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ARTICLE *in* ANALYTICAL CHEMISTRY · FEBRUARY 2014

Impact Factor: 5.64 · DOI: 10.1021/ac5001465 · Source: PubMed

analytical chemistry

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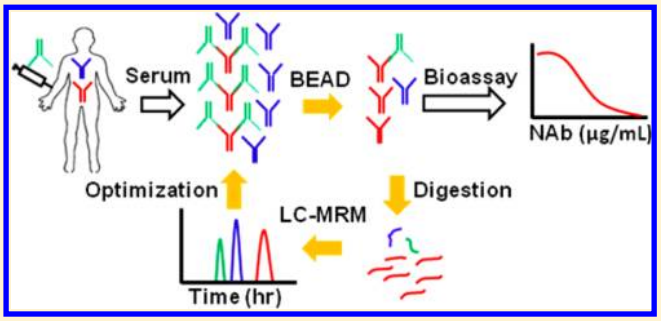
Innovative Use of LC-MS/MS for Simultaneous Quantitation of Neutralizing Antibody, Residual Drug, and Human Immunoglobulin G in Immunogenicity Assay Development

Hao Jiang,*[,§,†] Weifeng Xu,[§,†] Craig A. Titsch,[†] Michael T. Furlong,[†] Robert Dodge,[†] Kimberly Voronin,[‡] Alban Allentoff,[‡] Jianing Zeng,*[,†] Anne-Françoise Aubry,[†] Binodh S. DeSilva,[†] and Mark E. Arnold[†]

[†]Analytical and Bioanalytical Development and [‡] Discovery Chemistry Synthesis, Bristol-Myers Squibb, Princeton, New Jersey 08540, United States

ABSTRACT: Immunogenicity testing for antidrug antibodies (ADA) faces challenges when high levels of the drug are present in clinical patient samples. In addition, most functional cell-based assays designed to characterize the neutralizing ability of ADA are vulnerable to interference from endogenous serum components. Bead extraction and acid dissociation (BEAD) has been successfully applied to extract ADA from serum samples prior to conduction of cell-based assays. However, in the BEAD, certain amounts of the drug and endogenous serum components (so-called residual drug and serum components) from serum samples are carried over to final BEAD eluates due to formation of protein complexes with ADA or nonspecific binding with the beads. Using current enzyme-linked immunosorbent assay (ELISA)-based ligand-binding assays, it is difficult to evaluate the residual drug, which is complexed with excessive amounts of ADA and endogenous serum components in the BEAD eluates. Here, we describe an innovative application of LC-MS/MS for simultaneous detection of the residual human monoclonal antibody drug and endogenous human IgG and the neutralizing antibody positive-control (NAb-PC) in the BEAD eluates. In this study, the low levels of the residual drug and human IgG in the BEAD eluates indicate that the BEAD efficiently removed the high-concentration drug and serum components from the serum samples. Meanwhile, the NAb-PC recovery (~42%) in the BEAD provided an acceptable detection limit for the cell-based assay. This novel application of LC-MS/MS to immunogenicity assay development demonstrates the advantages of LC-MS/MS in selectivity and multiplexing, which provides direct and fast measurements of multiple components for immunogenicity assay development.



Antidrug antibodies (ADA) could have a significant impact on a biological drug's efficacy and potential patient safety. Neutralizing antibodies (NAb), a type of antidrug antibody, specifically block the biological activity of therapeutic proteins and thus result in the reduced efficacy of therapeutic protein products.[1−3] Functional cell-based assays, rather than competitive ligand binding assays (LBA), are the preferred format to evaluate the presence of the drug-associated NAb in a patient's serum samples,[4] but these assays are more susceptible to serum matrix effects and drug interferences.[5,6] Human serum contains many growth factors, cytokines, and other biological factors which may have a direct impact on the cells used in the assay. A high-concentration drug in a patient serum sample also directly affects the performance of the cell-based NAb assay. Therefore, sample cleanup to remove these serum components and the drug from serum samples prior to the cell-based assay is essential in the development of a NAb assay. Bead extraction and acid dissociation (BEAD) is an effective approach to purify and enrich the NAb from serum samples and has been demonstrated to improve sensitivity and increase drug tolerance.[7] However, during the BEAD, certain amounts of the drug and serum components may be still carried over to the cell-based assay due to two binding arms of the drug-associated ADA or through nonspecific binding to the beads in the BEAD treatment, both of which need to be evaluated in the NAb assay development. The NAb recovery also needs to be determined to ensure appropriate assay sensitivity. It would require a bioanalytical assay to determine the levels of the residual drug and endogenous IgG (an indicator of residual serum components) and the NAb in the BEAD eluates. To our knowledge, there is no report to date to measure residual monoclonal antibody (mAb) drug in the BEAD-treated serum, mainly due to the following two reasons. First, the antigen-binding sites of the residual drug may be blocked by the high-concentration NAb. In our case, the residual human IgG4 drug is ~300 ng/mL in the presence of μg/mL-level NAb positive control (NAb-PC), which makes the accurate measurement by the highly sensitive electrochemilumencent-bridging assay difficult. Second, currently no antibody is available that can specifically distinguish the Fc-region mutated (S229P) human

Received: December 10, 2013
Accepted: February 8, 2014
Published: February 8, 2014

Table 1. Characteristics and Mass Spectrometer Parameters for the Tryptic Peptides and Their Internal Standards

analyte ID[a]	amino acid sequence[b]	peptide location	feature	function in LC-MS/MS	MRM ion transition, m/z[c]	dwell time, ms	declustering potential (DP), V	collision energy (CE), eV	entrance potential (EP), V	collision cell exit potential (CXP), V
ASGI	ASGIXXXXXXMHWVR	CDR, V_H	unique to the drug	quantitation	825.5 (2+) > 1073.5 (1+, y_9)	30	100	40	10	20
SIL-f-ASGI	ASGIXXXXXXMHW{V*}RQAR	NA[e]	NA[e]	IS	828.5 (2+) > 1079.5 (1+, y_9)	20	100	40	10	20
YGPP	YGPP[Cys_CAM]PP[Cys_CAM]PAPEFLGGPSVFLFPPKPK	C_H2	unique to the drug	confirmatory	985.3 (3+) > 912.1 (3+, y_{25})	30	50	38	10	5
SIL-f-YGPP	SKYGPP[Cys_CAM]PP[Cys_CAM]PAPEFLGGPSVF{L*}FPPKPK	NA[e]	NA[e]	IS	987.6 (3+) > 914.4 (3+, y_{25})	20	50	38	10	5
VVSV	VVSVLTVLHQDWLNGK	C_H2	common in human IgG1, IgG3, and IgG4	quantitation	603.5 (3+) > 805.6 (2+, y_{14})	30	100	27	10	20
SIL-f-VVSV	STYRVVSV{L*}TVLHQDWLNGKEY	NA[e]	NA[e]	IS	605.8 (3+) > 809.1 (2+, y_{14})	20	100	27	10	20
VNSA (622)	VNSAAFPAPIEK[d]	C_H2	unique to mouse NAb-PC	quantitation	622.4 (2+) > 486.5 (1+, y_4)	30	80	38	10	20
SVSE (618)	SVSELPIMHQDWLNGK[d]	C_H2	unique to mouse NAb-PC	confirmatory	618.8 (3+) > 159.1 (2+, y_3)	30	80	26	10	17

[a]Tryptic peptides were named according to their first four amino acid codes in the sequences. SIL-f: stable isotopically labeled with flanking amino acids. [b]L*: [$^{13}C_6$, ^{15}N]Leucine, V*: [$^{13}C_5$, ^{15}N]Valine; the peptide fractions with underlines were tryptic peptides and monitored in LC-MS/MS assay; "XXXXXX": place holder for six amino acids that are BMS proprietary and confidential information; CAM, carboxyamidomethyl cysteine generated by reducing disulfide bound with dithiothreitol and alkylating with iodoacetamide. [c]The values in the parenthesis represent ion charges and fragmentation assignments [d]The sequence was proposed from in-silico tryptic peptides from mouse IgG [e]NA, not applicable.

dx.doi.org/10.1021/ac5001465 | Anal. Chem. 2014, 86, 2673−2680

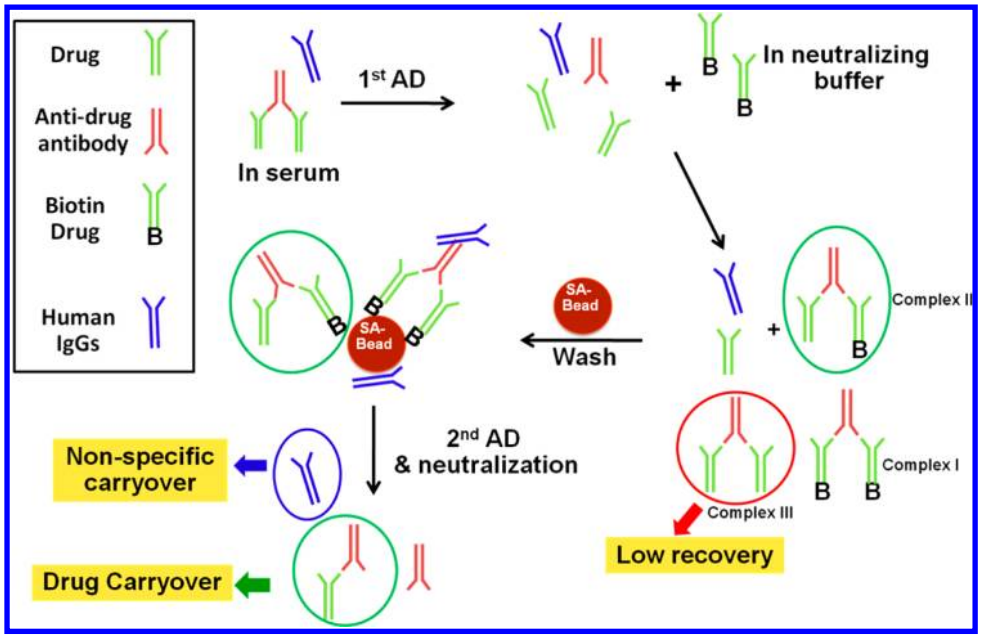


Figure 1. Mechanisms for residual drug and human IgG and low NAb recovery in the bead extraction and acid dissociation (BEAD). AD, acid dissociation.

IgG4 drug and endogenous human IgG4. LC-MS/MS is well-suited to these applications and has been demonstrated to be an orthogonal method to LBA in the quantitation of proteins in plasma or serum.[8−14] LC-MS/MS is selective, flexible, and capable of multiplexing to (a) distinguish the target peptides from other interfering peptides by combining the separating powers of chromatography and mass spectrometry, (b) adjust quantitation ranges by changing sample aliquot volumes, LC injection volumes, or MS parameters, and (c) simultaneously quantitate multiple target peptides derived from different proteins or a same protein using multiple reaction monitoring (MRM) mode. Here, we report a novel application of LC-MS/MS to assess key parameters (the levels of the residual drug and endogenous IgG and the NAb recovery) of the BEAD during method development of a cell-based NAb assay. The amounts of mAb drug, endogenous human IgG, and the NAb-PC in the BEAD eluates were simultaneously quantitated by a selective, sensitive, and accurate LC-MS/MS assay. The results provided useful information to evaluate the BEAD efficiency of removing serum components and extracting the NAb, which guides the optimization of the BEAD.

EXPERIMENTAL SECTION

Reagents and Chemicals. The drug (a reengineered mutated IgG4, molecular weight ∼150 kDa) and the NAb-PC (mouse monoclonal antibody against the drug, IgG1a/b) were obtained from Bristol-Myers Squibb (BMS) Research and Development. The internal standards (IS) were the synthetic peptides containing stable isotopically labeled (SIL) amino acids ([$^{13}C_6$,^{15}N]leucine or [$^{13}C_5$,^{15}N]valine) and flanking amino acids on the N-terminal and/or C-terminal, and these were designated as SIL-f-ASGI, SIL-f-YGPP, and SIL-f-VVSV (Table 1). Methanol (HPLC grade), acetonitrile (HPLC grade), isopropanol (HPLC grade), 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), phosphate-buffered saline (PBS) tablet, and Trizma base were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing grade trypsin (catalog no. V5111) was purchased from Promega (Madison, WI). Formic acid and glycine were obtained from EMD Biosciences (Gibbstown, NJ). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) cell culture medium were obtained from Gibco (Grand Island, NY). Biotin−drug was prepared in-house. Sea-Mag magnetic streptavidin-coated particles were purchased from Thermo (Indianapolis, IN). The blank BEAD matrix was prepared by mixing 100 mL of 300 mM glycine (pH 3.0), 22 mL of 400 mM HEPES (pH 8.2), and 13 mL of DMEM medium containing 10% FBS.

Bead Extraction and Acid Dissociation (BEAD). The BEAD procedure and the mechanisms for low NAb recovery and residual drug and endogenous IgG are illustrated in Figure 1. The experimental protocol was adapted from the literature[15] and further optimized in our laboratory. Human serum samples (100 μL) were treated with 100 μL of 400 mM glycine-HCl (pH 2.0) at room temperature for 1 h, followed by a 90 min treatment with 27.5 μL of a capture solution which contained 182 μg/mL of the biotin−drug in 1.8 M Trizma base (pH 8.8). After the incubation of the treated samples with 25 μL of streptavidin-coated magnetic beads (6 mg/mL) at room temperature for 1 h, the beads were washed three times with phosphate-buffered saline/Tween-20 (PBST) using a magnetic block to hold beads in the plate. Then, 90 μL of 300 mM glycine-HCl (pH 3.0) was added to each sample, and the samples were incubated at room temperature for 10 min. Eighty microliters of the supernatant was transferred to a new polypropylene plate containing 25 μL of neutralizing buffer (400 mM HEPES/DMEM medium with 10% FBS, 1:1.7, v/v). The final solutions were stored at −20 °C until LC-MS/MS analysis.

In the steps of the first acid dissociation and the biotin−drug replacing the unlabeled drug (Figure 1), three types of drug−NAb complexes can form due to the two binding arms of the NAb: one NAb with two biotin−drugs (Complex I), one NAb with one biotin−drug and one unlabeled drug (Complex II), and one NAb with two unlabeled drugs (Complex III). Both

dx.doi.org/10.1021/ac5001465 | Anal. Chem. 2014, 86, 2673−2680

Complex I and Complex II contain the biotin−drug and can be captured by streptavidin-coated beads allowing for their separation from the serum matrix upon washing. Using a second acid dissociation and neutralization, the NAb (from Complexes I and II) and the unlabeled drug (from Complex II) were both eluted from the beads. The unlabeled drug carried over by Complex II is called "residual drug". Complex III cannot be captured by the beads, which causes the loss of the NAb during the BEAD (i.e., low NAb recovery). Meanwhile, a certain amount of serum components such as human IgG may also be carried over due to nonspecific binding to the bead's surface or the NAb.

Selection of Quantitation Peptides and Confirmatory Peptides for the LC-MS/MS Assay. Three analytes (the drug, the NAb-PC, and human IgG) in this study are all IgG (~150 kDa). Tryptic peptides derived from the purified materials of the drug and the NAb-PC were screened by LC-MS/MS using an information-dependent acquisition (IDA) method (Q3MS survey scan coupled with MS/MS dependent scan). To select protein-specific peptides for LC-MS/MS analysis, the tryptic peptides with good LC-MS/MS sensitivity were further evaluated with the BEAD-treated blank matrix. Those peptide signals absent from the BEAD-treated blank matrix were identified as specific peptides for the proteins. These specific peptides were identified by interpreting in-silico tryptic peptides (PeptideMass, http://web.expasy.org/peptide_mass/) from each protein. The peptide uniquely present in the protein is called "unique peptides", and the one commonly present in a group of proteins (such as human IgG) is called "common peptides". The peptide chosen for quantitation of the intact protein is called "quantitation peptide", which is generally located in the complementarity-determining region (CDR). Another peptide from a different location of the same protein is called "confirmatory peptide", which is used to confirm the data accuracy of the "quantitation peptide" and the integrity of the protein primary structure in the samples. The data of the "quantitation peptide" were reported, and the data of the "confirmatory peptide" were used for evaluating the data agreement between two peptides. After selecting these peptides for the LC-MS/MS analysis, the MRM ion transition for each peptide was determined on the basis of the product ion spectra in which the most prominent product ion was selected. The MS parameters (gas flow-rate, temperature, electrospray voltage, declustering potential, collision energy, and collision cell exit-potential) were optimized by analyzing the tryptic peptides with varied values for each MS parameter.

Preparation of Calibration Standards, QCs, and Human Serum Samples. Calibration standards (50, 100, 200, 500, 1000, 2000, 5000, and 10 000 ng/mL) and QCs (50, 150, 850, 5000, 8000, and 100 000 ng/mL) were prepared from stock solutions of the drug (10 mg/mL) and the NAb-PC (5 mg/mL) by serial dilutions with the blank BEAD matrix. Identical nominal concentrations were assigned to the unique peptides ASGI (quantitation peptide) and YGPP (confirmatory peptide) for the drug, the unique peptides VNSA (quantitation peptide) and SVSE (confirmatory peptide) for the NAb-PC, and the common peptide VVSV (quantitation peptide) for the total of human IgG and the drug, because the peptide VVSV was derived from both the drug and endogenous human IgG (Table 1 and Figure 2). The concentration of the endogenous IgG was obtained by subtracting the measured drug concentration from the total concentration. To evaluate the BEAD efficiency of removing the drug and endogenous IgGs

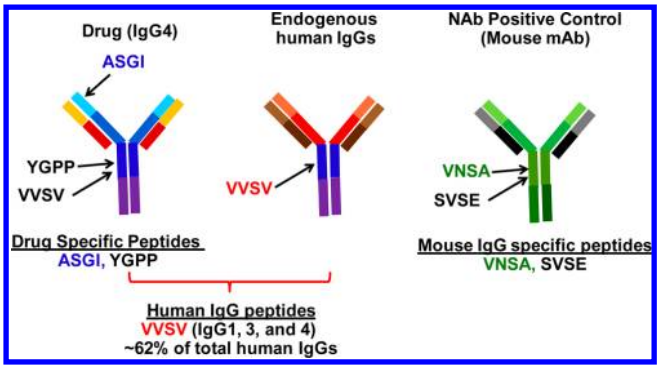


Figure 2. Quantitation peptides and confirmatory peptides for LC-MS/MS quantitation of the drug, human IgG, and NAb-PC.

and extracting the NAb, human serum samples were prepared with the NAb-PC in human serum at the concentrations of 0, 2, 5, 10, and 20 μg/mL (with or without spiking 100 μg/mL of the drug).

Trypsin Digestion of the BEAD Eluates. The blanks, calibration standards, QCs, and the BEAD eluates from the serum samples were processed according to our previously published LC-MS/MS method[14] with modifications. Aliquots of 25 μL of the samples were pipetted into the wells of a 96-well low-binding plate, followed by addition of 25 μL of SIL-f-IS solution (1 μg/mL in 50% methanol/water), except blank samples, to which 25 μL of 50% methanol/water was added. After addition of 10 μL of digestion buffer (1 M ammonium bicarbonate) and 10 μL of acetonitrile, the samples were reduced with 10 μL of 100 mM DTT at 60 °C for 30 min and alkylated with 10 μL of 250 mM IAA at 30 °C for 30 min. The digestion was initiated by adding 10 μL of 0.2 mg/mL trypsin (prepared in 0.1% formic acid/water solution) and incubating at 50 °C in a preheated thermomixer (1000 rpm) for 90 min. The digestion reaction was quenched by adding 25 μL of 10% formic acid in acetonitrile, and the samples were then filtered with a 96-well filter plate before LC-MS/MS analysis.

LC-MS/MS Assay. The protein digests (20 μL) were injected into a high-performance liquid chromatography system (model LC-30AD, Shimadzu Scientific Instruments, Inc., Columbia, MD) fitted with an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters Co., Milford, MA). The mobile phases of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) were delivered under a gradient program, consisting of 5% B to 42% B for 9 min. The flow-rate was 0.15 mL/min. The HPLC eluent was introduced into an AB Sciex 5500 triple-quadrupole mass spectrometer (Foster City, CA). The peptides were ionized in positive electrospray mode (curtain gas, 30 units; CAD gas, 8 units; gas 1, 40 units; gas 2, 50 units; ion spray voltage, +4000 V; temperature, 500 °C) and analyzed by the triple quadrupole analyzers in the MRM mode. The mass-dependent quadrupole parameters were listed in Table 1. The acquired chromatographic peaks were integrated using the Analyst software (version 1.5.1, AB SCIEX), and calibration curve regression and concentrations of the QCs and the BEAD eluates were calculated with Watson LIMS (version 7.3, Thermo Fisher Scientific, Inc.).

Cell-Based NAb Assay. The interleukin 2 (IL-2) releasing cell-based assay was applied to assess the amount of the NAb in human serum samples. Each BEAD eluate (40 μL) from human serum samples was incubated with 40 μL of the drug solution (500 ng/mL, i.e., the effective concentration of the dose−

dx.doi.org/10.1021/ac5001465 | Anal. Chem. 2014, 86, 2673−2680

response curve) in a NUNC edge plate for 20 min at room temperature. Freshly thawed 3A9 cells (5×10^4) were suspended in 40 μL of a complete DMEM medium containing 20 μM hen egg lysozyme (HEL) peptide and incubated at room temperature for another 20 min. Then, freshly thawed LK35.2 cells (2.5×10^4) suspended in 40 μL of complete DMEM medium containing 20 μM HEL peptide were added, mixed, and the whole plate was transferred to an incubator set at 37 °C, 5% CO_2, and 90% humidity for ~24 h. The supernatant (20 μL) of each well was collected after centrifugation for measuring the levels of IL-2 expressed by the cells using a mouse IL-2 alphaLISA kit (PerkinElmer, Waltham, MA). The luminescence signal was determined using an EnSpire plate reader (PerkinElmer, Waltham, MA).

RESULTS AND DISCUSSION

Optimization of the BEAD is a critical step in the method development of a cell-based NAb assay, because the BEAD removes interfering components from serum samples prior to the cell-based assay. In this study, three key parameters for the BEAD (the levels of the residual drug, the levels of endogenous IgG, and the NAb recovery) were assessed by LC-MS/MS to evaluate the BEAD efficiency of removing serum components and extracting the NAb.

LC-MS/MS for Quantification of the Drug, the NAb-PC, and Endogenous IgG. *Quantitation Peptides and Confirmatory Peptides.* After the peptide screening with LC-MS/MS, the quantitation peptides and the confirmatory peptides were selected for MRM quantitation. For the drug, a quantitation peptide ASGI located in the drug's CDR of the heavy chain variable region (V_H) and a confirmatory peptide YGPP in the drug's heavy chain constant region 2 (C_H2) were selected (Table 1). For the NAb-PC, five specific tryptic peptides were experimentally identified and named with their precursor ion's m/z (i.e., 622, 618, 551, 412, and 753), because the amino acid sequence of the NAb-PC (mouse IgG1a/b) was not known. Three tryptic peptides (622, 618, and 551) from the NAb-PC were identified on the basis of the information from in-silico tryptic digestion of mouse IgG constant region proteins. No tryptic peptides could be assigned to the peptides 412 and 753, suggesting that these two peptides might be derived from the variable regions of the NAb-PC. The quantitation peptide VNSA (622) and the confirmatory peptide SVSE (618) were selected for quantitation of the NAb-PC. The product ion of m/z 159.1 in the immonium ion range was used in the MRM transition for the peptide SVSE, because no other significant product ions provided good MS responses. However, this MRM transition still provided sufficient specificity and sensitivity based on the chromatographic results (compared with the results from blank samples) and the comparable concentration data to those from the quantitation peptide VNSA. For human IgG quantitation, the common peptide VVSV was selected to estimate human IgG concentration. This peptide was found in three of the four human IgG subclasses (IgG1, IgG3 and IgG4), representing approximately 68−81% of total human IgG found in human serum,[16] and has been used for quantitation of antibody drugs by LC-MS/MS.[12−14] No confirmatory peptide could be identified for the peptide VVSV. For internal standardization, SIL-f-ASGI was used as the internal standard for ASGI, VNSA, and SVSE. SIL-f-YGPP and SIL-f-VVSV were used as the internal standards for YGPP and VVSV, respectively.

Five of the eight peptides monitored in this study (Table 1) contain methionine (M) and tryptophan (W), both of which can be easily oxidized during storage and sample preparation. The oxidation of methionine to the sulfoxide form is of particular interest as it has been shown to occur in a wide variety of proteins and often reduces or eliminates biological activity. The stability of methionine residue in the drug was experimentally evaluated in our lab by monitoring the peptide ASGI (containing a methionine residue) and its oxidized form (containing a methionine sulfoxide residue) with an established LC-MS/MS method. The peptide ASGI is the best representative of all the derived peptides from the drug for evaluating the methionine oxidation, because it locates in the CDR of the mAb drug. In this experiment, serum samples were collected from two monkeys dosed with 50 mg/kg of the drug and stored for about 6 months. The serum samples were pretreated with and without reduction/alkylation reagents (dithiothreitol/iodoacetamide, DTT/IAA) prior to the trypsin digestion, because methionine sulfoxide can be reduced back to methionine by DTT. The oxidized form of the peptide ASGI was not detected in both the DTT/IAA-treated and the nontreated samples by LC-MS/MS, suggesting that the methionine residue in the mAb drug was stable during sample storage and preparation. Similarly, the oxidized form of the peptide ASGI was not detected in the BEAD-treated samples.

LC-MS/MS Assay Performance. The accuracy (% deviation from the nominal concentrations) and precision (% CV) for the QCs in five analytical runs were calculated using one-way analysis of variation (ANOVA) in Watson LIMS. The maximal inter- and intra-assay % CV values at different concentrations of QCs were ≤ 9.7 and 13.6, respectively (Table 2). The % deviation was within ± 8.6. The results of the confirmatory peptides YGPP and SVSE had good agreement with their corresponding unique peptides ASGI and VNSA (Figure 3), suggesting accurate quantitation with the LC-MS/MS assay and good integrity of the protein primary structures during sample storage and treatments. The low limit of quantitation (LLOQ) of 50 ng/mL for all quantitated peptides was determined, because a sufficient chromatographic response (signal-to-noise ratio >5) was observed in the LLOQ sample, and no interference peak was shown at the corresponding retention time as shown in the blank samples (Figure 4). To demonstrate assay specificity, the drug, the NAb-PC, or human IgG was spiked into the blank BEAD matrix and analyzed by the established LC-MS/MS method. The results showed no detectable signals in the other MRM channels, indicating that the analyte detection channels were specific for these target peptides and the lack of cross-interference (Table 3).

Table 2. Accuracy and Precision of the LC-MS/MS Assay

peptide ID	LLOQ (ng/mL)[a]	% dev[b]	inter-run (% CV)[b]	intra-run (% CV)[b]
ASGI	50	≤ ± 2.6	≤2.6	≤13.6
YGPP	50	≤ ± 3.6	≤8.0	≤6.6
VVSV	50	≤ ± 8.6	≤9.7	≤7.7
VNSA	50	≤ ± 4.2	≤3.9	≤6.2
SVSE	50	≤ ± 6.6	≤8.3	≤10.5

[a]The standard curve range was 50−10 000 ng/mL. The concentrations represent the intact proteins in the BEAD eluates. [b]The statistical data were obtained from six levels of QCs at 50, 150, 850, 5000, 8000, and 100 000 ng/mL.

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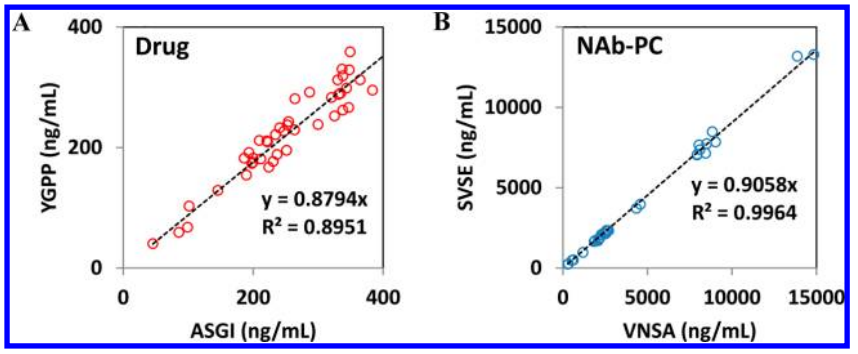

Figure 3. Comparison of the concentration data generated from the quantitation peptide and its confirmatory peptide. The linear regression equations and the plots describe the good correlation (slopes of 0.8794 for the drug and 0.9058 for the NAb-PC) between the concentrations of the confirmatory peptide and the quantitation peptide (YGPP vs ASGI for the drug and SVSE vs VNSA for the NAb-PC).

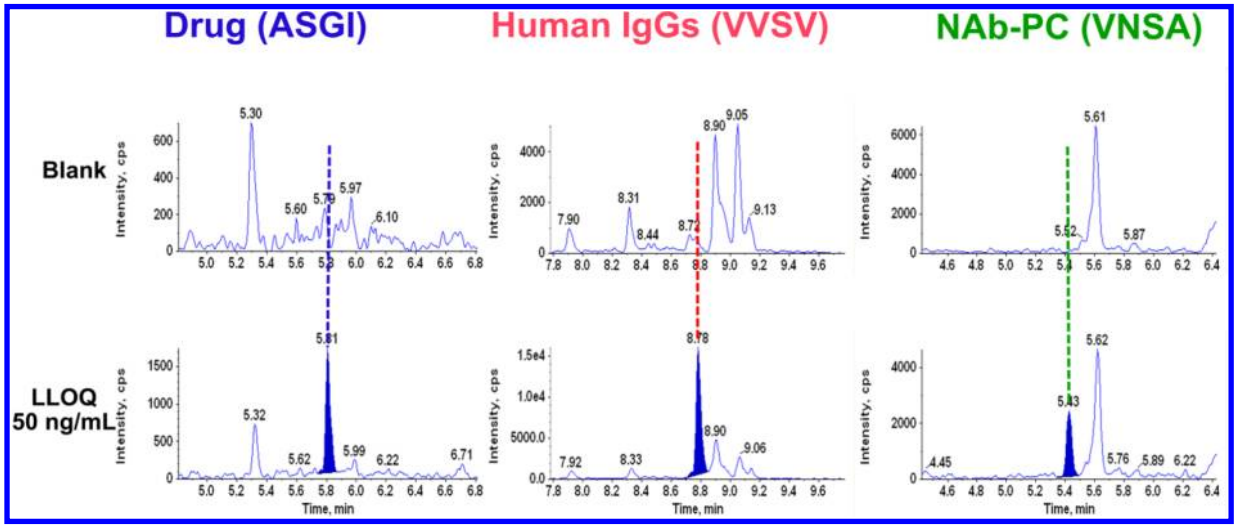

Figure 4. Representative chromatograms of the quantitation peptides at the low limit of quantitation (LLOQ) of 50 ng/mL and their blanks.

Table 3. LC-MS/MS Assay Specificity for the Drug, NAb-PC, and Human IgG

	concn (μg/mL)	NAb-PC concn[b] (μg/mL)	drug concn[b] (μg/mL)	human IgG concn[b] (μg/mL)
drug	1	N.D.[a]	1	N.D.[a]
	20	N.D.[a]	21	N.D.[a]
NAb-PC	1	1	N.D.[a]	N.D.[a]
	20	21	N.D.[a]	N.D.[a]
human IgG	1	N.D.[a]	N.D.[a]	0.62[c]

[a]N.D., not detectable. [b]Mean concentration from three replicates. [c]The value represents the concentration of human IgG containing the quantitation peptide VVSV, because the peptide VVSV is only contained in human IgG1, IgG3, and IgG4. The reference material of the intravenous immunoglobulin (IVIG), an extracted IgG product from human plasma, was spiked into the blank BEAD matrix at a concentration of 1 μg/mL. The result suggests that ~62% of the total human IgG contain the peptide VVSV.

Evaluation of the BEAD Efficiency of Removing Serum Components and Extracting the NAb. The concentrations of the residual drug and human IgG in the BEAD eluates are the parameters to evaluate the BEAD efficiency of removing the drug and endogenous serum components. The concentration of the NAb-PC in the eluates determines the NAb-PC recovery from the BEAD. These parameters guided selecting experimental conditions for the BEAD during the NAb assay development. In this study, elution buffer glycine-HCl (pH 2.0 vs pH 3.0), streptavidin beads (nonblocked vs preblocked with 1% bovine serum albumin) and biotin–drugs (in-house made vs vendor made) were evaluated during the method development. Those conditions, which provided lower concentrations of the residual drug and endogenous IgG (low carryover) and higher concentration of the NAb-PC (high recovery), were applied in the cell-based assay validation. The results suggested selecting the conditions of glycine-HCl (pH 3.0), preblocked beads, and in-house made biotin–drug in the validation of the cell-based assay. Here, we report representative results of the residual drug, residual IgG, and the NAb-PC, in the BEAD eluates from the final optimized BEAD method to elucidate their relationships with the drug and the NAb-PC in the serum samples. These findings provided good evidence to help understand the BEAD-removing serum-interfering components and the extraction of the drug-associated ADA, which assisted in optimization of the BEAD method. The benefit of the BEAD to the cell-based assay is also discussed.

Residual Drug and Human IgG in the BEAD Eluates. The human serum samples were treated with the BEAD and analyzed by LC-MS/MS. There was measurable residual drug (~0.1 μg/mL) even when the NAb-PC and the drug were not present in the serum samples (the empty circle at 0 μg/mL of NAb-PC in Figure 5A). It was suspected that the low but measurable residual drug was from the biotin–drug during the BEAD treatment, eluted as either a fragment of the biotin–drug

dx.doi.org/10.1021/ac5001465 | Anal. Chem. 2014, 86, 2673–2680

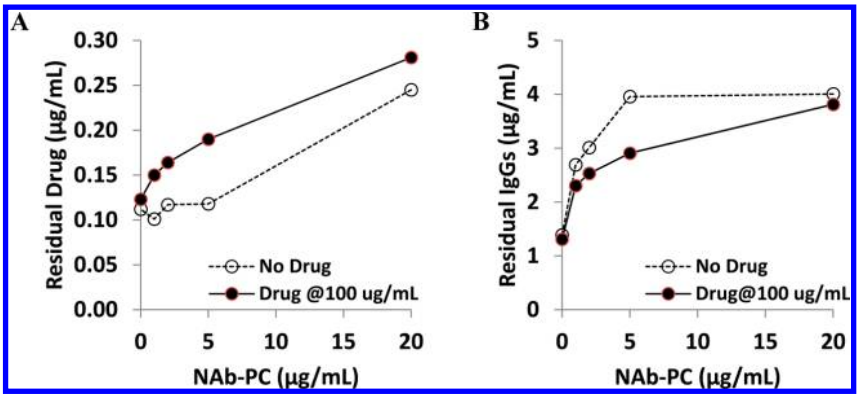

Figure 5. Effect of NAb-PC on the levels of residual drug (A) and human IgG (B) in the BEAD treatment. Human serum samples spiked with the NAb-PC at 0, 2, 5, 10, and 20 μg/mL with (empty circles) or without (solid circles) the drug (100 μg/mL of the drug) were treated with the BEAD, and the eluates were analyzed by LC-MS/MS. The levels of the residual drug and endogenous IgG were both slightly increased with the increases of the NAb-PC concentrations, suggesting that the NAb-PC increased the carryovers of the drug and endogenous IgG maybe due to protein−protein interactions. With addition of 100 μg/mL of the drug in the serum samples, the residual drug levels were slightly increased (p = 0.01), but the endogenous IgG levels were decreased (p = 0.06), the latter of which might be due to the competition of protein bindings between drug/NAb-PC and IgG/NAb-PC. Measurable drug (~0.1 μg/mL) in the zero samples (without the drug and/or NAb-PC) indicated that the background level of the residual drug came from the biotin−drug reagent in the BEAD treatment. Measurable endogenous IgG (~1.2 μg/mL) in the zero samples indicated IgG nonspecific binding on the beads.

or the unlabeled drug from the biotin−drug reagent. This observation was confirmed by a negative control experiment, in which the BEAD was conducted without addition of the biotin−drug and no residual drug was detected. The residual drug concentrations in the BEAD eluates increased with the increases of the NAb-PC concentrations but in a narrow range of 0.1−0.3 μg/mL. Addition of 100 μg/mL of the drug in the test samples led to only 0.03−0.06 μg/mL of the additional drug residue. This result suggested that 100 μg/mL of the drug in the serum samples slightly increase (p = 0.01, Student's t-test) the residual drug level in the BEAD eluates, because the BEAD efficiently removed the high concentration drug from the serum samples. In other words, the residual drug in the BEAD eluates mainly came from the biotin−drug reagent during the BEAD treatment.

Residual endogenous IgG was investigated in the same manner. The level of the residual IgG slightly increased (p = 0.06, Student's t-test) with the increases of the NAb-PC concentration, indicating the interaction between the NAb-PC and human IgG during the BEAD treatment. The prespiked drug (100 μg/mL, solid circles at 1, 2, and 5 μg/mL of the NAb-PC in Figure 5B) appeared to compete with human IgG binding to the NAb-PC, and thus decreased the IgG residue. When the NAb-PC concentration reached 20 μg/mL, such competition became less. Similar to the observation of the residual drug, there was ~1.4 μg/mL of the residual IgG (corresponding to ~0.01% of total serum IgG) when the NAb-PC was not present, suggesting that some IgG were nonspecifically bound to the beads. The low level of the residual IgG (<4 μg/mL, or <0.05% of total human IgG in serum) in the BEAD eluates indicated that the BEAD efficiently removed endogenous IgG from the serum matrix that could affect the cell-based assay.

NAb Recovery of the BEAD. The NAb-PC (a mouse IgG1a/b against the drug) was used as a substitute for human NAb to evaluate the NAb recovery in this study. The NAb-PC concentration in each BEAD eluates from the human serum samples spiked with the NAb-PC was quantitated with the LC-MS/MS assay. The ratio of the NAb-PC concentration in the BEAD-treated sample to that in the nontreated sample was used to represent the NAb-PC recovery. In Figure 6, the results showed good linear relationship between the NAb-PC concentrations in the BEAD-treated samples and those in the nontreated samples (the nominal concentrations). The slope of the linear curve represents the average ~42% recovery of the NAb-PC within the range of 0−20 μg/mL.

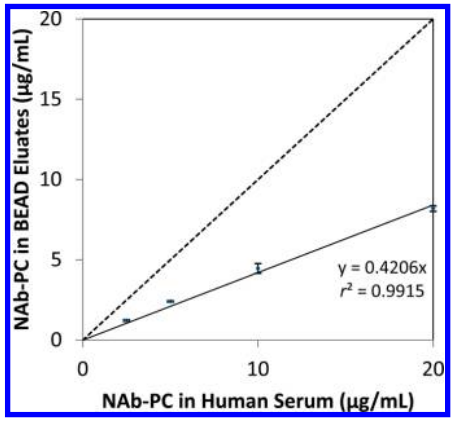

Figure 6. Relationship between the NAb-PC concentrations in the BEAD-treated samples and those in the nontreated samples (r^2, 0.9915). The slope of the linear curve represents the average ~42% recovery of NAb-PC in the BEAD treatment. The dotted line corresponds to theoretical 100% recovery. Samples were analyzed in triplicate at each concentration (% CV ≤ 12.7% for all concentration levels, the error bars represent standard deviations of three replicates).

Benefits of the BEAD to the Cell-Based NAb Assay. The purpose of applying the BEAD to serum samples prior to the cell-based assay is to remove the drug and serum components and extract the drug-associated ADA. The BEAD can efficiently remove the high concentration of the drug from patients' serum samples and significantly improve the drug tolerance for the NAb assay. In this study, the drug tolerance was 800 μg/mL at 20 μg/mL of the NAb-PC. In addition, the drug concentrations in patients' serum samples could be very different among patients or the samples from the same patient. The variable concentrations of the drug can generate different

dx.doi.org/10.1021/ac5001465 | Anal. Chem. 2014, 86, 2673−2680

responses in the cell-based assay and thus give different readouts of the NAb. Therefore, the residual drug concentrations in the BEAD eluates need to be accurately measured especially when the effective drug concentration in the cell-based assay is low or close to the concentrations of the residual drug. On the other hand, endogenous serum components could significantly suppress or enhance the detection signals (called serum matrix effects) in the cell-based assay. We observed significant serum matrix effects on the cell-based assay from 5, 15, and 30 serum matrixes which reduced the detection signals by 65, 92, and 92%, respectively. By applying the BEAD, ∼99% of endogenous serum components (based on the residual IgG level) were removed from the serum samples, and ∼42% of the NAb-PC was extracted. Furthermore, the impact of the residual drug and endogenous IgG was evaluated with the cell-based NAb assay. The cell-based assay was conducted in target-overexpressing 3A9 and LK35.2 cells in the presence of HEL peptide with the drug inducing a robust up-regulated expression of IL-2. In the treatment of the cells with the BEAD eluates, the extracted NAb-PC blocked the drug function and thus IL-2 production. The BEAD-treated samples (prespiked with 100 μg/mL of the drug in human serum) generated a comparable NAb curve to the NAb curves from the control samples without prespiked drug or serum matrix. This indicated that the low levels of the residual drug and endogenous IgG in the BEAD eluates had no significant effect on the cell-based assay, and the BEAD removed the drug interference and serum matrix effect.

■ CONCLUSIONS

We have developed a specific, sensitive, and accurate LC-MS/MS assay for simultaneous quantitation of the residual mAb drug and endogenous IgG and the NAb-PC in the BEAD eluates. The LC-MS/MS results provided useful information for the cell-based NAb assay development in regards to the efficiency of the BEAD at removing the drug and endogenous serum components from serum samples, the recovery of the NAb from the BEAD, the type of beads for a better recovery and limiting nonspecific carryover, and the reagents giving the best drug clearance and NAb recovery.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: hao.jiang@bms.com. Fax: 609-252-3845.
*E-mail: jianing.zeng@bms.com.

Author Contributions

[§]These authors contributed equally to the work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Dr. Qin C. Ji for review and comments.

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