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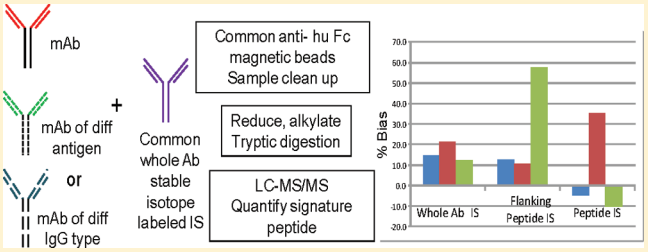
General LC-MS/MS Method Approach to Quantify Therapeutic Monoclonal Antibodies Using a Common Whole Antibody Internal Standard with Application to Preclinical Studies

Hongyan Li,[†] Robert Ortiz,[†] Linh Tran,[†] Michael Hall,[†] Chris Spahr,[‡] Ken Walker,[‡] John Laudemann,[‡] Sterling Miller,[‡] Hossein Salimi-Moosavi,[†] and Jean W. Lee*,[†]

[†]PKDM and [‡]Protein Sciences, Amgen Inc., Thousand Oaks, California 91320, United States

Supporting Information

ABSTRACT: Ligand binding assays (LBAs) are widely used for therapeutic monoclonal antibody (mAb) quantification in biological samples. Major limitations are long method development times, reagent procurement, and matrix effects. LC-MS/MS methods using signature peptides are emerging as an alternative approach, which typically use a stable isotope labeled signature peptide as the internal standard (IS). However, a new IS has to be generated for every candidate, and the IS may not correct for variations at all processing steps. We have developed a general LC-MS/MS method approach employing a uniformly heavy-isotope labeled common whole mAb IS and a common immunocapture for sample processing. The method was streamlined with automation for consistency and throughput. Method qualification of four IgG_2 and four IgG_1 mAbs showed sensitivity of 0.1 μg/mL and linearity of 0.1−15 μg/mL. Quality control (QC) data of these eight mAbs were accurate and precise. The QC performance of the whole molecule labeled IS was better than those of synthetic labeled IS peptides tested. The pharmacokinetic results of two mAbs (an IgG_2 and IgG_1 candidate) dosed in rats were comparable to those of LBA. The general LC-MS/MS method approach overcomes the limitations of current methods to reduce time and resources required for preclinical studies.



Monoclonal antibodies (mAb) have become commonplace biotherapeutics. More than 20 mAbs have been approved as drugs by the FDA, and nearly 300 mAbs are currently under development.[1] During early mAb drug development, the pharmacokinetic (PK) characteristics of multiple candidates are among the critical considerations for lead candidate selection. Typically, ligand binding assay (LBA), such as enzyme linked immunosorbent assay (ELISA), is used to measure the PK of a mAb candidate.[2] However, LBAs may have significant limitations, including procurement or generation of the binding reagents, interference from matrix components, and long development times.

Recently, tandem liquid chromatographic mass spectrometric (LC-MS/MS) methods have been applied to mAbs as an alternative to LBA for the bioanalysis of preclinical samples.[3] An enzymatic digestion (usually trypsin) of the mAb to peptides is required to enable straightforward quantification in the molecular range of conventional mass spectrometers such as triple quadrupole instruments. Generally, a peptide with a unique sequence (signature peptide) is chosen for quantification, representing the whole mAb (surrogate peptide). In some cases, multiple signature peptides are chosen for flexibility and added method robustness. Multidimensional chromatography can be used to separate the mAb signature peptide from those of the endogenous matrix; however, this often leads to long chromatographic times and frequent instrument maintenance. As an alternative, many of the published methods use an affinity step such as immunocapture, either inline or offline, which provides sample clean up as well as concentration of the sample to enhance sensitivity.[3a−c,e,f]

A synthetic stable isotope labeled internal standard (SIL-IS) of the signature peptide is often used to correct for variability in chromatography and MS ionization. Additional amino acid residues flanking the signature peptide or concatemers are also reported as being used to correct for variability of enzymatic digestion.[4] If the SIL peptide is added before digestion, it will also correct for any degradation (e.g., deamidation) that the surrogate peptide may undergo. However, the digestion efficiency of intact mAbs may differ substantially from those of concatemer peptides; thus, the peptide IS may not always reliably correct for variable digestion of the mAb. The ideal IS would be the whole mAb uniformly labeled with a stable isotopic amino acid, as illustrated by Heudi et al.[3e] However, substantial time and resources are required to generate a whole molecule SIL-IS for each new candidate. Therefore, we have developed a novel, general LC-MS/MS method approach using a common whole molecule SIL-IS and a common immuno-

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Table 1. Potential Surrogate Peptides of IgG_1 and IgG_2 mAbs Considered for Quantification[a]

peptide sequence	abbreviation	location	IgG_2	IgG_1	MS/MS transitions
SGTASVVC[CAM]LLNNFYPR	SGT	lc	Y	Y	899.5→272.2
SGTASVVC[CAM]L*L*NNFYPR	*SGT				905.5→272.2
NQVSLTC[CAM]LVK	NQV	HC	Y	Y	581.4→820.5
NQVSL*TC[CAM]L*VK	*NQV				587.3→832.5
GPSVFPLAPC[CAM]SR	GPS	HC	Y	N	644.4→800.5
GPSVFPL*APC[CAM]SR	*GPS				647.4→806.5
GLPAPIEK	GLP	HC	Y	N	412.8→654.4
GL*PAPIEK	*GLP				415.8→654.4

[a]Y: common peptide for the mAb group. N: Not common peptide. C[CAM]: Carboxyamidomethyl cysteine. For the *m/z* transitions of SIL-αDA-G2 IS peptides, a mass shift of +6 was used for each L*. For the NQVSL*TC[CAM]L*VK from the two synthetic SIL-peptides, a mass shift of +7 was used for each L* (not shown in the table).

capture for sample clean up and enrichment that is applicable to various mAbs in different matrixes.

For the common IS, we chose a mAb that is unlikely to exist in the preclinical species but possesses common peptide sequences with other mAbs. The human antidinitrophenol (DNP) IgG_2 mAb is not likely to be found in most test systems.[5] The whole molecule SIL-IS of clone anti-DNP-3A4-F-G2 (αDA-G2) was produced in cell culture, purified, and characterized. For sample clean up and enrichment, we used a common immunocapture of antihuman crystallizable fragment (anti-Fc) that recognizes human mAb biotherapeutics but not the endogenous immunoglobulins in the preclinical sample. We developed a general method approach and streamlined the process with automation for consistency and sample turn-around time within 1.5 days. The feasibility was tested, and the method was qualified with 4 mAbs of the same IgG_2 isotype as the SIL-IS: αDA-G2, antikeyhole limpet hemocyanin (KLH)-120.6-G2 (αK-G2), 827-435-G2 (827-G2), and anti-DNP-3B1-G2 (αDB-G2). In addition, the test was extended to 4 more mAbs of a different isotype IgG_1: anti-DNP-3A4-F-G1 (αDA-G1), anti-KLH-120.6-G1 (αK-G1), 655-341-G1 (655-G1), and anti-DNP-3B1-G1 (αDB-G1). We did not test an IgG_4 compound since this a minor subclass among the mAbs being developed. To show method application, one method was applied to the quantification of samples from rats dosed with αDA-G1 and αK-G2, and the results were compared to those analyzed by ELISA.

EXPERIMENTAL SECTION

Chemicals and Reagents. αDA-G2 was prepared and purified according to Doellgast et al.[5] The whole molecule SIL-IS of αDA-G2 was produced by incorporating [$^{13}C_6$,^{15}N]-leucine as an essential amino acid in cell culture. Purity was assessed by gel electrophoresis and peptide mapping. The 8 mAbs (αDA-G2, αK-G2, 827-G2, αDB-G2, αDA-G1, αK-G1, 655-G1, and αDB-G1) and biotinylated antibody against human Fc mAb clone 35 (b-Ab35) were prepared and purified at Amgen Inc. (Thousand Oaks, CA). Synthetic IS peptides with stable isotopic labeled leucine (L*), NQVSL*TCL*VK and REEMTKNQVSL*TCL*VKGFYPSD (6 flanking amino acids), were purchased from Midwest Bio-Tech, Inc. (Fishers, IN). Streptavidin magnetic beads (1 μm) and Dulbecco's phosphate buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). Acetonitrile (ACN), methanol (MeOH), and water (HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid (reagent grade) was from Aldrich, Inc. (St. Louis, MO). Control rat plasma and cynomolgus monkey serum were supplied by Bioreclamation Inc. (East Meadow, NY). Bovine serum albumin (BSA), iodoacetamide, and Tween 20 were purchased from Sigma (Saint Louis, MO). DTT was purchased from Thermo Scientific (Rockford, IL). HBS-EP buffer was obtained from GE Healthcare (Piscataway, NJ).

Preparation of Calibration Standards (STDs), Quality Control (QC) Samples, and IS Solutions. The STD concentrations of each mAb compound in rat plasma or cynomolgus monkey serum were 0.10, 1.5, 3.0, 9.0, and 15 μg/mL. The QC concentrations were 0.3, 5.0, and 10.0 μg/mL. The IS working solution was 36 μg/mL prepared in control blank matrixes for the whole molecule SIL-αDA-G2 and 1 and 2 μg/mL in 50% MeOH and 0.1% formic acid in water for the flanking and peptide SIL-IS, respectively.

Instrumentation. The quantitative analysis was performed on an ultra performance liquid chromatography (UPLC)-MS/MS system which consisted of an Acquity UPLC system (Waters, Milford, MA) coupled to an API 4000 triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) with a Turbo IonSpray ionization source. The analytical column was an Acquity UPLC BEH Shield RP C18 2.1 mm × 50 mm column with 1.7 μm particle size. A 0.2 μm precolumn filter unit was used to protect the analytical column. The mobile phases were 0.1% formic acid in ACN/water (5/95, v/v, mobile phase A) and 0.1% formic acid in ACN/water (95/5, v/v, mobile phase B). The LC gradient profile was as follows (min/% of mobile phase B): 0.0/2, 0.5/2, 4.0/40, 4.1/95, 4.6/95, 4.7/2, and 5.0/2. Total runtime was 5 min. The flow rate was 0.60 mL/min, and the column temperature was 50 °C. The autosampler temperature was set at 10 °C. Data was collected and processed using AB Sciex Analyst software (version 1.4.1). The ESI spray voltage was set at 5000 V. The source temperature was 500 °C. The curtain gas (CUR) was 30; nebulizer gas setting (GS1) was 40, and the auxiliary gas setting (GS2) was 50 (all arbitrary units). The ion transitions for MRM quantification determined from a tryptic digest are listed in Table 1. The synthetic peptide SIL-IS NQVSL*TC[CAM]-L*VK ion transition was 588.3→834.5.

Biological Sample Immunocapture and Digestion. b-Ab35 (3.3 mL; 1.2 mg/mL in 50% glycerol in PBS) was added to 100 mg of streptavidin beads in 6.7 mL of 0.1% Tween 20 in PBS and incubated overnight at 4 °C. The b-Ab35-coated magnetic beads were then washed with 0.01% Tween 20 in PBS and resuspended in HBS-EP buffer containing 1% BSA (1 mg beads/100 μL). The sample (200 μL), 20 μL of αDA-G2 SIL-IS, and 50 μL of b-Ab35-coated magnetic beads were introduced into a 96-well plate and incubated for 1 h at ambient temperature. During the incubation, the plate was

placed on a Tecan Evo 200 (Tecan, Switzerland) with shaking. The bead mixture was pipetted up and down three times every min to keep the beads suspended. The beads were washed three times with 0.01% Tween 20 in PBS and two times with PBS using a magnetic autowasher (Biotek, Winooski, VT) and then eluted with 200 μL of 50% MeOH and 3% formic acid in water. The eluate in the supernatant was transferred to a 96-well plate and dried with a HT-4X evaporator (Genevac, NY). For samples using the SIL-IS peptides, 50 μL of the IS was added before drying. The sample was reconstituted and incubated with 10 μL of 20 mM DTT in denaturing buffer (8 M urea, 250 mM Tris, pH 7.5) for 1 h at 37 °C with shaking to denature and reduce the disulfide bonds of the mAb analyte. Two microliters of 360 mM iodoacetamide in denaturing buffer were added and incubated on a shaker for 30 min at ambient temperature. The sample was diluted with 43 μL of 250 mM Tris buffer before adding 5 μL of 0.04 μg/mL trypsin and digested for 1 h at 55 °C. The reaction was stopped with 5 μL of 2% formic acid, and the plate was sealed. Ten microliters of the processed sample was injected for LC-MS/MS analysis.

Quantitative Analysis of QCs and Preclinical Samples. The calibration curve was established from the peak area ratios (analyte/IS) using 1/Concentration^2 weighted linear least-squares regression. Plasma samples were collected from Sprague−Dawley rats after dosing subcutaneously with αDA-G1 or αK-G2 at 5 mg/kg according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Amgen Inc. The samples were frozen and stored at −70 °C until analysis. The same set of PK study samples were analyzed by LC-MS/MS and ELISA (ELISA method in Supporting Information).

RESULTS AND DISCUSSION

Method Development. *Sample Clean Up for Sensitivity.* A major concern for an LC-MS/MS method to quantify mAb is insufficient sensitivity resulting from background noise and signal suppression due to highly abundant endogenous proteins such as albumin and immunoglobulins.[6] In order to decrease this chemical noise, three approaches of sample clean up have been reported: (1) depletion, such as the use of commercial albumin depletion kit, (2) general affinity capture, such as the use of solid phase Protein A, or (3) specific immunocapture using antibodies toward the mAb of interest.[3c,e,f,7] The albumin depletion method would be costly and not conducive to automation and therefore was not selected. Instead of Protein A mentioned in the second approach, we chose the general anti-Fc capture because the antibody clone 35 is highly specific to human IgGs, with discrimination from rodent and nonhuman primate IgGs. Anti-Fc Ab35 has been previously used in a solid phase platform to study the biotransformation of peptide-Fc fusion proteins in preclinical studies with MALDI-TOF MS.[8] In the current approach, streptavidin magnetic beads coated with b-Ab35 were used for increased capacity and automation compatibility. Additionally, b-Ab35 provided flexibility to assess bead capacity through easy adjustment of the ratio of b-Ab35 to the amount of streptavidin beads. The titration results showed that 20 μg of b-Ab35 per 200 μL of plasma sample was sufficient for 1 h immunocapture in the kinetic mode (Table S-1 and Figure S-1 in Supporting Information).

Considerations of the Common Internal Standard. The greatest assay accuracy and precision would result from the addition of an IS at the beginning of the sample processing to correct for variations in processing (immunocapture and digestion), extraction recovery, and ionization in the MS.[3e,f,9] The ideal IS for a mAb would be the same mAb that is uniformly incorporated with SIL amino acid residues of frequent occurrence.[3e,f] During early discovery, finding a common IS for the quantification of various mAbs could be highly beneficial as the generation of a large number of individual IS would be impractical. The following characteristics were considered for the common SIL-mAb IS: (1) the mAb should have common sequences in the constant regions of the heavy and light chains (HC and lc) with the candidate mAbs, (2) the whole molecule SIL-IS can be produced and purified from the cell culture of the mAb by incorporation of an essential SIL-amino acid, and (3) there is minimal contribution of the SIL-IS to the unlabeled analyte signal in the mass spectrometer (i.e., high incorporation of the SIL-amino acid).

One of the anti-DNP mAb clones, αDA-G2, has the potential to satisfy the above conditions as a common IS. L* labeled αDA-G2 was produced in cell culture, purified, and characterized. The peptide map of the SIL-IS showed uniform labeling on the whole molecule of αDA-G2 with a consistent mass shift of +6 and not the expected +7 from [$^{13}C_6$,^{15}N]-leucine. This phenomenon was repeatable while the mechanism is unknown. We hypothesize that the ^{15}N may be removed by the action of Leu transaminase with low probability of reamination of the ^{15}N label.[10] Since the loss of one amu was complete and consistent during production, the tracked masses of the IS peptides were invariable and did not impact the suitability of this IS.

Identification and Optimization of Surrogate Peptides for Therapeutic Human mAbs Quantification. Our strategy of developing a general LC-MS/MS method approach was first to test quantification of 4 mAbs of the same IgG_2 subclass and then extend to 4 mAbs of another isotype, i.e., IgG_1. Surrogate peptides were identified by in silico tryptic digestion analysis, and among these, 5 common sequences for both IgG_2 and IgG_1 were identified that could be potentially used as surrogate peptides for quantification. These 5 peptides (abbreviations in parentheses) were from both κ-light and IgG_1 and IgG_2 heavy chains. From the light chain (lc), there were the following: SGTASVVCLLNNFYPR (SGT), DSTYSLSSTLTLSK, and TVAAPSVFIFPPSDEQLK; from the heavy chain (HC), there were DTLMISR and NQVSLTCLVK (NQV). Two additional HC peptides were identified as common peptides for the IgG_2 group: GPSVFPLAPCSR (GPS) and GLPAPIEK (GLP). However, DTLMISR was excluded due to the potential variable oxidation of methionine. DSTYSLSSTLTLSK and STSESTAALGCLVK were not chosen due to weak ion signals, and TVAAPSVFIFPPSDEQLK was not chosen due to poor chromatographic peak shape. The MS/MS transitions of the four remaining potential signature peptides are listed in Table 1.

These 8 mAbs were individually digested as described in the Supporting Information. The digests were infused into the ionization source of the mass spectrometer. The m/z for doubly charged peptide ions were calculated for 4 potential surrogate peptides (Table 1) and utilized as the precursor ions for MS/MS experiments for peptide identification. For SIL-αDA-G2, identical tryptic peptides were expected except that each Leu was heavy-labeled which provided a 6 Da shift in mass. Likewise, the same MRM parameters optimized for NQVSLTC[CAM]LVK were applied to the two synthetic

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peptide ISs in which isotope labeled [$^{13}C_6$,^{15}N]-leucine provided a 7 Da shift in mass instead.

SGTASVVC[CAM]LLNNFYPR and NQVSLTC[CAM]-LVK were identified definitively as shown in Figure 1. The most abundant product ions were m/z 272.3 (y_2^+) and m/z 820.4 (y_7^+) from peptides SGT and NQV, respectively.

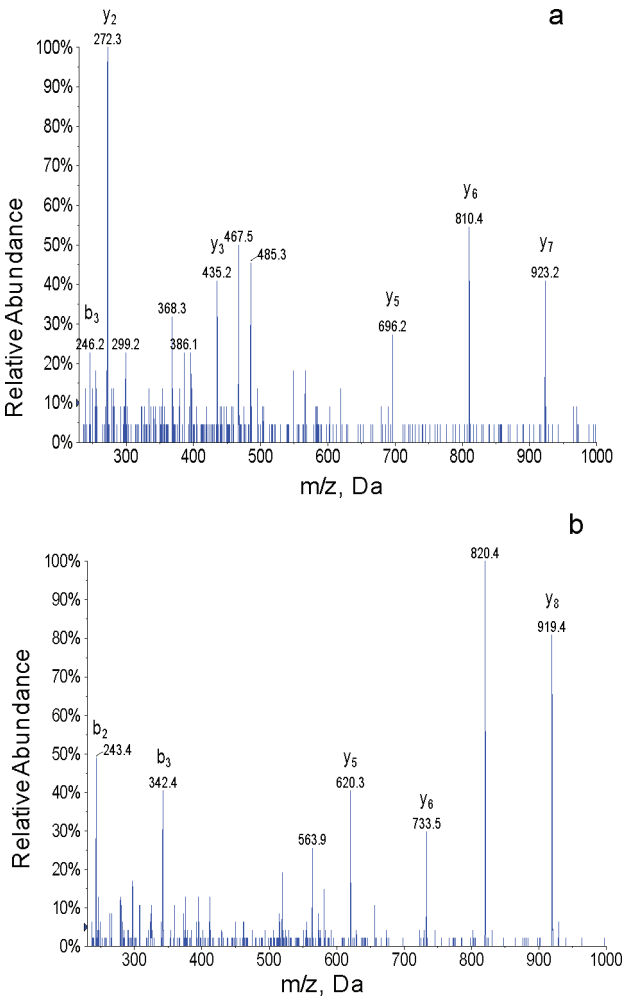

Figure 1. MS/MS spectra of the quantitative surrogate peptides. (a) SGTASVVC[CAM]LLNNFYPR. (b) NQVSLTC[CAM]LVK.

Tryptic Digestion and Liquid Chromatography Optimization. High throughput capability for this method requires a short tryptic digestion time and fast chromatographic separation. Surrogate peptide ion intensity was optimized with respect to digestion time and temperature. For αDA-G2, digestion at 55 °C for 1 or 2 h provided comparable output to that of 37 °C for 4 h. Attempts to use microwave digestion for even shorter digestion time were not successful (Table S-3 in Supporting Information). Efficient digestion of mAbs requires denaturation by chaotropes (e.g., guanidine-HCl or urea). However, these agents may also inhibit trypsin activity. In this method, a simple dilution step that decreased urea to ≤2 M with Tris buffer combined with incubation at 55 °C led to efficient trypsin digestion in 1 h. The short digestion time also minimized potential urea-related carbamylation. Cysteine-containing tryptic peptides can be problematic as quantitative peptides; however, consistent and reproducible results were obtained for both SGT and NQV after cystine reduction with DTT and alkylation with iodoacetamide, indicating the feasibility of the use of surrogate tryptic peptides with cysteine residues.

In order to further decrease the number of steps in the analytical process, no desalting step was performed after the digestion. The use of a divert valve allowed crude digests to be analyzed directly by LC-MS/MS without the need for desalting. UPLC was chosen as the chromatographic platform as it provides highly efficient chromatographic separations combined with a fast run time and increased sensitivity. A BEH Shield RP C18 column (2.1 mm × 50 mm) was found to provide excellent assay sensitivity and reproducibility for the two surrogate peptides, which were well retained and eluted as sharp and symmetric peaks within a 5 min run time.

Method Qualification. *Sensitivity.* The final purity of SIL-IS was about 95% as estimated by the amounts of labeled and unlabeled Leu from several tryptic peptides (Figure S-2 in Supporting Information). The high purity is crucial to minimize cross talk of the SIL-IS with the unlabeled analyte in the mass spectrometer. The signal contribution from the unlabeled peptides was insignificant when a surrogate peptide with two Leu's was used (such as the NQV or SGT peptide). The LLOQ of 0.1 μg/mL was achieved when the amount of IS was kept at ~3 μg/mL or lower when *NQV or *SGT peptide was used as shown in Figure S-2 (Supporting Information) and confirmed by method qualification. When a surrogate peptide with one Leu was used, such as the GPS or GLP peptide, the sensitivity was estimated to be 0.5 μg/mL. Even at this higher LLOQ, the sensitivity is sufficient for most preclinical studies to cover the expected concentration range throughout a PK time course.

Linearity. STD curve linearity of mAbs using common surrogate peptides of NQV, SGT, GPS, and GLP were evaluated. The peak area ratios of the each surrogate peptide over the same SIL-IS peptide (NQV/*NQV, SGT/*SGT, GPS/*GPS, and GLP/*GLP) or over *NQV (SGT/*NQV, GPS/*NQV, and GLP/*NQV) were correlated to the STD concentrations using linear regression. The correlation coefficients R^2 for the various regressions of each mAb were consistently >0.99, except for three, which were >0.97 (Table S-3 in Supporting Information). Therefore, linearity was shown with surrogate peptides against the same corresponding SIL-IS peptide or against the *NQV IS peptide. For the NQV/*NQV method that was used for method qualification of 8 mAbs, the mean regression equation was $Y = 0.522(0.048)X + 0.022(0.0098)$ for IgG_2 and $Y = 0.431(0.037)X + 0.025(0.015)$ for IgG_1 with SD in parentheses. The slopes could be different when the surrogate peptides against a different SIL-IS peptide are used for regression. It should be cautioned that each method should be evaluated carefully if a different SIL-IS peptide is used.

Accuracy and Precision. The accuracy and precision of QCs for the mAbs are presented in Table 2, using the peak area ratios of NQV/*NQV for calculation. The results show that the accuracy was acceptable with % bias and % CV meeting the general LBA criteria of ±20% of the nominal value.

For all 8 mAbs, the use of the common whole Ab IS allowed accurate and precise quantification as evinced by the QC recoveries (Table 2) and similar squares of correlation coefficient (i.e., R^2 of the linear regression in Table S-3, Supporting Information). In terms of extrapolation to the quantification of other mAbs and the overall general use of the method, it can be reasonably argued that the method approach can be applied to all IgG_2 candidates using exactly the same

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Table 2. Precision and Accuracy of QCs[a]

	% CV			% bias		
	LQC	MQC	HQC	LQC	MQC	HQC
827-G2	2.81	3.97	7.07	−12.3	5.31	−1.34
αK-G2	9.54	2.79	0.977	−16.7	−4.28	2.57
αDB-G2	6.81	8.60	2.93	−1.12	2.4	−3.48
αDA-G2	6.21	2.35	0.341	10.8	2.8	1.70
655-G1	18.7	6.86	10.1	16.6	3.03	−8.26
αK-G1	11.2	4.02	4.47	−16.4	−5.65	−13.9
αDB-G1	17.6	7.36	7.35	−12.8	2.61	2.05
αDA-G1	8.81	7.84	7.08	−6.48	−7.95	1.00

[a]QC concentrations at low (LQC), mid (MQC), and high (HQC) were 0.3, 5, and 10 μg/mL, respectively. Results from $N = 3$ at each QC level.

surrogate peptides in the common regions of the HC or lc as the SIL αDA-2 IS. For the extension to a different isotype (e.g., IgG_1), as long as the proper STD curves and QCs are analyzed, the method can be used for cross-isotype analysis. If more surrogate peptides were desired other than the two common surrogate peptides for IgG_1, the best course of action is to generate a whole Ab IgG_1 SIL IS similar to that for IgG_2.

Comparison of Whole Molecule SIL-IS vs Peptides IS. Synthetic SIL-IS without or with flanking amino acids have been applied previously to the quantification of bioactive peptides and proteins.[4a,b,d] Due to high structural complexity, Abs may behave differently than other proteins in the digestion and other processing steps. Therefore, we compared the QC accuracy and precision performance using the SIL-IS of the whole molecule Ab to those with the synthetic peptides for three mAbs. Three sets of QCs each from αDA-G2, αDA-G1, or αK-G2 were analyzed in three replicates. The two synthetic peptide SIL-ISs were introduced to the immunocapture extract prior to the dry down step. Figure 2 shows that, overall, all three mAb were quantified accurately and precisely using the whole molecule SIL-IS. For the flanking peptide SIL-IS, the αDA-G2 QCs were also well within ±20%. The mid and high QC values of the αDA-G1 were near the ±20% threshold but not the LQC or the QCs of the αK-G2. For the peptide SIL-IS, the QC values of αDA-G2 were marginally acceptable, with higher variability and imprecision for αDA-G1 and the worst results for αK-G2. The whole molecule IS peak area counts within the runs appeared to be more consistent (15.5% CV) than those of the flanking peptide IS (28.1% CV) or the peptide IS (27.7% CV), $N = 32$.

If the immunocapture and digestion steps were optimized and the reproducibility was under control day-to-day and between analysts, then the synthetic peptide ISs may be adequate. The emphasis in this manuscript is that, with the whole Ab IS, fluctuations in these processing steps can be easily corrected for without the need to make sure that one has absolute control over the fidelity of all the processing steps.

Application to the Matrix of a Different Species. STDs and QCs of αDA-G2 were prepared in cynomolgus monkey serum and run side-by-side with STDs and QCs prepared in rat

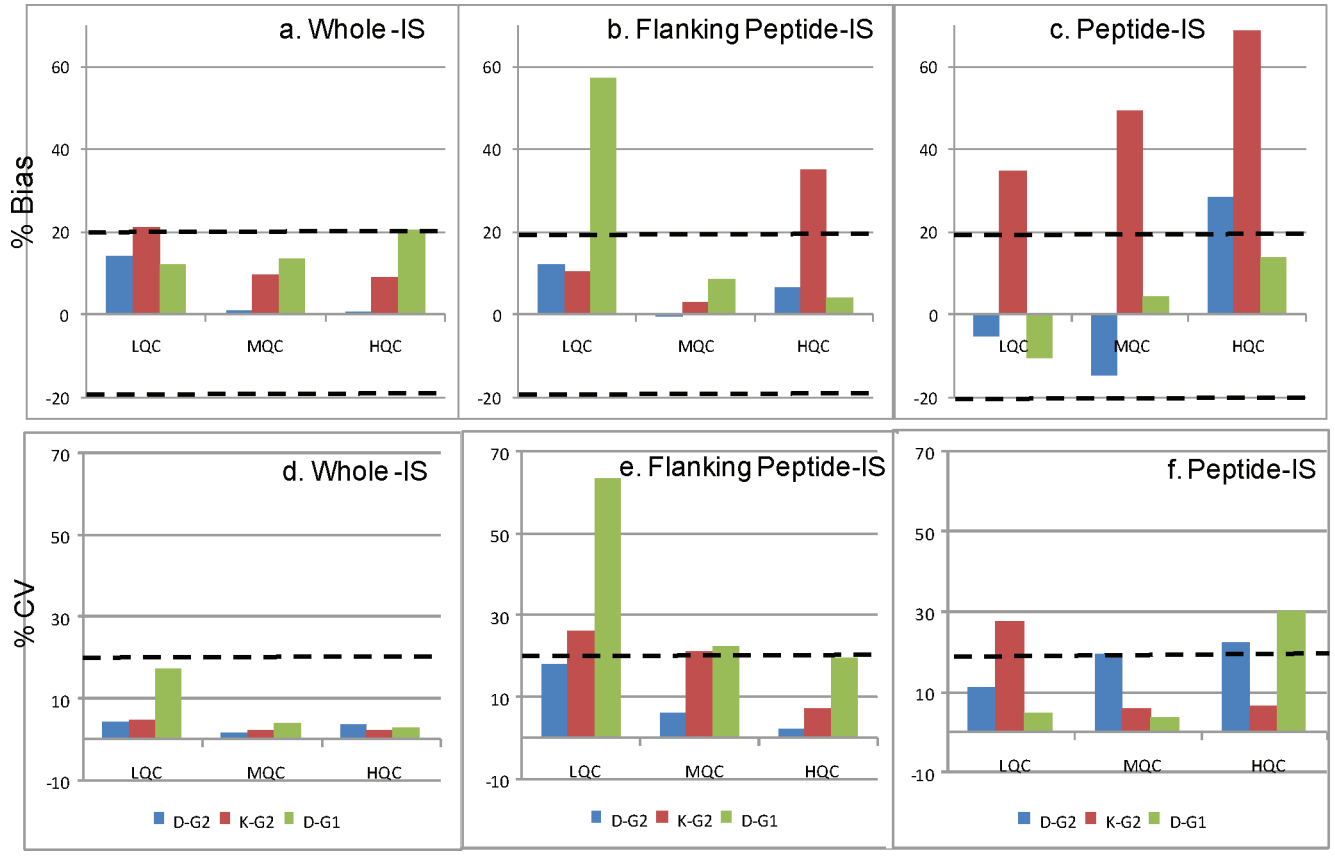

Figure 2. Accuracy and precision of QCs from 3 mAbs obtained with three different SIL-IS's. (a, b, c): Accuracy of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively. (d, e, f): Precision of whole SIL-IS, flanking SIL-IS, and peptide SIL-IS, respectively. The dashed lines of 20% are the thresholds of acceptance commonly used by LBAs. αDA-G2, αK-G2, and αDA-G1 QCs are represented by blue, red, and green color bars, respectively.

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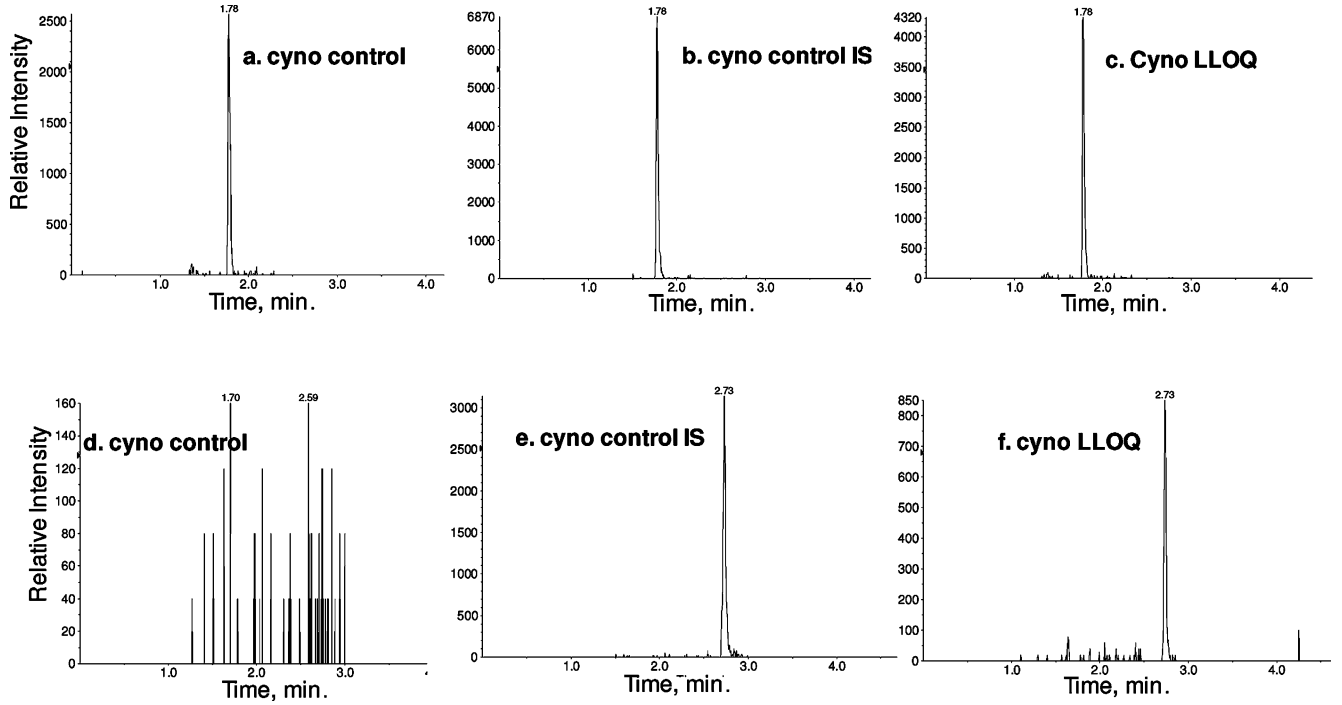

Figure 3. Chromatograms of NQVSLTC[CAM]LVK (a to c, retention time = 1.78 min) vs SGTASVVC[CAM]LLNNFYPR (d to f, retention time = 2.73 min) signature peptide in cynomolgus monkey control serum. (a, d) No addition to blank control serum; (b, e) IS added; (c, f) 0.1 μg/mL αDA-G2 added.

plasma. A high background was observed with the blank control monkey sample (Figure 3a–c), which led to the loss of sensitivity and a nonlinear STD curve using the NQV peptide. Instead of undertaking the arduous task of identifying and eradicating the background interference of this surrogate peptide, we chose instead to track a different surrogate peptide SGT, which provided a cleaner background with sufficient sensitivity (Figure 3d–f) and good linearity from 0.1 to 15 μg/mL for the cynomolgus monkey serum comparable to that of the rat plasma. The linear regression of the standard curve was as follows: $Y = 0.551X + 0.00678$, with $R^2 = 0.9958$. QC samples were accurately quantified with % bias of 20.3, 3.0, and −3.8 for the low, mid, and high QC, respectively. Thus, the whole molecule SIL-IS provided the flexibility to change to a more suitable surrogate peptide with LC-MS in the MRM mode, allowing the method to be reoptimized within an hour. In contrast, such a matrix effect could take weeks to resolve for an ELISA method and at least a week to identify and synthesize another IS for an LC-MS/MS method using a peptide SIL-IS.

Application to Preclinical Studies. *Comparison of PK Results from LC-MS/MS and ELISA.* Samples from Sprague–Dawley rats dosed with αDA-G1 and αK-G2 were analyzed by ELISA and LC-MS/MS methods. The concentrations from LC-MS/MS correlated well to those of ELISA over a wide range of 0.2–50 μg/mL (Figure S-3 in Supporting Information). The correlation plot slopes were close to 1.00, with R^2 of 0.988 and 0.967 for αDA-G1 and αK-G2, respectively.

The PK parameters derived from the LC-MS data agreed very well with those of the ELISA. The AUC_{inf} was 15.7 and 13.5 h*mg/mL for αDA-G1 by LC-MS and ELISA, respectively, and 35.2 and 30.0 h*mg/mL for αK-G2 by LC-MS and ELISA, respectively. The clearance was 0.319 and 0.37 mL/h/kg for αDA-G1 by LC-MS and ELISA, respectively, and 0.142 and 0.166 mL/h/kg for αK-G2 by LC-MS and ELISA, respectively. The good agreements are also shown by the time–concentration profiles between the LC-MS and ELISA methods (Figure 4). Therefore, the general LC-MS method approach using the common whole molecule SIL-IS and immunocapture was demonstrated for the bioanalysis of mAbs of a different isotype (IgG_1 instead of IgG_2) or against a different antigen (anti-KLH instead of anti-DNP).

In this example, the similarity of the PK data to those of ELISA could be the result of the use of the same immunocapture reagents for both methods. However, such agreement is dependent on the ELISA method specificity governed by the detector reagents and the signature peptide used for the quantification in the LC-MS/MS method, which can be different case-by-case.

■ CONCLUSIONS

A general LC-MS/MS method approach using a common whole mAb SIL-IS and immunocapture was developed, qualified, and applied to mAb quantification in preclinical samples. The method was accurate and precise, with quantitative results comparable to those of ELISA. The processing steps of immunocapture, reduction, alkylation, and tryptic digestion were optimized and streamlined to improve throughput with adequate consistency. The common whole Ab molecule SIL-IS was able to correct for variations from the beginning of sample processing to ionization in the mass spectrometer. It performed better than the synthetic peptide IS with or without flanking amino acids in correcting for assay variability. It also allowed rapid method development with flexible choice of a suitable surrogate peptide for new application, such as to a different species or different mAb. Most importantly, this universal, flexible, and readily implementable LC-MS/MS method approach can reduce method development time and the resources required for

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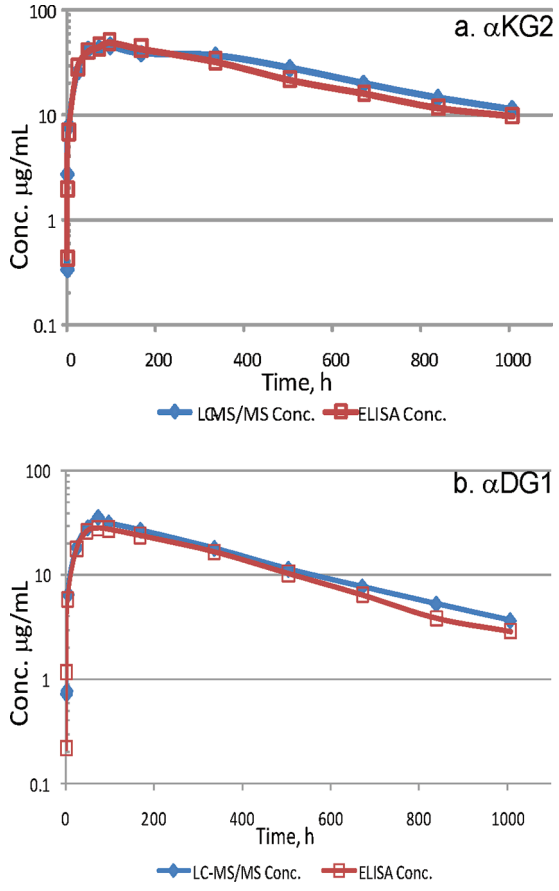

Figure 4. PK time−concentration profiles determined by ELISA and LC-MS/MS methods using common SIL-IS of anti-DNP, IgG_2. (a) Anti-KLH IgG_2; (b) anti-DNP, IgG_1. Red lines: ELISA. Blue lines: LC-MS/MS methods.

multiple candidates in different biological matrixes during the preclinical stages when specific LBA reagents are not available. Once the candidate is selected, specific binding reagents can then be developed for late preclinical and clinical studies. ELISA may be the preferred analytical technique for clinical sample analysis because it is more cost-effective and the turnaround times are faster than LC-MS/MS methods. As LBAs have been established over many years as the analytical tool for large molecule drugs, bridging experiments analyzing the same sample sets by both methods will be required to understand the assay specificity and potential interferences.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jwlee@amgen.com. Tel.: 805/447-9463. Fax: 805/499-9072. Address: One Amgen Center Drive, MS 30E-3-B, Amgen Inc., Thousand Oaks, CA 91320.

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