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Research paper

Multiplexed monitoring of therapeutic antibodies for inflammatory diseases using Fab-selective proteolysis nSMOL coupled with LC-MS

Noriko Iwamoto[a,b], Megumi Takanashi[b], Kotoko Yokoyama[b], Atsushi Yonezawa[c,d], Masaya Denda[c,d], Motomu Hashimoto[e], Masao Tanaka[e], Hiromu Ito[f], Minoru Matsuura[g], Shuji Yamamoto[g], Yusuke Honzawa[g], Kazuo Matsubara[c], Takashi Shimada[a,b,*]

[a] Shimadzu Bioscience Research Partnership, Shimadzu Scientific Instruments, 21720 23rd Drive Southeast #250, Bothell, Washington 98021, USA
[b] Leading Technology of Bioanalysis and Protein Chemistry, Shimadzu Corporation, Med-Pharm Collaboration Building of Kyoto University, 46-29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
[c] Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[d] Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
[e] Department of Advanced Medicine for Rheumatic Diseases, Graduate School of Medicine, Kyoto University, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[f] Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[g] Department of Gastroenterology & Hepatology, Graduate School of Medicine, Kyoto University, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

Monoclonal antibodies have accelerated the availability of treatment options for many diseases in which the molecular mechanism has been elucidated in detail. Therefore, an assay that can universally analyze antibodies for clinical pharmacokinetics and cross-sectional studies would be indispensable.

We have developed a universal antibody bioanalysis with a Fab-selective tryptic reaction, named nano-surface and molecular-orientation limited (nSMOL) proteolysis, that collects the specific antibody signature peptides in biological samples. Using the nSMOL method, we have fully validated the bioanalysis of many antibodies, Fc-fusion proteins, and their biosimilars.

Inflammatory immune diseases often require long-term clinical management because of the remission and relapse observed. Accurate antibody monitoring in systemic circulation could contribute to the improvement of clinical outcomes. Because several biopharmaceuticals can be selected as practical treatment options, the assay development that quantitates many antibodies simultaneously would be applicable in many theraprutic monitoring.

In this study, we have validated the LC-MS bioanalysis method for seven-mixed antibodies (Infliximab, Adalimumab, Ustekinumab, Golimumab, Eculizumab, Etanercept, and Abatacept) using the nSMOL normal reaction condition and two-mixed antibodies (Tocilizumab and Mepolizumab) using the acidified reduction acceleration condition, as reported in our previous papers. Moreover, this multiplexed assay has been verified using clinical patient samples. The nSMOL approach enables the quantitation of several immunosuppressive antibodies simultaneously in human serum, and nSMOL can potentially be applicable to the drug-drug interaction assays or therapeutic antibody monitoring of several inflammatory immune diseases to optimize administration.

1. Introduction

The molecular mechanism of inflammatory immune diseases is initiated by the destructive and consecutive stimulation of many cytokines in patient endothelia and articulation. For example, the role of tumor necrosis factor (TNF) in inflammatory responses has been indicated that TNF is partly activated to effector/mediator pathway to accelerate tissue injury.(Elliott et al., 1994; Smeets et al., 2003; Haraoui, 2004) Thus, the blockade of cytokine signaling by neutralizing cytokines and/or their receptors can be an effective therapeutic

* Corresponding author at: Shimadzu Bioscience Research Partnership, Shimadzu Scientific Instruments, 21720 23rd Drive Southeast #250, Bothell, Washington 98021, USA.
E-mail address: tashimada@shimadzu.com (T. Shimada).

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Table 1
Monoclonal antibodies approved for inflammatory immune disease.

Antibody	Structure and subclass	Target	Indications
Infliximab (Elliott et al., 1994)	Chimeric IgG1κ	TNFα	RA, BS, psoriasis, AS, KD, CD, UC
Adalimumab (Weinblatt et al., 2003)	Fully human IgG1κ	TNFα	RA, BS, psoriasis, AS, CD, UC, hidradenitis, JIA
Ustekinumab (Reddy et al., 2007)	Fully human IgG1κ	IL-12, IL-23 p40 subunit	psoriasis, CD
Golimumab (Kay et al., 2008)	Fully human IgG1κ	TNFα	RA, UC
Eculizumab (Flood-Page et al., 2003)	Humanized IgG2/4κ	C5	hemoglobinuria, HUS, MG
Etanercept (van der Poll et al., 1997)	Extracellular ligand-binding domain of TNF receptor II (TNFR) fused on Fc	TNFα	RA, JIA
Abatacept (Verwilghen et al., 1994)	Extracellular domain of CTLA-4 fused on Fc	CD80/86	RA, JIA
Tocilizumab (Mihara et al., 2005)	Humanized IgG1κ	IL-6 receptor (IL-6R)	RA, JIA, Castleman, Takayasu arteritis, GCA
Mepolizumab (Hart et al., 2001)	Fully human IgG1κ	IL-5	asthma, EGPA

TNF: tumor necrosis factor, CTLA: cytotoxic T-lymphocyte-associated antigen, IL: interleukin, C5: complement component 5, RA: rheumatoid arthritis, BS: Behcet's syndrome, AS: ankylosing spondylitis, KD: Kawasaki disease, CD: Crohn's disease, UC: ulcerative colitis, JIA: juvenile idiopathic arthritis, HUS: hemolytic uremic syndrome, MG: myasthenia gravis, GCA: giant cell arteritis, EGPA: eosinophilic granulomatosis with polyangiitis.

strategy.(Curtis and Singh, 2011) The primary option in this therapy is to select monoclonal antibodies and Fc-fusion proteins against cytokine signaling molecules for the disease management. However, many studies have suggested that there are multiple pathways and phenotypes associated with each inflammatory disease. Targeting the TNF pathway can potentially treat rheumatoid arthritis (RA), and Infliximab has been developed as an TNF pathway blockade antibodies.(Temekonidis et al., 2003) However, the downstream molecules for TNF receptor type I and II are quite different. In a comparative clinical trials using TNF blockade agents, Infliximab and Etanercept, the efficacy of these treatment options was different in Crohn's disease (CD) and juvenile chronic arthritis (JCA).(Lovell et al., 2000; Rutgeerts et al., 2004; Delaunay et al., 2005; Laas et al., 2008) From these studies, many antibodies and protein pharmaceuticals have been developed as immunosuppressive agents (summarized in Table 1).

The clinical response of an inflammatory disease is observed to influence drug concentration in circulation. Therefore, dosage adjustments for individual patients may be helpful for maintaining drug efficacy. And the blood level of the drugs can predict long-term disease management.(den Broeder et al., 2002; Ternant et al., 2015) While there are multiple options of biopharmaceuticals for inflammatory diseases, the current clinical indicators are only scientifically evidenced in several studies for RA or CD.(Mulleman et al., 2009; Vande Casteele et al., 2015) Many options cannot be accurately supplied enough, and the dosing decisions are still left to clinician's experiences. Recent studies have shown that Infliximab efficacy correlates with blood trough concentrations. According to the standard of the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR), Infliximab trough concentration of clinical remission threshold has reported at approximately 1 μg/ml in circulation.(Maser et al., 2006; Mochizuki et al., 2007; Mulleman et al., 2009) These recommendations can be an important therapeutic decision in practical care to keep dosage, increment/decrement, or switch to another biopharmaceuticals.

Biopharmaceuticals are exogenous proteins that evoke the immune responses, leading to the production of anti-drug antibodies (ADAs). (Moots et al., 2017; Balsa et al., 2018) Inflammatory diseases generally require long-term therapeutic management. Therefore, monitoring of the clinical responses should be a key for effective treatment outcomes. An ADA assay, as well as trough concentrations of antibody, can determine whether a good clinical response has occurred. The immunogenicity risk of producing ADAs is high for murine antibodies, but also occurs for humanized and fully human antibodies. And the production of ADAs also has the possibility derived from the aggregates and/or particle size of antibodies.(Ratanji et al., 2014; Kubiak et al., 2016; Rane et al., 2019) ADA titers are associated with blood concentration of antibody drugs and have a potential correlation with decreasing efficacy.

Inflammatory immune diseases have a variety of clinical observations, and several clinical departments will practically care for patients. The use of antibody monitoring data might improve treatment outcomes. Therefore, a flexible and universal assay method is required for monoclonal antibodies and protein pharmaceuticals. Up to date, we have developed a novel liquid chromatography-mass spectrometry (LC-MS) assay named nano-surface and molecular-orientation limited (nSMOL) proteolysis. This method can bioanalytically validate many monoclonal antibodies in human serum/plasma to apply a regulated LC-MS analysis for antibodies and protein pharmaceuticals of cancer and inflammatory diseases.(Iwamoto et al., 2014; Iwamoto et al., 2016; Iwamoto et al., 2018a; Iwamoto and Shimada, 2018; Iwamoto et al., 2018b; Iwamoto et al., 2018c; Iwamoto et al., 2019) Because the Fab domain is oriented towards the reaction solution by collecting via Fc loop in resin pore, nSMOL can selectively proteolyze on the antibody Fab regions by limiting the access of trypsin to the antibody substrate using the difference between modified trypsin on the surface of nanoparticles with a 200 nm diameter and the IgG collection resin in a 100 nm pore. In this reaction, complementarity-determining regions (CDRs)-containing peptides can be effectively collected and quantitated by LC-MS analysis while maintaining the antibody sequence specificity and minimizing the large excess of tryptic peptides from the IgG frameworks and protease contamination. Because antibody monitoring using LC-MS can be quantified from biological samples by measuring antibody-specific signature peptides, in principle, this analytical method should be possible for all antibodies and Fc-fusion proteins. Furthermore, the simultaneous and continuous characterization of multiple antibodies is applicable. This approach could contribute to the cross-sectional monitoring of antibodies for inflammatory diseases. Thus far, there is no published assay that simultaneously analyzes monoclonal antibodies of inflammatory immune diseases and their method validation set out by the Food and Drug Administration (FDA) guidance criteria. This method has the potential to provide important information for the clinical decisions that have not yet been properly defined, such as the reference of drug-drug interactions, switching, withdrawal, reduction, and increase of practical antibody therapy. In our paper, we reported the validation of the multiplexed and simultaneous nSMOL LC-MS assay in a mixture containing seven antibodies under the normal protocol and two antibodies in acidified reduction exposure condition for low sensitivity antibodies.(Iwamoto et al., 2019) Moreover, we verified this method using clinical samples.

2. Materials and methods

2.1. Chemicals

Modified trypsin-immobilized glycidyl methacrylate (GMA)-coated nanoferrite particle FG beads with surface activation by N-

hydroxysuccinimide was purchased from Tamagawa Seiki (Nagano, Japan). Toyopearl AF-rProtein A HC-650F resin was from Tosoh Bioscience (Tokyo, Japan). Infliximab, Ustekinumab, and Golimumab were taken from solution remainder of Mitsubishi Tanabe Pharma (Osaka, Japan). Adalimumab was from AbbVie (North Chicago, IL). Eculizumab was from Alexion Pharma (Boston, MA). Etanercept was from Pfizer (New York, NY). Abatacept was from Bristol-Myers Squibb (New York, NY). Tocilizumab was from Chugai Pharmaceutical (Tokyo, Japan). Mepolizumab was from GlaxoSmithKline (Brentford, UK). Individual male and female control human serum was from Kohjin Bio (Saitama, Japan). Modified porcine trypsin and P14R (14-Pro and Arg) internal standard synthetic peptide was from Sigma-Aldrich (St. Louis, MO). n-octyl-β-D-thioglucopyranoside (OTG) was from Dojindo Laboratories (Kumamoto, Japan). Ultrafree-MC GV centrifugal 0.22 μm filter was from Merck Millipore (Billerica, MA). Other reagents, buffers, and solvents were from Sigma-Aldrich and Wako Pure Chemical Industries (Osaka, Japan).

2.2. Structural confirmation of signature peptides

Nine antibodies and Fc-fusion proteins (Infliximab, Adalimumab, Ustekinumab, Golimumab, Eculizumab, Etanercept, Abatacept, Tocilizumab, and Mepolizumab, each 20 μg) were denatured in 9 M of urea and 2 mM of Tris(2-carboxyethyl)phosphine (TCEP) at room temperature for 30 min. Next, the individual antibodies were diluted 10-fold in a 25 mM Tris-HCl solution (pH 8.0) and digested using modified trypsin (1 μg) at 37 °C for 16 h. The proteolytic reaction was quenched by the addition of the formic acid solution at a final concentration of 0.5%. For the nSMOL reaction, 20 μg of each antibody was collected with 25 μl of PBS-substituted AF-rProtein A resin 50% slurry in 180 μl of PBS containing 0.1% OTG with gentle vortexing at 25 °C for 5 min. Protein A resin was collected in an Ultrafree filter device. It was first washed twice using 300 μl of PBS containing 0.1% OTG, and then twice using 300 μl of PBS by filter centrifugation (10,000 x*g* for 1 min). Finally, the solution was substituted with 80 μl of 25 mM Tris-HCl (pH 8.0). nSMOL proteolysis was carried out using 1 μg of modified trypsin on the surface of FG-beads with gentle vortexing at 37 °C for 16 h under a saturated vapor atmosphere. After proteolysis, the reaction was stopped by adding formic acid at a final concentration of 0.5%. The peptide solution was collected by centrifugation (10,000 x*g* for 1 min) to remove Protein A resin and trypsin FG-beads. The structure of tryptic peptides from each antibody were determined by high-resolution liquid chromatography-linear ion trap time-of-flight (IT-TOF) MS (Nexera X2 ultra high performance liquid chromatograph and LCMS-IT-TOF, Shimadzu, Kyoto, Japan) and matrix-assisted laser desorption/ionization (MALDI) TOF MS (AXIMA Performance, Shimadzu), the parent and fragment ions were assigned using an in-house Mascot Proteome Server version 2.6 and Distiller peak processing software (Matrix Science, London, UK). The allowance of peptide *m/z* tolerance was set within 0.05 Da for LCMS-IT-TOF MS and 0.2 Da for MALDI-TOF MS. The LC-MS conditions were as follows: solvent A, 0.1% aqueous formic acid; solvent B, acetonitrile with 0.1% formic acid; column, L-column2 ODS, 2.1 × 150 mm, 2 μm, 10 nm pore (Chemicals Evaluation and Research Institute, Tokyo, Japan); column temperature, 40 °C; flow rate, 0.2 ml/min; gradient program, 0–5 min: %B = 3, 5–35 min: %B = 3–30 gradient, 35–46 min: %B = 95, 46–55 min: %B = 3. MS and MS/MS spectra were obtained using desolvation line and heat block at 250 and 400 °C, respectively. Nebulizer nitrogen gas flows were set to 3 l/min. The drying gas pressure was 100 kPa. Ion accumulation time was 30 msec for MS, and 70 msec for MS/MS analysis. The MS/MS analysis was performed using the automated data dependent mode. Argon pulse time into the ion trap cell was 125 μsec. The electrode of the collision-induced dissociation (CID) cell was set at −1.5 V. The MALDI MS conditions were as follows: reflectron high-resolution mode from *m/z* 600 to 4500 mass acquisition range, high-purity recrystallized α-cyano-4-hydroxycinnamic acid (Shimadzu GLC, Tokyo, Japan) as a MALDI matrix, externally calibrated by protonated mass signals of a five peptide mixture, bradykinin fragment 1–7 (monoisotopic m/z 757.40), angiotensin II (m/z 1046.54), P14R synthetic peptide (m/z 1533.86), ACTH fragment 18–39 (m/z 2465.20), and insulin oxidized B chain (m/z 3494.65), internally calibrated by protonated signals from the tryptic autolysis fragments (m/z 842.51 and m/z 2211.10).

2.3. Prediction of the signature peptides

Amino acid sequences were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) and Drug Bank, Infliximab (KEGG entry no. D02598, Drug Bank accession no. DB00065), Adalimumab (D02597, DB00051), Ustekinumab (D09214, DB05679), Golimumab (D04358, DB06674), Eculizumab (D03940, DB01257), Etanercept (D00742, DB00005), Abatacept (D03203, DB01281), Tocilizumab (D02596, DB06273), and Mepolizumab (D04923, DB06612). Multiple sequence alignment analysis was performed by the ClustalW algorithm on GENETYX software (GENETYX, Tokyo, Japan) using the amino acid sequences. For Infliximab, the alignment was performed using the sequences of the chimeric antibody. For Eculizumab, Tocilizumab, and Mepolizumab, this analysis was performed using the sequences of humanized antibodies. For Adalimumab, Ustekinumab, and Golimumab, this was performed using the sequences of fully human antibodies. And for Etanercept and Abatacept, the alignment analysis was performed on the Tumor necrosis factor receptor 2 (SwissProt accession TNR1B_HUMAN) and Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4_HUMAN) shown in our previous report.(Iwamoto et al., 2018b) In this analysis, theoretical tryptic peptides with no overlap to the sequence of immunoglobulin framework, hinge regions, positions of cysteine residue, and Fc-fusion insertion were aligned.

2.4. Setting conditions for multiple reaction monitoring (MRM) of each signature peptide

The peptide quantitation was analyzed using an LC-electrospray ionization-MS (LC-ESI-MS) with triple quadrupole (Nexera X2 and LCMS-8050/8060, Shimadzu). The LC-MS conditions were as follows: solvent A, 0.1% aqueous formic acid; solvent B, acetonitrile with 0.1% formic acid; column, Shim-pack GISS C18, 2.1 × 50 mm, 1.9 μm, 20 nm pore (Shimadzu); column temperature, 50 °C; flow rate, 0.4 ml/min; gradient program for seven antibody mix in normal nSMOL conditions, 0–1.5 min: %B = 1, 1.5–5.5 min: %B = 1–40 gradient, 5.5–6.5 min: %B = 95 with flow rate 0.8 ml/min, 6.5–7.5 min: and %B = 1 with flow rate 0.4 mi/min; gradient for two antibody mix in acidified reduction acceleration nSMOL conditions, 0–1.5 min: %B = 1, 1.5–4.5 min: %B = 1–30 gradient, 4.5–5.5 min: %B = 95 with flow rate 0.8 ml/min, 5.5–6.5 min: %B = 1. MS spectra were obtained with ESI probe temperature, desolvation line, and heat block of 300 °C, 250 °C, and 400 °C, respectively. Nebulizer, heating, and drying gas flows were set to 3, 10, and 10 l/min, respectively. The dwell time was set to 10 msec for each transition. MRM monitor ions of peptide fragments were imported from the measured values of the structure-assigned fragments by high-resolution LC-MS analysis. CID Ar partial pressure in the Q2 cell was set to 270 kPa. Candidate MRM transition *m/z* were computationally set, and the electrode voltage of Q1 pre bias, collision cell Q2, and Q3 pre bias, and the most abundant m/z of the parent and fragment ion were performed using the fully tryptic peptides of each antibody and their synthetic peptides by the optimization support software (LabSolutions, Shimadzu).

2.5. Preparation of the sample for each antibody validation by nSMOL proteolysis

In the current study, we performed a bioanalytical validation for all nine antibodies in human serum using the nSMOL method as described in our previous report with a minor improvement. The scheme of the

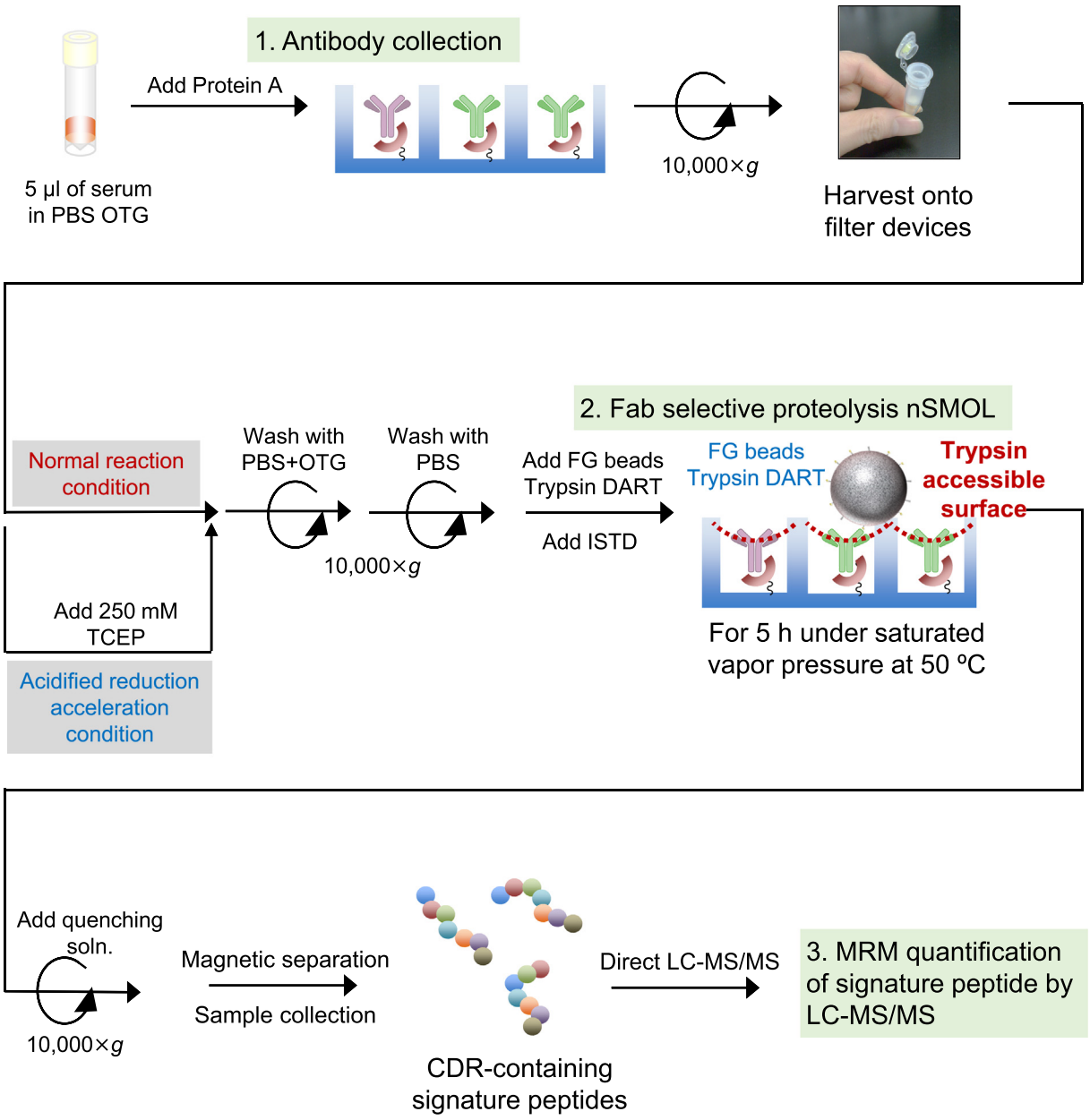

Fig. 1. Protocol of nSMOL proteolysis under normal and acidified reduction conditions.

protocol is shown in Fig. 1. The nSMOL proteolysis coupled with the LC-MS/MS method was validated in accordance with the Guideline on Bioanalytical Method Validation in Pharmaceutical Development from Notification 0711–1 of the Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau, the Ministry of Health, Labour and Welfare, dated May 24, 2018. The objective of a full validation is to demonstrate the assay performance, e.g. selectivity, lower limit of quantification (LLOQ), low concentration of quality control (LQC), middle concentration of quality control (MQC), high concentration of quality control (HQC), calibration curve, accuracy and precision, matrix effect, carry-over, dilution integrity, and stored and processed sample stability. Briefly, all validation sample sets were prepared and stored at −80 °C for 24 h or longer before each validation assay. A 5 µl aliquot of each antibody-spiked human serum was diluted 10-fold in PBS (pH 7.4) containing 0.1% OTG to avoid non-specific binding to the resin and plastic materials. The IgG fraction from serum sample was collected by 12.5 µl of PBS-substituted AF-rProtein A resin (50% slurry) in 95 µl of PBS containing OTG with gentle vortexing at 25 °C for 5 min. For acidified reduction acceleration nSMOL, the resin was also treated with 75 µl of 250 mM TCEP-HCl aqueous solution at room temperature for 30 min with gentle vortexing. Protein A resin was harvested onto an Ultrafree filter and first washed twice with 300 µl of PBS containing OTG to remove other serum proteins except for IgGs, and then with 300 µl of PBS to remove detergents that inhibit column separation, carryover, and ionization of peptides in ESI interface. Each washing substitution was directly performed by centrifugation (10,000 xg for 1 min) on filter devices. After these washing steps, Protein A resin was substituted with 80 µl of the nSMOL reaction solution. nSMOL proteolysis was carried out using 5 µg trypsin on FG-beads with gentle vortexing at 50 °C for 5 h in a saturated vapor atmosphere for uniform contact between Protein A resin and FG beads nanoparticles. After nSMOL proteolysis, the reaction was quenched by adding formic acid at a final concentration of 0.5%. The peptide solution was collected by centrifugation (10,000 ×*g* for 1 min) and magnetic separation to remove Protein A resin and trypsin FG-beads. These analytes were transferred into low protein binding polypropylene vials (TORAST-H

Table 2
Linear quantitation range of each antibody and its signature peptides in human serum.

	Linear quantitation range [μg/ml]	Signature peptide	Position
Antibodies for nSMOL normal condition			
Infliximab	0.391–100	SINSATHYAESVK	H-CDR2 aa. 55–67
Adalimumab	1.56–100	APYTFGQGTK	L-CDR3 aa. 94–103
Ustekinumab	0.391–100	RRPGQGYFDFWGQGTLVTVSSSSTK	H-CDR3 aa. 99–123
Golimumab	1.56–100	SNWPPFTFGPGTK	L-CDR3 aa. 92–104
Eculizumab	1.56–100	LLIYGATNLADGVPSR	L-CDR2 aa. 46–61
Etanercept	0.391–100	VFCTK	N-terminus aa. 42–46
Abatacept	0.391–100	MHVAQPAVVLASSR	N-terminus aa. 1–13
Antibodies for nSMOL acidified reduction acceleration condition			
Tocilizumab	0.781–100	VTMLR	H-CDR2 aa. 68–72
Mepolizumab	1.56–100	DPPSSLLR	H-CDR3 aa. 98–105

aa: amino acid.

Table 3
Validated MRM transition for each signature peptides and internal standard P14R.

Antibodes	MRM transition [m/z]	Q1 [V]	Collision [V]	Q3 [V]
Infliximab	469.65 ➔ 603.80 (y11++)	−46	−16	−34
Adalimumab	535.30 ➔ 901.40 (y8+)	−40	−21	−26
Ustekinumab	691.20 ➔ 539.25 (b9++)	−40	−31	−28
Golimumab	718.45 ➔ 1048.45 (y10+)	−44	−24	−42
Eculizumab	830.45 ➔ 515.10 (y5+)	−40	−41	−26
Etanercept	299.30 ➔ 498.20 (y4+)	−30	−12	−19
Abatacept	489.25 ➔ 420.20 (y4+)	−30	−16	−30
Tocilizumab	310.20 ➔ 520.40 (y4+)	−30	−13	−20
Mepolizumab	442.70 ➔ 672.40 (y6+)	−36	−23	−30
P14R IS	512.10 ➔ 292.30 (b3+)	−30	−19	−34

Bio Vial, Shimadzu GLC), and then analyzed by LC-MS. The linear quantitation range and optimized MRM condition for each signature peptide of each antibody in human serum samples are summarized in Tables 2 and 3.

2.6. Verification of simultaneous analysis intercompared with the single antibody assay using QC and clinical samples

After the validation assay was developed for each antibody and Fc-fusion protein, the verification and reproducibility of the quantitation data was carried out by intercomparing the data calculated from the calibration curve of mixed antibodies and the data from the single calibration curve of each antibody. In this study, we have verified seven-mixed antibodies (Infliximab, Adalimumab, Ustekinumab, Golimumab, Eculizumab, Etanercept, and Abatacept) under normal reaction condition, and two-mixed antibodies (Tocilizumab and Mepolizumab) under acidified reduction acceleration condition. For further verification, we performed the intercomparison analysis using clinical samples of Infliximab, Adalimumab, and Tocilizumab between the current data calculated from the mixed calibration curve and the data randomly selected from each single calibration curve in the past year.

2.7. Clinical sample information

Forty-five Japanese patients with rheumatoid arthritis (RA) or inflammatory bowel disease (IBD) were enrolled in this study at Kyoto University Hospital from November 2017 to January 2019. Infliximab, Adalimumab, or Tocilizumab were administered continuously for the treatment of RA or IBD in these patients (14 patients treated with infliximab, 16 patients treated with adalimumab, and 15 patients treated with tocilizumab). Serum samples (2 ml) were collected before the administration of therapeutic antibodies, and the baseline levels were measured by individual and multiple assay methods. This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by Kyoto University Graduate School and the Faculty of Medicine, Ethics Committee (R0357 for RA patients and R0012/R1632 for IBD patients).

3. Results and discussion

3.1. The normal and acidified reduction acceleration nSMOL assay conditions

In Fig. 1, we showed the two nSMOL protocols in normal and acidified reduction acceleration conditions. We generally use the normal reaction condition for nSMOL antibody assay, and seven antibodies (Infliximab, Adalimumab, Ustekinumab, Golimumab, Eculizumab, Etanercept, and Abatacept) have been carried out the all validation and intercomparison test using this condition. The acidified reduction acceleration condition has been developed for the purpose of improving the nSMOL reaction efficiency for low sensitivity antibodies. The proteolysis of an antibody is normally proceeded in an nSMOL reaction, in our multiple studies, there were local regions resistant to the trypsin reaction in several monoclonal antibodies. As a result, signature peptides with extremely low yield may be observed. The low yield could be a result of structural rigidity, packing, or the vicinity of conserved disulfide positions. If these regions overlap near the CDR regions, signature peptides with high yield could be difficult to collect in the nSMOL reaction. And under the acidified reduction acceleration condition, signature peptides on light chain are considered to be decreased the recovery in principle since light chain Fab will be dissociated in the acid reduction exposure. This discussion has been supported by our other verification studies (data not shown). So, we use this acidified condition only when effectively collecting the signature peptides from antibodies on heavy chain. To advance this issue, Tocilizumab and Mepolizumab which corresponded to the low sensitivity antibodies were separately analyzed using acidified reduction acceleration conditions.

3.2. Amino acid sequence alignment and confirmation of interference from human serum matrix

The signature peptides for each antibody were predicted by confirming the sequence specificity, insertion, and deletion using ClustalW multiple amino acid sequence alignment analysis in Fig. 2. For linear correlation between MRM signal response and each antibody concentration set in human serum without interference from endogenous IgGs, we have analyzed and decided all candidate signature peptides and their MRM transitions including CDR-containing peptides of each antibody using a serial dilution of each antibody in human serum. The signature peptides APYTFGQGTK in CDR3 on light chain, RRPGQGYFDFWGQGTLVTVSSSSTK in CDR3 on heavy chain, and SNWPPFTFGPGTK in CDR3 on light chain were selected from the fully human antibodies, Adalimumab, Ustekinumab, and Golimumab, respectively (Fig. 2a and b). Same as above, the signature peptides LLIYGATNLADGVPSR in CDR2 on light chain, VTMLR in CDR2 on heavy chain, and DPPSSLLR in CDR3 on heavy chain were selected from humanized antibodies, Eculizumab, Tocilizumab, and Mepolizumab, respectively

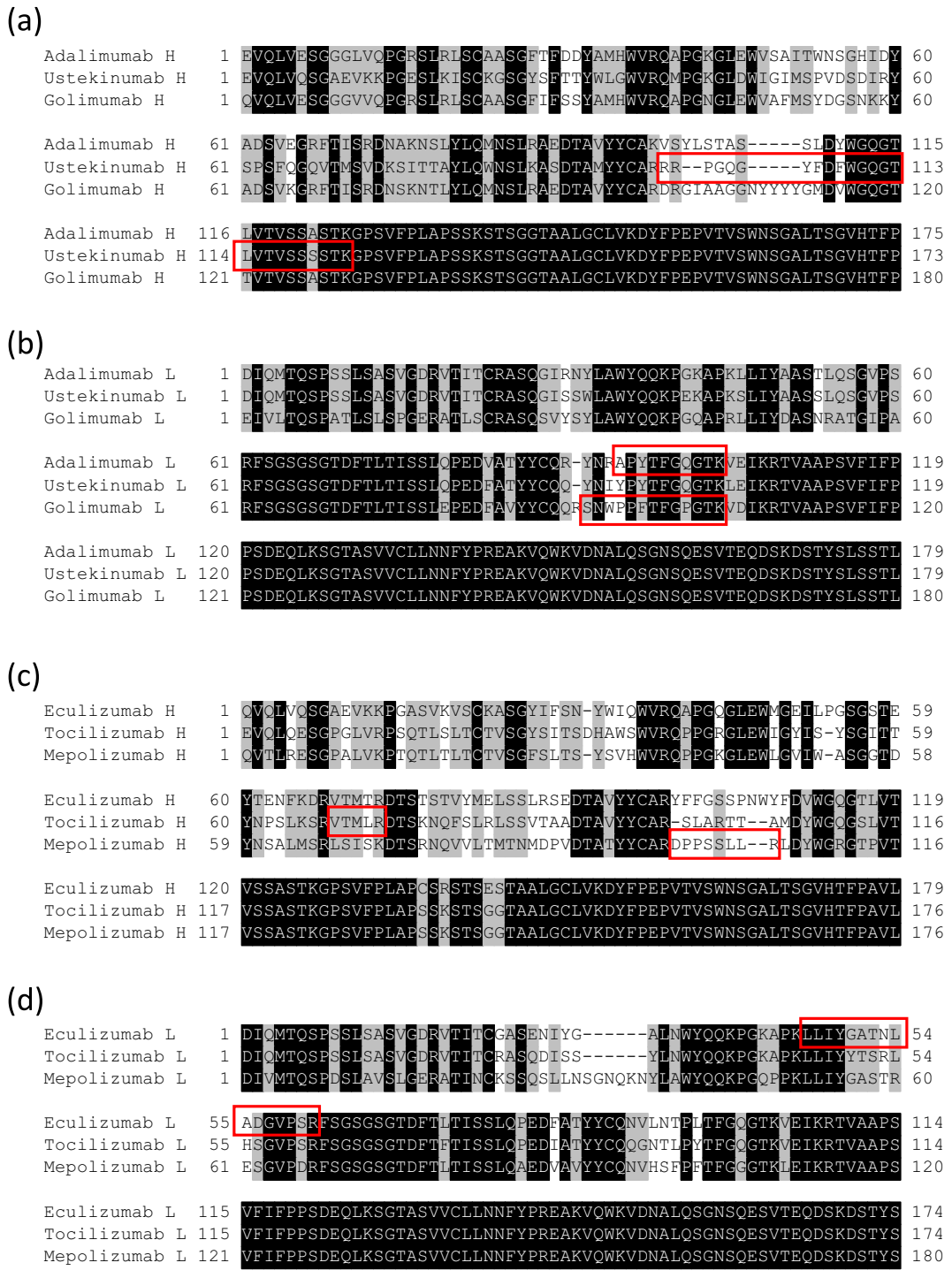

Fig. 2. ClustalW sequence alignment to predict the signature peptides in each antibody. (a) Heavy chain of Adalimumab, Ustekinumab, and Golimumab, (b) light chain of Adalimumab, Ustekinumab, and Golimumab, (c) heavy chain of Eculizumab, Tocilizumab, and Mepolizumab, and (d) light chain of Eculizumab, Tocilizumab, and Mepolizumab are shown. Black residues are the identical amino acids in the common framework, and gray shows similar amino acids. Non-labeled amino acids and insertions show the specific regions in each antibody of the candidate signature peptides with CDR-containing. Each selected signature peptides are shown in red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2c and d). The signature peptides of Infliximab (SINSATHYAESVK), and the Fc-fusion proteins Etanercept (VFCTK) and Abatacept (MHVAQPAVVLASSR) have been described in our previous validation reports.(Iwamoto et al., 2018c; Iwamoto et al., 2018b; Iwamoto et al., 2019) In Fig. 3a and b, representative MRM chromatographs are shown. The MRM intensity was dependent on the collection yield and ionization efficiency of individual peptides, and the multiplexed nSMOL assay quantitate each antibody with sufficiently separation profile.

3.3. nSMOL bioanalysis assay validation for each antibody

3.3.1. Calibration curve reproducibility

The calibration linearity and reproducibility ($N = 3$) were evaluated by the analysis set of 9 calibration standards (zero sample, 0.391,

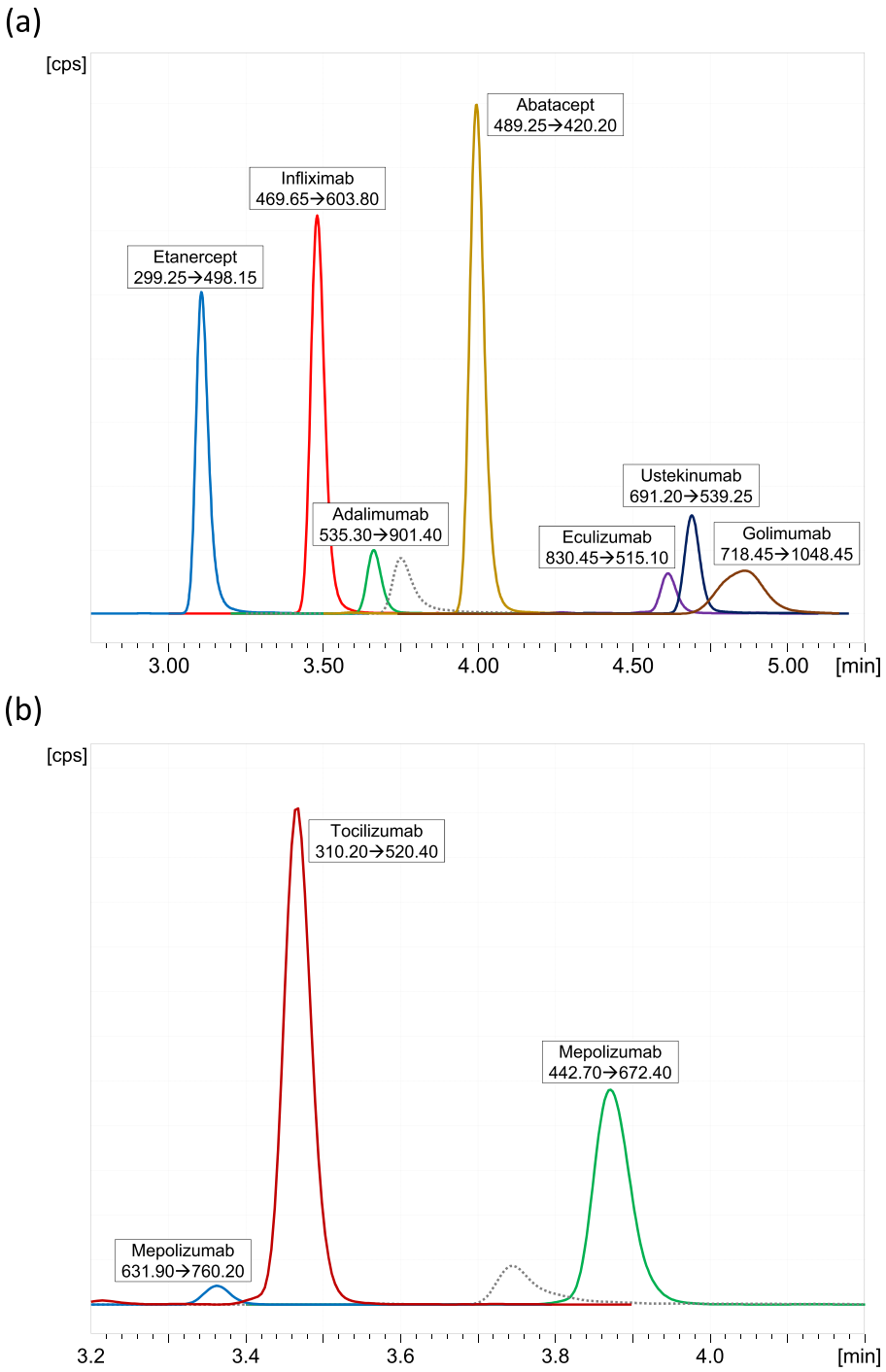

Fig. 3. Representative MRM chromatogram of (a) seven-mixed antibodies (Infliximab, Adalimumab, Ustekinumab, Golimumab, Eculizumab, Etanercept, and Abatacept) in normal reaction condition, and (b) two-mixed antibodies (Tocilizumab and Mepolizumab) in acidified reduction acceleration conditions. MRM chromatograms from signature peptides of each antibody are described in the figure with each MRM transition information. The dashed gray line shows the MRM of P14R internal standards (*m/z* 512.10 ➔ 292.30).

0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, and 100 μg/ml) using linear regression fitting. The calibration plot was weighted by the 1/Area2. The averaged accuracy (%) of each antibody under normal nSMOL condition was intercompared with seven-mix antibody assay data and single assay (Table 4), with 97.7–108% accuracy at LLOQ and 91.8–107% at other concentration set, respectively. Similarly, the accuracy (%) using two-mix assay under acidified reduction acceleration condition is summarized in Table 5, with 99.5–102% at LLOQ and 93.9–109% at other concentration, respectively. The accuracy data on the calibration test were confirmed to be within 15% of error at all concentration set in both the mixed and single assay.

3.3.2. *Precision and accuracy in inter- and intra-assay*

Precision and accuracy data were determined by the analysis of sample set at LQC and HQC concentrations for seven-mix assay (Table 6) and two-mix assay (Table 7). The intra-day and inter-day precision and accuracy were determined by analyzing five QC sample sets on three different days. The accuracy (%) of seven-mix assay was 87.9–109% at LLOQ, and at 89.0–110% HQC, respectively. And that of two-mix assay was 90.4–101% at LLOQ, and 92.3–107% at HQC,

Table 4
Average accuracy (%) of calibration curve reproducibility (N = 3) under normal reaction conditions.

Antibodies	Cal. set	Nominal concentration [μg/ml]								
		0.391	0.781	1.56	3.13	6.25	12.5	25	50	100
Infliximab	7-mix	103	96.9	100	96.1	99.1	101	103	102.3	104
	Individual	104	91.8	110	94.7	104	102	101	99.5	99.0
Adalimumab	7-mix	ND	ND	106	98.7	97.3	98.9	102	98.2	105
	Individual	ND	ND	108	92.7	97.0	104	105	98.2	99.6
Ustekinumab	7-mix	103	99.0	95.5	104	97.0	104	98.8	103	100
	Individual	105	94.9	103	102	100	102	97.1	100	102
Golimumab	7-mix	ND	ND	97.7	98.5	105	101	98.1	103	96.9
	Individual	ND	ND	98.4	100	99.8	104	99.4	102	97.5
Eculizumab	7-mix	ND	ND	103	95.2	100	98.7	98.8	97.8	110
	Individual	ND	ND	98.3	102	108	104	93.6	101	99.3
Etanercept	7-mix	100	100	99.8	100	97.1	103	103	101	98.7
	Individual	99.5	99.3	101	107	99.6	102	100	99.4	95.2
Abatacept	7-mix	103	97.5	98.5	96.1	98.2	101	104	100	105
	Individual	98.6	104	97.4	105	98.4	101	98.3	99.0	101

Cal: calibration.

Table 5
Average accuracy (%) of calibration curve reproducibility (N = 3) under acidified reduction acceleration conditions.

Antibodies	Cal. set	Nominal concentration [μg/ml]								
		0.391	0.781	1.56	3.13	6.25	12.5	25	50	100
Tocilizumab	2-mix	ND	102	95.3	102	99.0	106	105	96.5	104
	Individual	ND	101	95.1	98.8	98.6	106	106	109	93.9
Mepolizumab	2-mix	ND	ND	99.8	102	98.2	103	101	99.6	98.4
	Individual	ND	ND	99.5	99.8	104	102	96.5	105	97.9

Table 6
Precision and accuracy data calculated by seven-mix antibody standard (Infliximab, Adalimumab, Ustekinumab, Golimumab, Eculizumab, Etanercept, and Abatacept) compared to each individual data under normal reaction conditions.

[μg/ml]	Antibody	Set	Run 1 (N = 5)			Run 2 (N = 5)			Run 3 (N = 5)			N = 15		
			Mean	CV%	Acc%	Mean	CV%	Acc%	Mean	CV%	Acc%	Mean	CV%	Acc%
7-mixed	Infliximab	1.17	1.14	8.96	97.4	1.11	4.09	94.6	1.18	3.12	101	1.14	3.11	97.6
		80	73.6	4.18	92.0	73.6	3.16	92.0	82.0	4.34	102	76.4	0.815	95.5
	Adalimumab	4.69	4.12	2.47	87.9	4.28	3.54	91.3	4.72	5.98	101	4.37	2.13	93.3
		80	70.8	3.18	88.5	74.1	2.62	92.6	83.0	1.38	104	76.0	0.748	95.0
	Ustekinumab	1.17	1.19	6.51	102	1.27	4.25	109	1.15	8.58	97.9	1.20	1.84	103
		80	77.4	4.41	96.7	78.3	2.30	97.8	77.8	2.26	97.2	77.8	1.21	97.3
	Golimumab	4.69	4.83	4.87	103	4.73	3.32	101	4.35	1.99	92.7	4.64	1.60	98.9
		80	85.0	2.58	106	76.4	5.96	95.5	73.8	2.69	92.3	78.4	1.81	98.0
	Eculizumab	4.69	4.34	9.56	92.5	4.60	6.25	98.1	4.34	1.39	92.6	4.43	4.06	94.4
		80	72.0	3.28	90.0	72.4	3.51	90.5	71.5	2.23	89.4	72.0	0.700	90.0
	Etanercept	1.17	1.19	4.05	102	1.18	5.29	101	1.18	5.97	101	1.18	0.955	101
		80	78.7	3.64	98.4	71.2	2.59	89.0	75.4	6.14	94.2	75.1	1.87	93.9
	Abatacept	1.17	1.15	1.63	98.6	1.14	3.72	97.7	1.24	3.83	106	1.18	1.30	101
		80	76.8	4.47	96.0	75.9	5.54	94.8	85.1	1.17	106	79.3	2.11	99.1
Individual	Infliximab	1.17	1.17	9.72	100	1.13	4.30	96.2	1.14	3.49	97.8	1.15	3.53	98.0
		80	82.3	4.19	103	78.4	3.16	98.0	87.8	4.35	110	82.8	0.835	104
	Adalimumab	4.69	4.38	2.34	93.4	4.21	3.39	89.9	4.73	6.10	100.8	4.44	2.20	94.7
		80	71.8	3.17	89.8	69.9	2.61	87.4	76.8	1.38	96.1	72.9	0.844	91.1
	Ustekinumab	1.17	1.15	6.53	98.2	1.16	4.64	98.8	1.19	8.38	102	1.16	1.97	99.5
		80	74.6	4.41	93.3	77.4	2.30	96.8	78.9	2.26	98.6	77.0	1.13	96.2
	Golimumab	4.69	5.07	4.55	108	4.37	3.89	93.2	4.84	1.96	103	4.76	1.43	102
		80	83.6	2.57	105	75.7	5.95	94.6	80.6	2.69	101	80.0	1.69	100
	Eculizumab	4.69	4.50	9.90	96.0	4.43	6.43	94.5	4.33	1.60	92.2	4.42	4.27	94.2
		80	77.2	3.29	96.5	71.6	3.52	89.5	74.5	2.22	93.1	74.4	0.677	93.0
	Etanercept	1.17	1.19	4.07	101	1.17	5.53	100	1.15	5.86	98.2	1.17	0.890	99.9
		80	78.8	3.64	98.5	74.1	2.59	92.7	75.3	6.14	94.2	76.1	1.80	95.1
	Abatacept	1.17	1.23	1.57	105	1.19	3.82	102	1.16	3.96	99.4	1.19	1.27	102
		80	80.4	4.47	101	81.0	5.54	101	82.8	1.17	103	81.4	2.25	102

CV: Coefficient of Variation, Acc: Accuracy.

Table 7
Precision and accuracy data calculated by two-mix antibody standard (Tocilizumab and Mepolizumab) compared to each individual data under acidified reduction acceleration condition.

[μg/ml]	Antibody	Set	Run 1 (N = 5)			Run 2 (N = 5)			Run 3 (N = 5)			N = 15		
			Mean	CV%	Acc%	Mean	CV%	Acc%	Mean	CV%	Acc%	Mean	CV%	Acc%
2-mixed	Tocilizumab	2.34	2.33	4.26	99.7	2.11	3.08	90.4	2.19	4.13	93.8	2.21	0.800	94.6
		80	79.6	8.87	99.5	79.4	7.76	99.3	83.2	2.84	104	80.7	3.09	101
	Mepolizumab	4.69	4.75	4.68	101	4.75	2.70	101	4.29	1.97	91.6	4.60	1.53	98.0
		80	81.1	5.82	101	85.0	4.95	106	84.6	6.60	106	83.5	0.83	104
Individual	Tocilizumab	2.34	2.22	2.88	95.1	2.28	4.30	97.3	2.28	9.35	97.4	2.26	3.46	96.6
		80	73.8	3.14	92.3	85.2	5.51	107	84.9	1.68	106	81.3	2.08	102
	Mepolizumab	4.69	4.72	3.63	101	4.67	4.78	99.7	4.48	3.55	95.5	4.63	0.739	98.6
		80	76.1	1.51	95.1	81.9	7.33	102	82.0	4.71	103	80.0	3.04	100

Table 8
Processed sample stability of seven-mix antibody analysis under normal reaction conditions.

Antibody	Processed after [h]	24		48	
Infliximab	Nominal concentration [μg/ml]	1.17	80	1.17	80
	Mean	1.15	73.7	1.16	76.8
	Acc%	98.3	92.1	99.1	96.0
Adalimumab	Nominal concentration [μg/ml]	4.69	80	4.69	80
	Mean	4.75	81.5	4.76	78.0
	Acc%	101	102	101	97.5
Ustekinumab	Nominal concentration [μg/ml]	1.17	80	1.17	80
	Mean	1.19	77.3	1.24	80.2
	Acc%	101	96.6	106	100
Golimumab	Nominal concentration [μg/ml]	4.69	80	4.69	80
	Mean	4.48	80.7	4.77	91.1
	Acc%	95.5	101	102	101
Eculizumab	Nominal concentration [μg/ml]	4.69	80	4.69	80
	Mean	4.40	72.6	4.37	75.6
	Acc%	93.8	90.1	93.2	94.5
Etanercept	Nominal concentration [μg/ml]	1.17	80	1.17	80
	Mean	1.19	82.1	1.16	80.2
	Acc%	102	103	99.1	100
Abatacept	Nominal concentration [μg/ml]	1.17	80	1.17	80
	Mean	1.17	78.3	1.14	82.4
	Acc%	100	97.9	97.4	103

Table 9
Processed sample stability of two-mix antibody analysis under acidified reduction acceleration conditions.

Antibody	Processed after [h]	24		48	
Tocilizumab	Nominal concentration [μg/ml]	2.34	80	2.34	80
	Mean	2.24	79.7	2.19	79.5
	Acc%	95.9	99.7	93.4	99.4
Mepolizumab	Nominal concentration [μg/ml]	4.69	80	4.69	80
	Mean	4.66	78.6	4.64	84.3
	Acc%	99.3	98.2	99.0	105

respectively. These data of the same day set ($N = 5$, each) and 3 day set ($N = 15$) was also confirmed to be within 15% error at all concentration.

3.3.3. Processed sample stability

The processed sample stability after the nSMOL reaction at 5 °C for 24 and 48 h was demonstrated at the concentration of LQC and HQC for seven-mix assay (Table 8) and two-mix assay (Table 9). The accuracy (%) of seven-mix assay was 93.8–101% for 24 h and 93.2–106% for 48 h at LLOQ, 90.1–103% for 24 h and 91.1–103% for 48 h at HQC, respectively. And that of two-mix assay was 95.9–99.3% for 24 h and 93.4–99.0% for 48 h at LLOQ, and 98.2–99.7% for 24 h and 99.4–105% for 48 h at HQC, respectively. These data were also confirmed to be within 15% error at all conditions.

3.4. Intercomparison between simultaneous and single assay using clinical patient samples

We have verified the intercomparison results of seven-mix and single assay for Infliximab (Fig. 4a, $N = 14$) and Adalimumab (Fig. 4b, $N = 16$), as well as two-mix and single assay for Tocilizumab (Fig. 4c, N = 15) using clinical patient samples. A Pearson correlation analysis by linear regression fitting showed that all sample set had good correlation between simultaneous antibody assay and individual single antibody assay, and each data of comparison group had high reproducibility and low variation plotted within the 95% confidence intervals. Furthermore, the slope correlations in each comparison set were close to 1.000 and the peak area cps itself has also highly reproducible. Our multiplexed and simultaneous nSMOL assay was adequately applicable to clinical studies even in mixed antibody assay conditions.

4. Conclusion

In conclusion, we have developed an LC-MS bioanalytical validation of nine therapeutic antibodies for inflammatory immune diseases using nSMOL strategy. And using these validated conditions, we demonstrated that nSMOL satisfied the validation guidance criteria even in the multiplexed and simultaneous determination of mixed antibodies in QC and clinical patient samples. A universal assay method that measures multiple monoclonal antibodies in biological samples at the same time could monitor therapeutic antibodies and cross-sectional analysis in multimodal inflammatory diseases at central core facilities.

Antibody levels in systemic circulation could inform not only individual observations of clinical responses, but also the analysis of secondary loss of efficacy and the development of dosing criteria. In addition, this method can greatly reduce assay cost reduction at a core lab. Moreover, our approach could provide an optimal regulated assay method for therapeutic drug monitoring (TDM) applications, and therapeutic antibodies for which TDM will be required in the future.

Declaration of interest

MH, MT, and HI belong to the department that is financially supported by Tanabe-Mitsubishi, Chugai, UCB Japan, and Ayumi Pharma. MH has received research grants and/or speaker fee from Tanabe-Mitsubishi, Astellas, Eisai, and Bristol-Myers. MT has received research grants from Astellas, AbbVie, Pfizer, and Taisho-Toyama. HI has received a research grant and/or speaker fee from Bristol-Myers, Astellas, and Asahi-Kasei. The other authors declare that there is no conflict of interest regarding this study.

Author contributions

NI and TS supervised the study design, developed the mass spectrometry analysis, and drafted the manuscript. MT and KY carried out the validation assays of each antibody bioanalysis. AY, MD, and KM

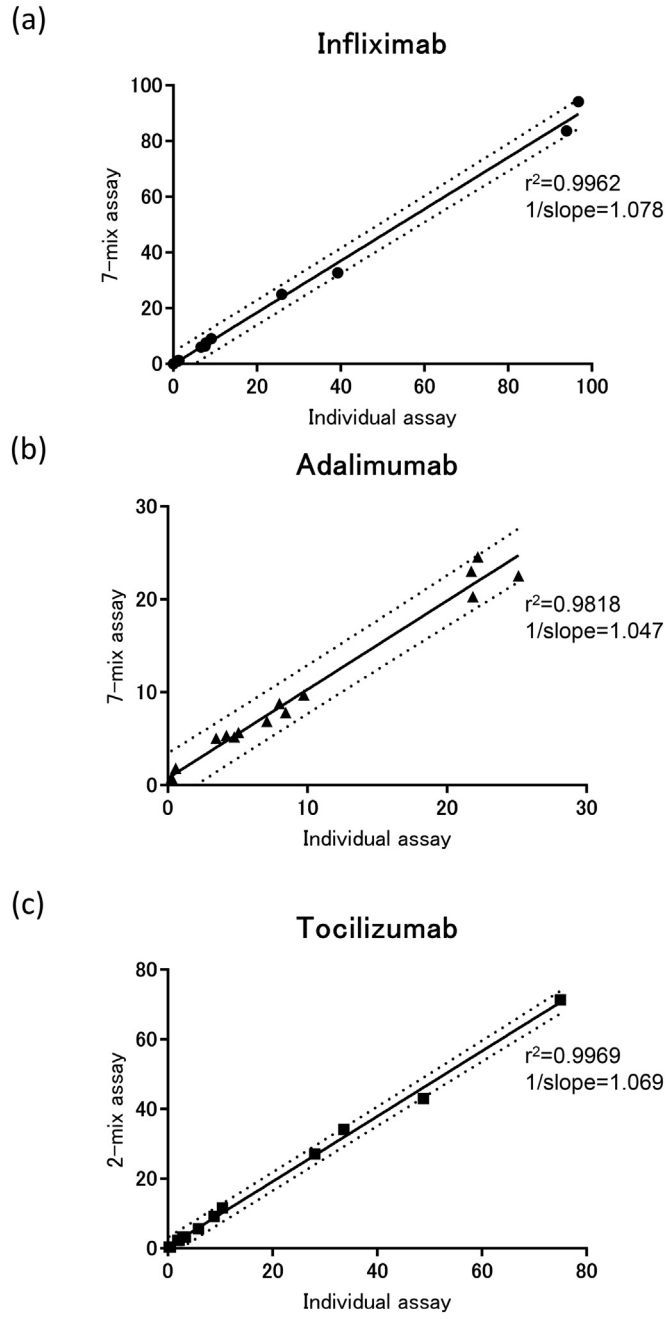

Fig. 4. Verification of quantitative data using calibration standards from the mixed antibodies compared with the individual assay in clinical patient sample. (a) Infliximab (●, $N = 14$) and (b) Adalimumab (▲, $N = 16$) treated patient samples in normal reaction conditions. (c) Tocilizumab (■, $N = 15$) treated patient samples in acidified reduction acceleration conditions. Pearson correlation coefficient (r2) and 1/slope value are described in the figure. The solid line shows the linear regression fitting, and the dashed line shows the 95% confidence interval. The horizontal and vertical axis shows the concentration [μg/ml] of each antibody in the clinical serum samples.

participated in the clinical study design and statistical analysis. MH, MT, and HI practiced the clinical care and managed the samples from RA patients. MM, SY, and YH practiced the clinical care and managed the samples from IBD patients. All authors read and approved the final manuscript.

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