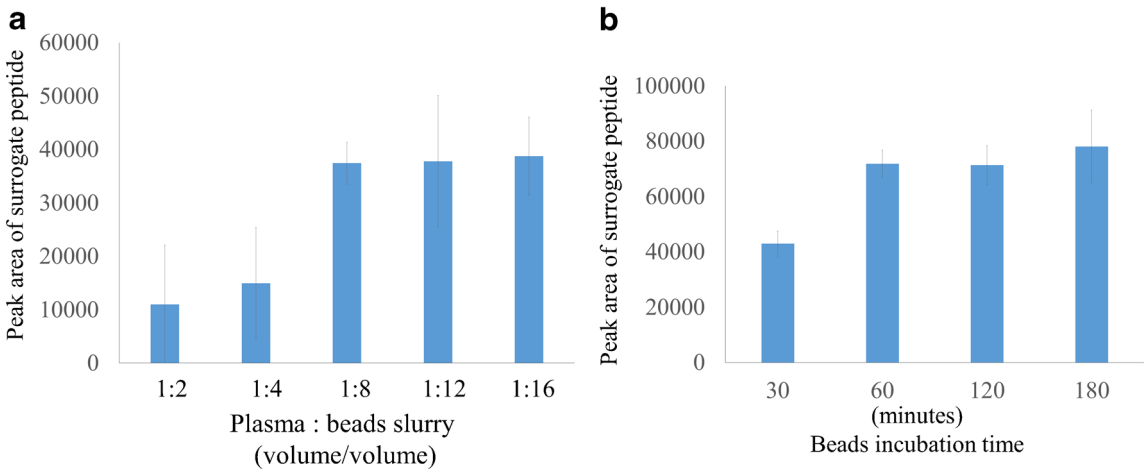


Fig. 2 Optimization of the protein G purification procedure. **a** Effect of bead volume on signal intensity of a surrogate peptide of bevacizumab. Five microliters of plasma was mixed with different volumes of beads in five different groups. **b** Effect of bead incubation time on signal intensity of a surrogate peptide of bevacizumab. Five microliters of plasma, 40 μL of bead solution, and 420 μL of PBS were mixed to evaluate incubation time

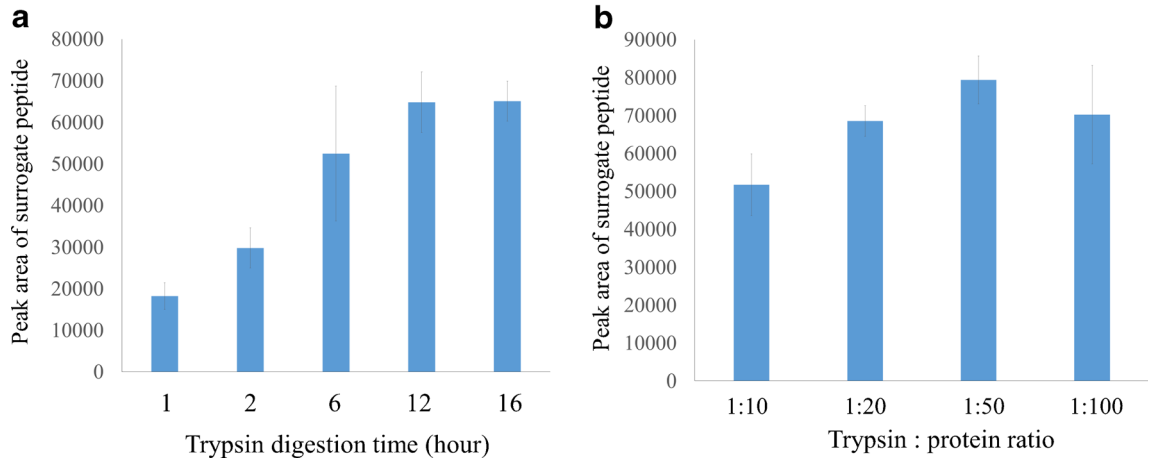


Fig. 3 Optimization of the trypsin digestion procedure. Effect of **a** trypsin digestion time and **b** trypsin amount on the signal intensity of a surrogate peptide of bevacizumab

Linearity, limits of detection, and limits of quantification

Bevacizumab-spiked plasma samples were used to validate the established protocol. The linear range for quantification of bevacizumab in human plasma was designed according to the therapeutic range. Method linearity was evaluated from 30 to 1000 μg mL^{-1}. The calibration curve was obtained with a weighting factor of $1/X$ and by linear regression analysis. The coefficient of determination was greater than 0.99, and the equation of this calibration curve is $y = 0.003460X-$

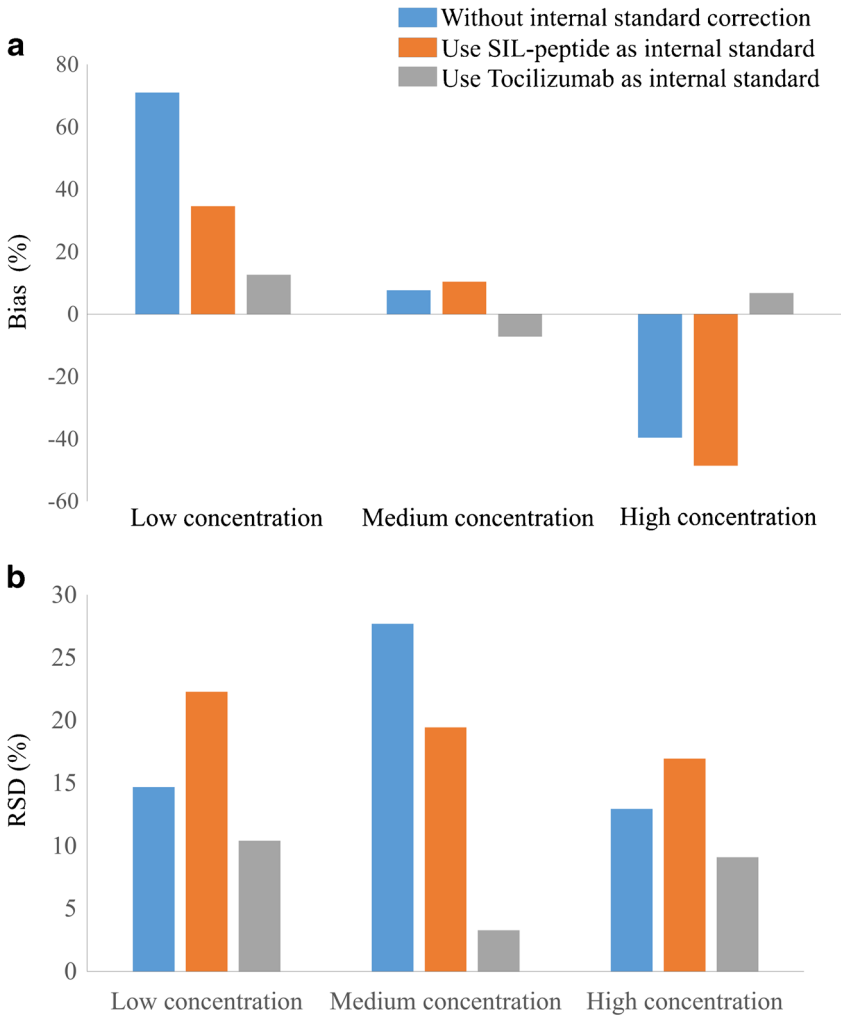


Fig. 4 **a** Accuracy and **b** precision for three concentrations of QC samples with or without internal standard correction. The accuracy and precision with internal standard corrections was calculated based on two types of internal standards, including SIL peptide and tocilizumab

Table 2 Accuracy and precision of bevacizumab quantification in plasma samples at three concentrations

Concentration (μg mL^{-1})	Accuracy (% recovery)	Precision	
		Repeatability (% CV)	Intermediate precision (% CV)
30	112.7 ± 4.5	5.2	12.9
300	92.8 ± 3.2	1.2	3.2
1000	106.8 ± 4.2	2.7	9.5

0.039664. The LOD and LOQ were 4 and 10 μg mL^{-1} in plasma samples, respectively.

Accuracy and precision

The quantification accuracy, repeatability and intermediate precision were all evaluated at three concentrations. Accuracy evaluation used plasma samples obtained from both healthy volunteers and patients with infection diseases. Previous study has indicated immunoglobulin levels will be increased in infection conditions [25]. Considering the possible physiological variation of endogenous IgG which may affect accuracy of bevacizumab quantification, we used plasma samples from both healthy volunteers and patients with infection disease for evaluation of method accuracy. The accuracy was tested by spiking plasma samples with bevacizumab standard at three concentration levels and was expressed as percentage recovery. The overall percentage recoveries of three concentration levels were within 92.8 ± 3.2 to 112.7 ± 4.5%. Repeatability and intermediate precision in terms of peak area ratios (surrogate peptide/IS) at three concentration levels were lower than 5.2 and 12.9% RSD, respectively. Accuracy and precision test results are shown in Table 2.

Stability, matrix effect, and extraction recovery

The previous study indicated that bevacizumab was stable in plasma samples at − 20 °C for 15 days [17]. Process stability was evaluated in this study. Processed bevacizumab-spiked plasma samples were placed in autosampler for 24 h at 4 °C. The results indicated that all surrogate peptide signals were stable (RSD < 3%) after 24 h storage at 4 °C for three tested concentrations. The matrix effects were tested at three concentration levels, and the results were between 94 and 125%. The extraction recoveries of protein G trapping procedure were tested at three concentration levels, and the recoveries were within 83.6 to 98.8%.

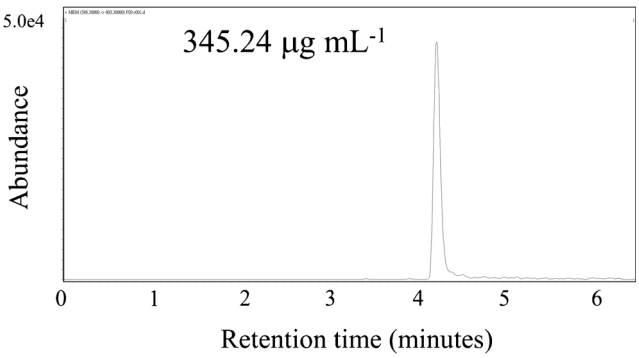


Fig. 5 A representative chromatogram of a plasma sample obtained from a breast cancer patient with brain metastases. The bevacizumab dose was 15 mg kg^{-1}

Clinical sample analysis

To evaluate the applicability of the established protocol, we applied it to quantify five plasma samples that were obtained from patients who were under bevacizumab treatment due to brain metastases of breast cancer. These patients all received bevacizumab at a dose of 15 mg kg^{-1}. Figure 5 shows one representative chromatogram from the analysis of one patients' plasma sample. The concentrations of bevacizumab and the clinical characteristics of the five patients are listed in Table 3. These results indicate that the method is effective for the quantification of bevacizumab in patient plasma samples.

Discussion

This study used protein G trapping and in-solution digestion coupled with LC-MS/MS to quantify bevacizumab in human plasma samples. ELISA is a commonly used analytical method for mAb quantification. Although it shows advantage in high throughput analysis, cross reactivity caused quantification problem was frequently being discussed [11, 12, 26, 27]. The amino acid sequence of humanized antibodies is 93–95% human. Since they show highly structure similar to endogenous IgGs, the endogenous IgGs may be possible interfere the results of ELISA method [27, 28]. Compared to the ELISA-based method, one of the most critical advantages of LC-MS/MS platform is that LC-MS/MS can provide better selectivity. To render the established LC-MS/MS method more applicable and economic in the clinical laboratory for therapeutic drug monitoring, protein G beads were used for sample

Table 3 The concentrations of bevacizumab and clinical characteristics of 5 breast cancer patients with brain metastases

Number	Age	Sex	Height (cm)	Weight (kg)	Dosage	Disease status	Bevacizumab (μg mL^{-1})
1	53	F	157	38	15 mg kg^{-1}	IV	345.2 ± 20.5
2	65	F	145	52.5	15 mg kg^{-1}	IV	468.6 ± 3.0
3	35	F	159	58	15 mg kg^{-1}	IV	651.7 ± 3.0
4	64	F	153	48.6	15 mg kg^{-1}	IV	302.4 ± 9.6
5	63	F	152	51	15 mg kg^{-1}	IV	352.2 ± 15.7

purification. Compared to other antibody-based purification methods, the protein G method does not require specific antibodies to purify target proteins. Additionally, the cost for protein G purification is much lower compared to using customized antibodies. Although the protein G method is less selective, our study results revealed that IgG variations in each individual had a minimal effect on quantification accuracy.

To provide accurate quantification results, a suitable internal standard is necessary to calibrate possible variations that occur during sample preparation. Previous studies adopted extended SIL peptides as the internal standards. However, one limitation of this method is that the digestion efficiency between the extended SIL peptide and a monoclonal antibody may be different; as a result, extended SIL peptides may not accurately mimic variations in the digestion procedure. The other limitation is that this extended SIL peptide cannot correct for the fluctuation in the protein purification step, such as with protein G trapping used in this study. This finding is because the extended SIL peptide cannot be trapped by the protein G beads. Compared to the extended SIL peptide, a mAb-based internal standard with greater structural similarity to bevacizumab would provide better calibration performance [29]. Considering that it is too expensive to synthesize an SIL-mAb, we use tocilizumab as an internal standard. As the results shown in Fig. 4, tocilizumab successfully corrected the variations from the initial stage of protein trapping to the trypsin digestion and thereby significantly improve the precision of the method. A similar concept has been proposed by Hongyan et al. In their study, they applied a mAb as a common IS in preclinical studies as this mAb does not exist in preclinical species but has peptide sequences that are common with target analytes. This common IS method should select general IgG sequences as their quantification surrogate peptides. However, as the majority of developed therapeutic mAbs are humanized IgGs, these general sequences also appear in endogenous IgGs. Therefore, their method is limited to preclinical studies and not for human samples. In contrast to their common IS, using tocilizumab as the IS is applicable to human sample analysis and provides good calibration performance at low cost.

Finally, the application to actual samples revealed that bevacizumab concentrations fluctuated significantly between individuals. The concentration difference may reach twofold, even though they received the same dose. One phase I trial has reported bevacizumab concentration in plasma samples after drug administration at 15 mg kg^{-1}. The bevacizumab concentrations were ranged from 100 to 400 μg mL^{-1} from day 0 to day 14 [30]. In our study, the plasma sample was taken at day 1 after drug administration, and our detected concentration levels were similar with their reported results. However, that phase I study did not discuss the concentration fluctuation between each test individuals. Several recent studies indicated that the efficacies vary in samples from patients undergoing bevacizumab treatment [26, 31, 32]. The other concern with the use of bevacizumab is side effects. Higher concentrations of bevacizumab may lead to side effects such as hemorrhage and phlebitis [33]. Therefore, a wider application of TDM for bevacizumab treatment could improve the therapeutic efficacy and reduce the side effects. The protocol proposed in this study, including protein G trapping and in-solution digestion with tocilizumab calibration, provided an effective, economic, and readily assessable strategy for clinical laboratories to conduct TDM of bevacizumab to achieve personalized therapy.

Conclusions

In this study, we developed an LC-MS/MS method to quantify bevacizumab in human plasma using protein G trapping and in-solution digestion for sample pretreatment. We selected readily available and economical materials for sample preparation to facilitate its wider use in clinical fields. Protein G was used to trap the target analyte (bevacizumab) and minimize sample complexity. The IgG-based drug-IS tocilizumab exhibited good calibration performance. The validation results demonstrated that the method is accurate and could be used for pharmacokinetic studies and therapeutic drug monitoring for bevacizumab. Currently, the majority of available mAb drugs in the clinical field belong to the IgG class, and some IgG4 antibody drugs are currently undergoing clinical trials [34]. As the IgG class of drugs is now gaining more attention in the clinic, the general and simple protocol presented here may be applicable to other IgG class mAb drugs to improve the safety and effective use of mAb drugs.

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Compliance with ethical standards Plasma samples including healthy volunteers and patients were collected at the National Taiwan University Hospital and obtained with informed consent. The study was approved by the institutional review board of the National Taiwan University Hospital.

Conflict of interest The authors declare that they have no conflict of interest.

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