

Antibody drug quantitation in coexistence with anti-drug antibodies on nSMOL bioanalysis



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ABSTRACT

Therapeutic monoclonal antibodies (mAbs) are developed for treatment of diverse cancers and autoimmune diseases. For expansion of mAbs approval against unapproved diseases and pharmaceutical development, pharmacokinetics study is very important. Bioanalysis provides one of the most essential index against pharmacokinetics information. So far, we developed useful method for bioanalysis of mAbs in plasma or serum, nSMOL: nano-surface and molecular-orientation limited proteolysis. This method can provide accurate and reproducible value of mAbs content in plasma. Quantification of mAbs using ELISA is strongly influenced by endogenous ligand or anti-drug antibodies. In this report, we exhibited the role of nSMOL proteolysis coupled to LC-MS/MS analysis against quantification of mAbs bound to some binding molecules. The ligands against mAbs do not affect quantification of mAbs concentration in plasma using nSMOL proteolysis. On the other hands, some anti-drug antibodies (ADA), such as idiotypic antibodies, inhibit quantification of mAbs using nSMOL proteolysis. Acid dissociation has some efficacy in accurate value of quantitation of ADA binding mAbs using nSMOL proteolysis coupled to LC-MS/MS analysis. Accordingly, we consider that nSMOL method will contribute to understanding of mAb PK data and therapeutic reference combining with ADA measurements.

Introduction

Large amounts of monoclonal antibodies are continually encountered in circulation, the host may mount significant anti-drug antibodies (ADAs) response. Recently, humanized antibodies have increased to produce because of preventing the immunogenicity, but humanization could not reduce enough immunogenicity [1]. ADAs might be expected to diminish the effectiveness of mAbs as a therapeutic drug, and perhaps have other metabolic consequences. For example, Bevacizumab has reported to be able to induce antibodies (human anti-Bevacizumab or anti-drug antibodies) in patients receiving Bevacizumab [2]. Ranibizumab, a monoclonal antibody fragment targeting VEGF, also induced anti-drug antibodies in 17% of treated patients [3]. Furthermore, ADAs against Nivolumab were determined in samples collected during Nivolumab treatment [4]. However, this is highly dependent on the sensitivity of the assay. On April 22, 2016, guidance on immunogenicity testing of therapeutic proteins was released from FDA [5], so that evaluation of immunogenicity will be essential for development of mAb drugs [6,7]. The detection of ADAs is performed using a bridging electrochemiluminescence immunoassay,

which provides a semi-quantitative assessment of immunogenicity for ADA. Immunogenicity of mAbs is needed for approval.

Immunogenicity interfered with the accuracy assessment by enzyme-linked immunosorbent assay (ELISA) method. Thus, ADAs may interfere with the quantification of mAbs levels in PK/PD analysis. Assay tolerance to such interference may depend on assay platform and reagents. Various approaches have been developed to improve ADAs tolerance in mAbs analysis but limited success was observed. Then, we provide the information about quantification of ADAs binding mAbs using nSMOL proteolysis coupled to LC-MS/MS bioanalysis.

As observed with other antibodies, the pharmacokinetics of Bevacizumab is well described. Overall, in all clinical trials, Bevacizumab disposition was characterized by a low clearance, a limited volume of the central compartment, and a long elimination half-life. This enables target therapeutic Bevacizumab plasma levels to be maintained with a range of administration schedules (such as once every 2 or 3 weeks). Moreover, Nugue G et al. suggested that serum Bevacizumab concentration is useful clinical pharmacodynamics marker [8]. Hence, credible methodologies for measurements of mAb in plasma are crucial for the assessment of exposure–response

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Table 1
The list of ADAs.

ADA name	Immunogen	Format	Affinity (Kd, nM)
HCA168	Trastuzumab	Human Fab	0.02 nM
HCA176	Trastuzumab	Human IgG1	0.4 nM
HCA177	Trastuzumab	Human IgG1	0.02 nM
MAB11130	F (ab)2 fragment of Trastuzumab	Mouse IgG1	NA
HCA185	Bevacizumab	Human IgG1	0.4 nM
HCA182	Bevacizumab	Human Fab	0.4 nM
MAB11128	F (ab)2 fragment of Bevacizumab	Mouse IgG1	NA
8G6G3D8	Nivolumab	Mouse IgG	NA

relationships in support of efficacy and safety evaluations, and dose selection for chemotherapeutic clinicians.

For the quantification of therapeutic mAbs in biological specimens, classical ligand binding assays such as ELISA is the most widely used technique. However, in some cases, an immunological-based assay is not the most appropriate method for quantifying mAbs. For example, mAbs can be analogs of endogenous IgGs with a minor change to their amino acid sequence, the standard ELISA unable to differentiate between endogenous and exogenous variants. In these cases, mass spectrometry-based methodologies are available for quantify the mAbs, which offer superior selectivity over an immunoassay, and significantly shorten method development times.

On the other hand, liquid chromatography-mass spectrometry (LC-MS) has become one of the most widely used methods in pharmaceutical laboratories. Recently, tandem LC-MS (LC-MS/MS) have been applied to mAbs as an alternative to ELISA for the bioanalysis of pre-clinical samples [9]. Although LC-MS/MS provides high sensitivity and high specificity for quantifying target analytes in complicated biological matrices, the associated severe matrix effects result in large quantification errors. To address this issue, we proposed a novel strategy for decreasing contaminant from various biological matrix using nSMOL proteolysis [10]. The nSMOL method is designed as solid-solid proteolysis for Fab-selective limited proteolysis. This developed proteolysis has made it possible not only to minimize sample complexity, but also to maintain the sequence specificity of peptides in CDRs. In this article, we investigate whether ADAs affect the interference of quantification values of mAbs using nSMOL strategy.

Materials and methods

Reagents and materials

Trypsin-immobilized glycidyl methacrylate (GMA)-coated nano-ferite particle FG bead with surface activation by NHS group was purchased from Tamagawa Seiki (Nagano, Japan). Toyopearl AF-rProtein A HC-650F resin was from Tosoh (Tokyo, Japan). nSMOL Antibody BA Kit was from Shimadzu (Kyoto, Japan). Trastuzumab and Bevacizumab was obtained from Chugai Pharmaceutical (Tokyo, Japan). Nivolumab

was obtained from Ono Pharmaceutical (Osaka, Japan). Individual male and female human EDTA-2K treated-plasma was purchased from Kohjin Bio (Saitama, Japan). Modified trypsin was purchased from Sigma-Aldrich (St. Louis, MO). n-Octyl- β -D-thioglucopyranoside (OTG) was purchased from Dojindo Laboratories (Kumamoto, Japan). P14R, internal standard synthetic peptide, was purchased from Sigma-Aldrich. Ultrafree-MC GV centrifugal 0.22 μ m filter was purchased from Merck Millipore (Billerica, MA). Recombinant human ErbB2 Fc chimera and recombinant human His-tagged PD-1 were purchased from R&D systems (Minneapolis, MN). Recombinant human vascular endothelial growth factor (VEGF-A₁₆₅) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-Trastuzumab antibodies (HCA169, HCA176, and HCA177) and anti-Bevacizumab antibodies (HCA182 and HCA185) were purchased from Bio-Rad (Raleigh, NC). MAB11128 and MAB11130 were purchased from Abnova (Taipei, Taiwan). Anti-Nivolumab was purchased from Genscript (Piscataway, NJ) (Table 1). Other reagents, buffers, and solvents were purchased from Sigma-Aldrich and Wako Pure Chemical Industries.

nSMOL proteolysis

In this study, the concentration of mAbs in plasma samples was quantified using nSMOL proteolysis coupled to LC-MS/MS analysis. nSMOL method was described in our previous report [10] and we briefly explain in this report. An aliquot of mAb-spiked human plasma was diluted 10-fold in PBS containing 0.1% OTG. The Ig fraction in plasma was collected with Protein A resin (50% slurry) with gentle vortexing at 25 °C for 15 min. Non-specific absorption on Protein A resin was washed twice with 300 μ l of PBS containing 0.1% OTG and then with 300 μ l of PBS, and then washed resin suspended in 75 μ l of 25 mM Tris-HCl (pH8.0) containing 10 fmol/ μ l P14R. nSMOL proteolysis was carried out using 10 μ g modified-trypsin immobilized FG-beads with gentle vortexing at 50 °C for 5 h in saturated vapor atmosphere. After nSMOL proteolysis, reaction was quenched by adding 10% formic acid at a final concentration of 0.5%. The peptide solution was recovered only by centrifugation (10,000 \times g for 1 min) with to remove Protein A resin and trypsin immobilized FG-beads. These analytes were transferred into low protein binding polypropylene vials, and then performed LC-MS analysis without other solid-phase purification, evaporative concentration, and reconstitution. nSMOL method has been already validated prior to analysis by using Trastuzumab, Bevacizumab, or Nivolumab-spiked plasma [11–13].

Binding of ADAs or ligands to mAbs

The ADA- or ligand-treated mAbs were incubated for 30 min at room temperature with gently vortexing under indicated conditions. After incubation, 10 μ l of human plasma added to each samples and quantified mAbs concentrations using nSMOL proteolysis coupled with LC-MS/MS analysis.

Table 2
MRM transition of signature candidate peptides from Trastuzumab, Bevacizumab, and Nivolumab.

Selected peptide	Transition mass (m/z)	Q1 [V]	Collision [V]	Q3 [V]	Role
Trastuzumab IYPTNGYTR	542.8 \rightarrow 404.7	–20	–18	–30	Quantitation
	542.8 \rightarrow 808.4	–20	–18	–28	Structure
	542.8 \rightarrow 610.3	–20	–25	–22	Structure
Bevacizumab FTFSLDTSK	542.3 \rightarrow 797.4	–38	–18	–30	Quantitation
	542.3 \rightarrow 898.5	–38	–20	–34	Structure
	542.3 \rightarrow 650.3	–38	–19	–34	Structure
Nivolumab ASGITFSNSGMHWVR	550.8 \rightarrow 661.5	–24	–18	–24	Quantitation
	550.8 \rightarrow 746.4	–26	–16	–28	Structure
	550.8 \rightarrow 785.4	–26	–25	–30	Structure

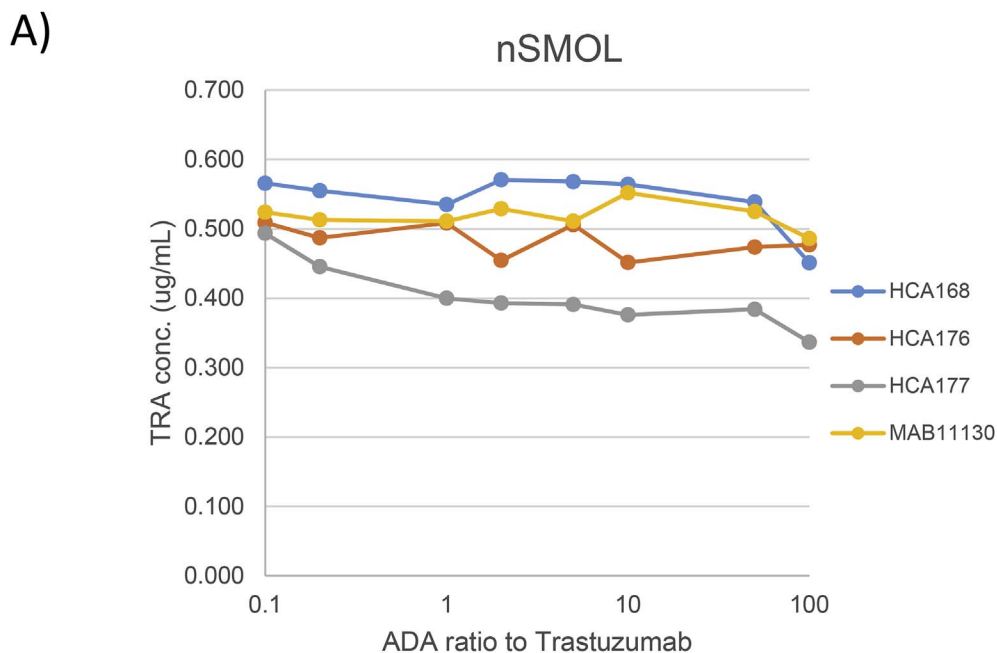
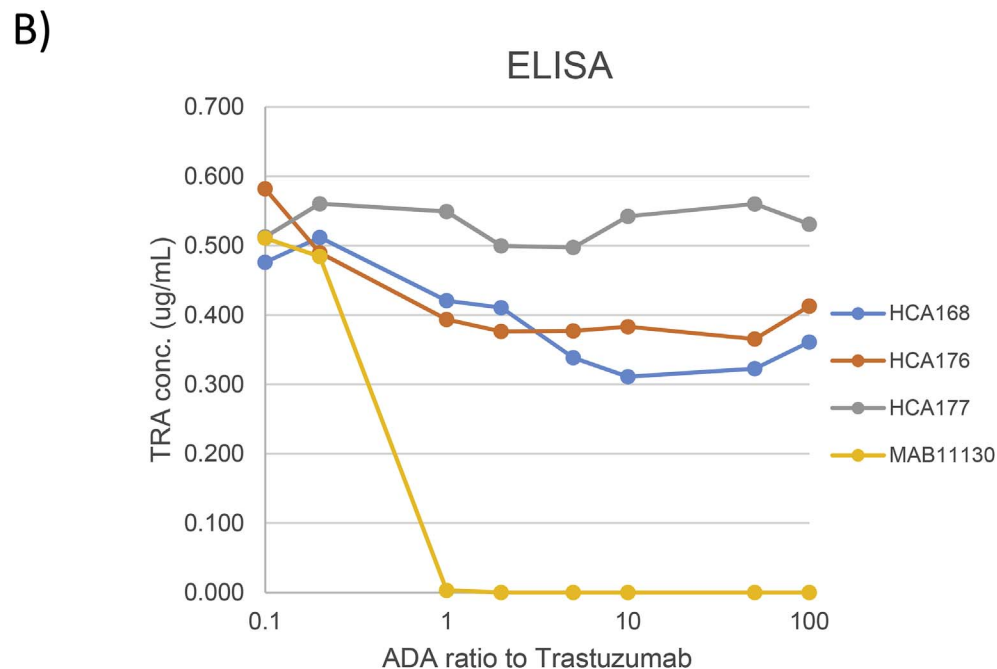


Fig. 1. ADA interference of Trastuzumab bioanalysis using nSMOL and ELISA. Concentrations of Trastuzumab coexistent with indicated ratios of ADA against Trastuzumab (0.5 $\mu\text{g}/\text{mL}$) were obtained by nSMOL (A) or ELISA (B). HCA168, HCA176, and HCA177 are human monoclonal antibodies. MAB11130 is mouse monoclonal antibody.



Acid dissociation reaction

For examination of acid dissociation effect on ADA-binding mAbs measurement, the samples were tested under nonacidified or acidified conditions. No additional pretreatment was applied to the nonacidified samples. The acid dissociation reaction was performed for 1 h at room temperature with one in two dilutions of sample in 0.5 M Glycine-HCl (pH2.6). Then the neutralization step was carried out by addition of 1 M Tris-HCl (pH 8.6), protein A resin, and PBS containing 0.1% OTG. After 30 min incubation with gently vortexing, nSMOL reaction was performed as described.

LC-MS/MS analysis

The nSMOL proteolysis samples were injected into a high-performance liquid chromatography system (LC-30AD Nexera, Shimadzu, Japan) fitted with an SHIM pack (2.1 X 50 mm, 2 μm , Shimadzu). The mobile phases of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) were used as follows: 0.4 mL/min; gradient program, 0–2 min: %B = 1, 2–4.5 min: %B = 1–40 gradient, 4.5–6 min: % B = 95 with flow rate 1 mL/min, 6–6.5 min: %B = 1 with flow rate 1 mi/min, and 6.5–7 min: %B = 1.

MS spectra were obtained with ESI probe temperature, desolvation line, and heat block at 300 $^{\circ}\text{C}$, 250 $^{\circ}\text{C}$ and 400 $^{\circ}\text{C}$, respectively. Gas

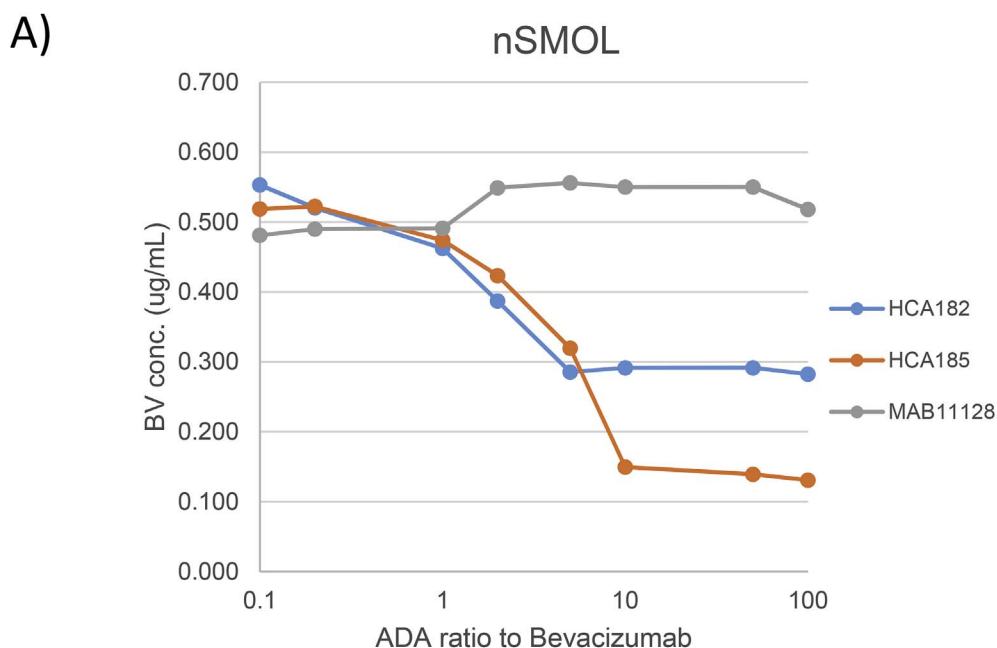
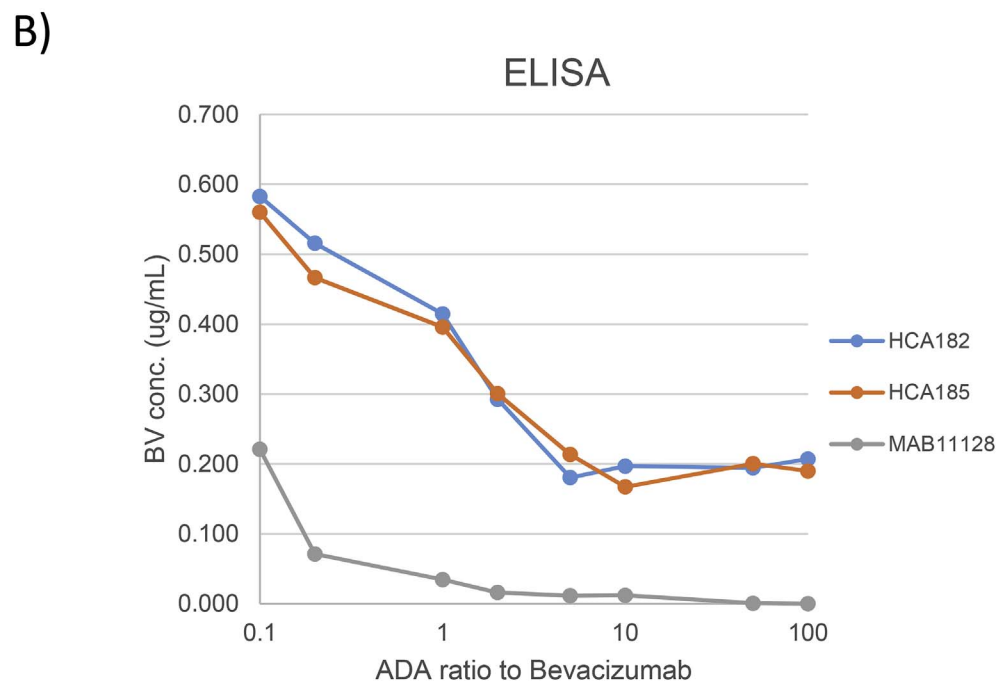


Fig. 2. ADA interference of Bevacizumab bioanalysis using nSMOL and ELISA. Concentrations of Bevacizumab coexistent with indicated ratios of ADA against Bevacizumab (0.5 $\mu\text{g/mL}$) were obtained by nSMOL (A) or ELISA (B). HCA182 and HCA185 are human monoclonal antibodies. MAB11128 is mouse monoclonal antibody.



flows of nebulizer, heating, and drying nitrogen were set to 3, 10, and 10 l/min, respectively. The dwell time was set at 10 msec for each transition. CID Ar partial pressure in the Q2 cell was set to 270 kPa. The electrode voltage of Q1 pre bias, collision cell Q2, Q3 pre bias, and parent and fragment ion m/z were optimized using the LabSolutions software (Shimadzu). For MRM transition, one fragment ion of b- or y-series were selected for quantitation, and two ions were for structural confirmation according to the optimized MRM ion yield (Table 2).

Enzyme-linked immunosorbent assay (ELISA)

Trastuzumab and Bevacizumab concentrations in plasma were

measured by ELISA reagents (Somru Bioscience, PE Canada for both antibodies and Matrics Biotek, Turkey for Trastuzumab).

Statistical analysis

Data were analyzed for statistical significance and indicated as *P* values. Data were analyzed with t-test when comparing means of two independent groups. $P < 0.01$ was considered statistically significant ($*P < 0.01$).

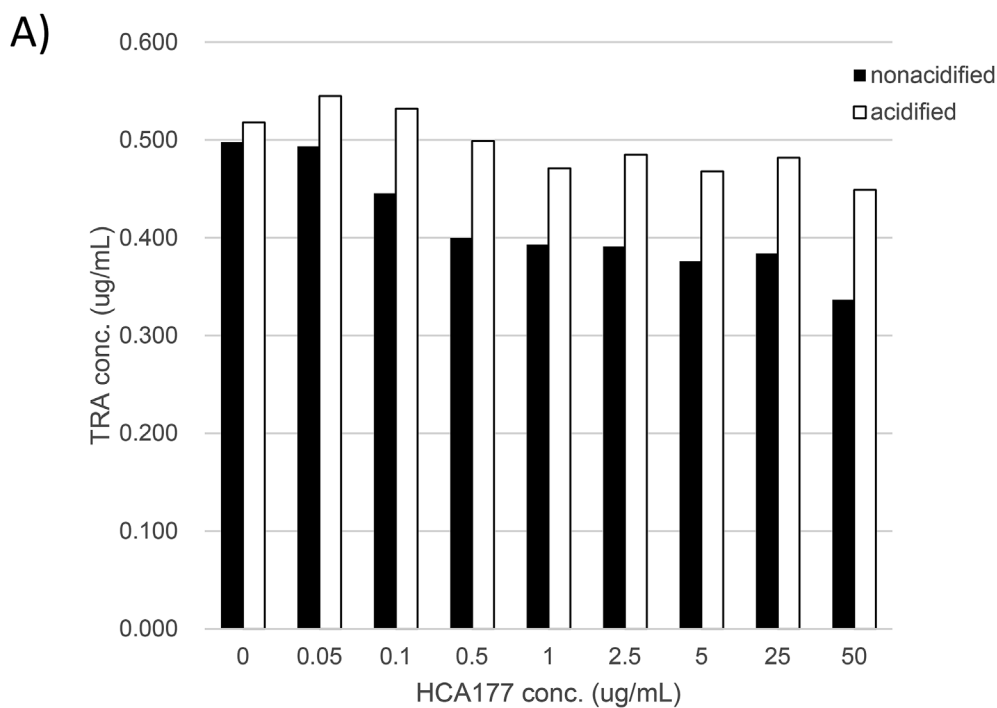
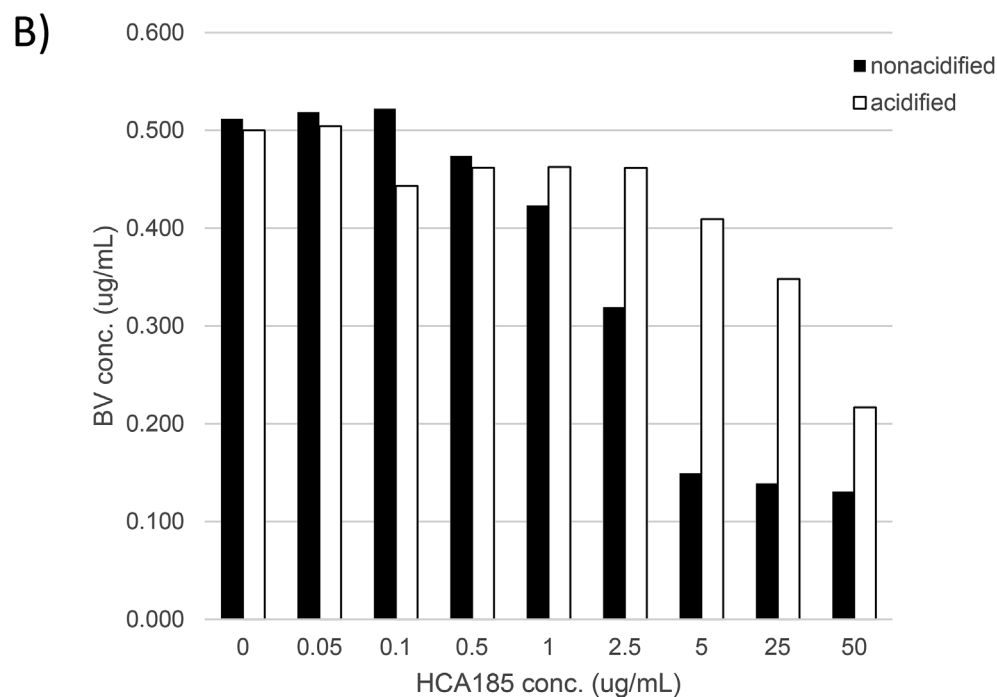


Fig. 3. Acid dissociation effect on quantification of ADA-binding Trastuzumab (A) or Bevacizumab (B) using nSMOL. Concentrations of nonacidified- and acidified-treated plasma samples were obtained by nSMOL.



Results

Quantification of ADA-binding trastuzumab using nSMOL and ELISA method

The plasma concentrations of ADA-bound Trastuzumab were measured by both nSMOL and the ELISA methods. Fig. 1A summarizes Trastuzumab concentration values obtained by the two methods. The HCA168, HCA176 or MAB11130 binding Trastuzumab concentrations obtained by nSMOL method showed approximately within the guideline limit of error $\pm 15\%$ against theoretical values. However, quantitation of Trastuzumab, which reacted with equal amounts or more of

HCA177, obtained by nSMOL method exhibited over the guideline limit of error $\pm 15\%$ against theoretical values at the concentration of Trastuzumab $0.5 \mu\text{g/mL}$. Similarly, at the concentration of Trastuzumab $50 \mu\text{g/mL}$, equal amount of HCA177 significantly interfered with quantification of Trastuzumab by nSMOL method (Table S1). HCA168 and HCA177 are same clone and paratope specific, high affinity, anti-idiotypic antibody, but format is different. As expected from the data, an idiotypic and high affinity IgG against Trastuzumab resulted in the strongest inhibition of quantified values obtained by nSMOL method. On the other hand, MAB11130 resulted in the strongest inhibition of quantified values obtained by the ELISA method but not in nSMOL method (Fig. 1B). These results suggested that the quantitation by

ELISA directly receives quantitative inhibition depending on the binding site. The capture antibody of ELISA kit we used is an idiotype antibody of Trastuzumab. Therefore, we inferred that some ADAs do not interfere with Trastuzumab quantification by the ELISA method, since ADA-bound Trastuzumab can bind to the capture antibody depending on the binding site of ADA to Trastuzumab. With other type of ELISA kit using ligand antigen ErbB2 as a capture molecule, Trastuzumab quantification was disturbed by every ADA bond in a concentration-dependent manner (Fig. S1). These results suggested that every ADA bind to Trastuzumab and interfere with binding to ErbB2.

Quantification of ADA-binding bevacizumab using nSMOL and ELISA

Fig. 2 summarizes Bevacizumab concentration values obtained by the two methods. Both HCA182 and HCA185, anti-Bevacizumab antibodies, interfered with Bevacizumab quantification using nSMOL. At the concentration of Bevacizumab 0.5 µg/mL, about twice and more times of HCA182 and HCA185 ratio to Bevacizumab is maximum amount of inhibitory effect on accurate quantity (Fig. 2A). HCA185 interfered with the quantitative value of Bevacizumab by nSMOL method more than HCA182. HCA182 and HCA185 are same clone and anti-idiotypic antibody. Similarity to Trastuzumab, IgG isotype of anti-Bevacizumab antibody has more inhibition effect than Fab isotype. MAB11128 inhibited quantitative values only in the ELISA measurement but not in nSMOL method. At 50 µg/mL of Bevacizumab, equal amount of HCA182 or HCA185 underestimated the total Bevacizumab concentration obtained by nSMOL (Table S2).

The effect of acid dissociation on ADA-inhibited accurate quantification value by nSMOL

Binding of ADAs can be released by adding acid buffer. The ADA-released antibody drug might overcome the inhibition effect on quantification drug concentration by nSMOL method. Fig. 3A showed the effect of acid dissociation on HCA177 binding Trastuzumab quantification results obtained by nSMOL method. The acid dissociation caused improvement of the concentration results accuracies of the 5- to 100-fold ADAs binding Trastuzumab. The inhibition effects of HCA185 bond on quantification of Bevacizumab using nSMOL method were partly recovered by acid dissociation (Fig. 3B).

These results demonstrated that within 50% decline of accuracy against total drug concentration obtained by nSMOL method was recovered by acid dissociation reaction in nSMOL method.

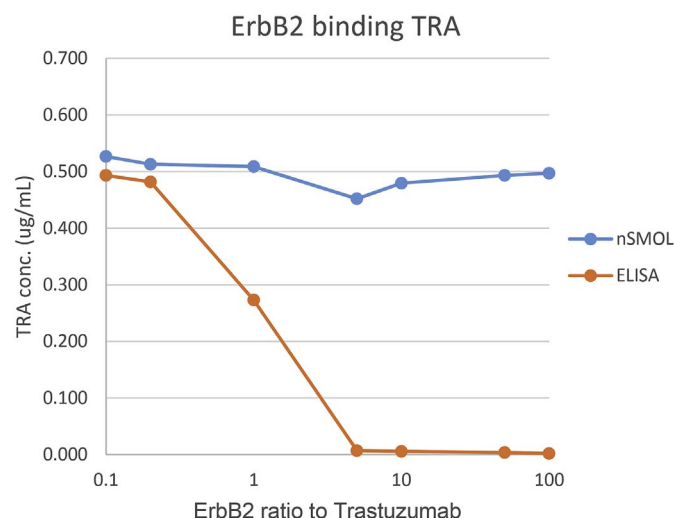


Fig. 4. Quantification of ErbB2 binding Trastuzumab using nSMOL and ELISA. Concentrations of Trastuzumab coexistent with indicated ratios of ErbB2 were obtained by nSMOL (●) or ELISA (◇).

Accurate quantification of ligands binding mAbs using nSMOL

The target molecules of Trastuzumab, Bevacizumab, and Nivolumab are ErbB2, VEGF-A, and PD-1, respectively. As a result of quantitating ErbB2-reacted Trastuzumab by ELISA, the quantitative value was inhibited in a concentration-dependent manner of ErbB2. However, the concentration results obtained by nSMOL method did not interfere with quantification of ErbB2-bound Trastuzumab (Fig. 4). Likewise, quantitative values by nSMOL method were not affected in antibody drugs in which ligands were bound to Bevacizumab and Nivolumab (Figs. 5 and 6). However, in the ELISA method, Bevacizumab quantitative values were inhibited by VEGF coexistence. The drug antibodies bound to every concentration of each target molecules showed accurate drug concentration results by quantification using nSMOL method.

Discussion

The mAbs are being expanded as therapeutic application, resulted in requirement of assay methods for such protein drugs in preclinical and clinical studies. Moreover, development of biosimilars to the innovator

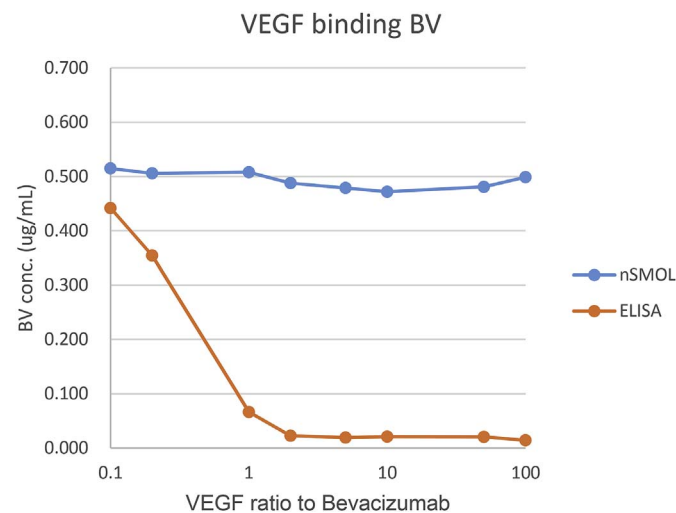


Fig. 5. Quantification of VEGF binding Bevacizumab using nSMOL and ELISA. Concentrations of Bevacizumab coexistent with indicated ratios of VEGF-A₁₆₅ were obtained by nSMOL (●) or ELISA (◇).

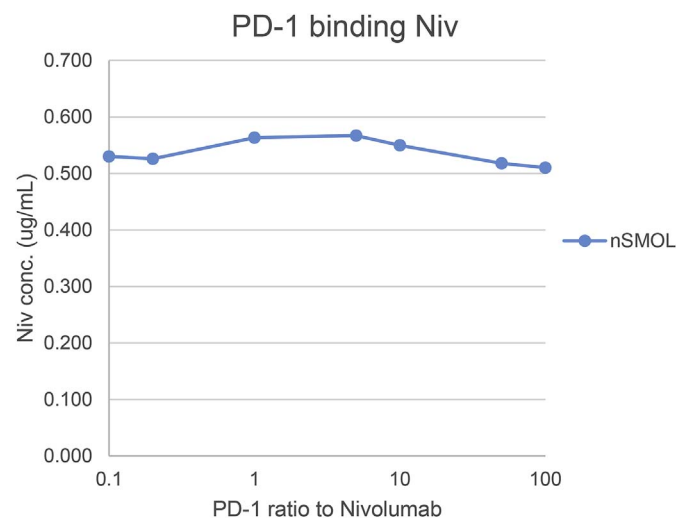


Fig. 6. Quantification of PD-1 binding Nivolumab using nSMOL. Concentrations of Nivolumab coexistent with indicated ratios of PD-1 were obtained by nSMOL (●).

drug and new generation medicine, such as Nivolumab against cancer immune therapy, is accelerated. Bioanalytical methods were contributed to not only the quantification of therapeutic mAbs but also their application in clinical pharmacokinetic studies. From the above background, the establishment of accurate methods for mAbs bioanalysis is surely critical.

Currently, ADAs were observed in mAb treated rheumatoid patients as one of the factor of secondary failure [14]. However, the therapy of various cancers may change by virtue of immune checkpoint inhibitor, such as Nivolumab and Pembrolizumab. Immune checkpoint inhibitors improve overall survival of patients. As a result, long-term administration of these immune checkpoint inhibitors may become normal treatment of cancer. In that case, it is expected that the probability of ADA production against anticancer drugs will increase.

The standardized methodology for determination the plasma concentration of biologics was ELISA. Recently, we have developed new methodology for mAbs bioanalysis. nSMOL is the method for mAbs bioanalysis using LC-MS/MS and the only method that fulfills all criteria of guideline. However, the effect of ADAs on the quantification of mAbs by nSMOL method that remains to be uncovered.

In this study, we revealed whether each ADA or ligand against Trastuzumab, Bevacizumab, and Nivolumab influence quantification by nSMOL method. The some idiotypic antibodies against Trastuzumab or Bevacizumab affected the accuracy of quantification results. On the other hand, none-idiotypic antibodies against Trastuzumab, Bevacizumab, and Nivolumab and each ligand didn't inhibit the quantification results by nSMOL method. Fig. S2 exhibits Nivolumab concentration values obtained by nSMOL method. The 8G6G3D8 antibody, ADA against Nivolumab, did not inhibit the quantification values at every concentration. Furthermore, acid dissociation has a positive effect on ADA complexed to drug dissociation. Accuracy of quantitative value using some immunoassay platforms including ELISA, MSD, Gyrolab, and AlphaLISA is improved by acid dissociation [15]. But quantification using nSMOL has not been evaluated the acid dissociation effect. In this paper, we clarified that acid dissociation restores the inhibition of quantitative values using nSMOL method by ADA.

The ELISA method is used as a protein quantification method, but the detection antibodies which contribute the method differs depending on the ELISA kit. We demonstrated that none-idiotypic antibodies and ligands substantially inhibited quantification results by ELISA.

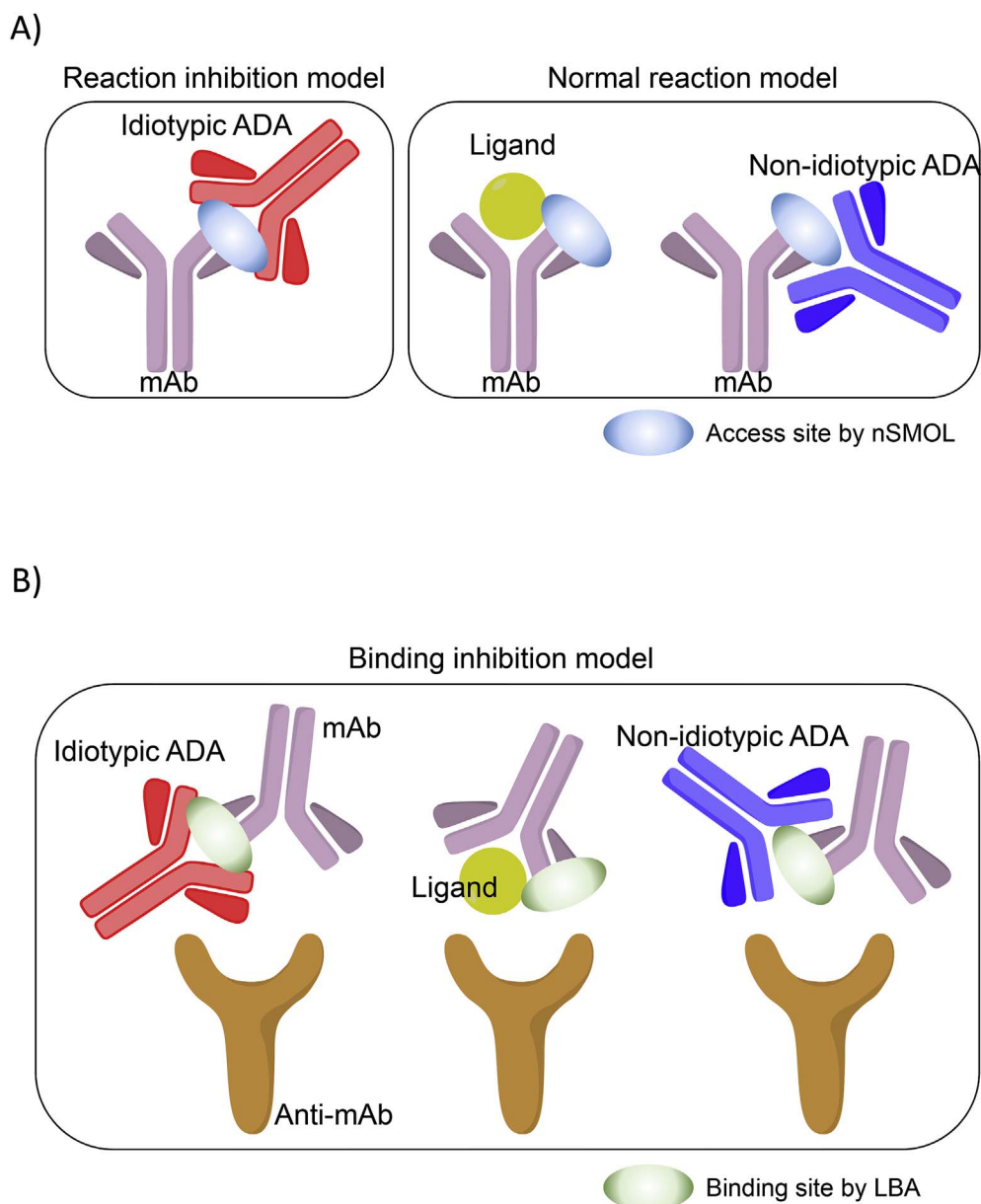


Fig. 7. Bioanalysis assay inhibition model in the coexistence of anti-drug antibodies or specific ligands.

a) Inhibition model in nSMOL analysis. b) Inhibition model in LBA analysis.

However, the idiotypic antibodies showed little interference with the measured value using one kit, but significantly inhibited the quantitative value using other kit.

In the current study, we suggest that the nSMOL method affects quantitative values only when idiotypic antibody is bound, but doesn't affect otherwise. The nSMOL selectively “reacts” to Fv region in IgG. Consequently, in nSMOL method, it was suggested that the binding materials to the Fv region, such as idiotypic antibody, of the antibody drug affected the quantitative values as in Fig. 7. However, in ligand binding assay, immobilized anti-mAb specifically “binds” to its specific recognition site. So, in the coexistence of idiotypic antibodies, ADAs, and ligands, primary antibody will be undergone the binding inhibition by their steric hindrances.

Now, since the guideline of immunogenicity of mAbs is issued by FDA and EMA, quantification of ADAs is also an indispensable item in PK study. The ADAs are assumed to be composed of heterogeneous polyclonal antibodies and low concentration including in plasma of therapeutic patient. Accordingly, the actual ADAs have little effect on bioanalysis of nSMOL proteolysis combined LC-MS/MS. We anticipate that the nSMOL method also contributes to PK study along with that information and the promotion of personalized medicine of mAbs.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2017.11.002>.

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