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Quantitative LC/ESI-SRM/MS of antibody biopharmaceuticals: use of a homologous antibody as an internal standard and three-step method development

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Abstract Monoclonal antibody-based therapeutic agents (antibody drugs) have attracted considerable attention as a new type of drug. Concomitantly, the use of quantitative approaches for characterizing antibody drugs, such as liquid chromatography (LC)-mass spectrometry (MS), has increased. Generally, selective quantification of antibody drugs is done using unique peptides from variable regions (V_H and V_L) as surrogate peptides. Further, numerous internal standards (ISs) such as stable isotope-labeled (SIL)-intact proteins and SIL-surrogate peptides are used. However, developing LC-MS methodology for characterizing antibody drugs is time-consuming and costly. Therefore, LC-MS is difficult to apply for this purpose, particularly during the drug discovery stage when numerous candidates must be evaluated. Here, we demonstrate an efficient approach to developing a quantitative LC/electrospray ionization (ESI)-selected reaction monitoring (SRM)/MS method for characterizing antibody drugs. The approach consists of the following features: (i) standard peptides or SIL-IS are not required; (ii) a peptide from the homologous monoclonal antibody serves as an IS; (iii) method development is monitored using a spiked plasma sample and one quantitative MS analysis; and (iv) three predicted SRM assays are performed to optimize quantitative SRM conditions such as transition, collision energy, and declustering potential values. Using this strategy, we developed quantitative SRM methods for infliximab, alemtuzumab, and bevacizumab with sufficient precision (<20%)/accuracy (<±20%) for use in the drug discovery stage. We have also demonstrated that choosing a higher homologous peptide pair (from analyte mAb/IS mAb) is necessary to obtain the sufficient precision and accuracy.

Abbreviations

CDR	Complementarity-determining region
CE	Collision energy
CXP	Collision cell exit potential
DP	Declustering potential
D-PBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
ESI	Electrospray ionization
EP	Entrance potential
IAA	Iodoacetamide
IS	Internal standard
LBA	Ligand binding assay
LC	Liquid chromatography
mAb	Monoclonal antibody
MS	Mass spectrometry
pSRM	Predicted SRM
QC	Quality control
SRM	Selected reaction monitoring
SIL	Stable isotope-labeled
V_H	Heavy-chain variable region
V_L	Light-chain variable region

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Introduction

Interest in monoclonal antibody (mAb)-based therapeutic agents (antibody drugs) has grown substantially over the last three decades. The specificities of antibody drugs exceed those of low molecular weight drugs and the development of antibody engineering makes possible the production of custom mAbs [1]. The long biological half-lives (immunoglobulin G1, IgG1, $t_{1/2}$ 21 days) are attributed to a recycling mechanism mediated by the neonatal Fc receptor [2–4]. Therefore, the pharmacological activities of antibody drugs are expected to persist longer than those of low molecular weight drugs. Antibody drugs are therefore widely used, despite their high cost. For example, adalimumab, infliximab, rituximab, bevacizumab, and trastuzumab were among the 10 top selling drugs in 2014 as reported by Genetic Engineering & Biotechnology News [5]. Moreover, the higher success rates of antibody drugs [6] have encouraged the pharmaceutical industry to conduct in-house research and development over licensing. The research and development of antibody drugs requires quantification methods to evaluate candidate mAbs for clinical and preclinical studies. In particular, the drug discovery stage urgently requires accurate and precise high-throughput quantification methods because of the large numbers of candidates that must be evaluated.

Ligand binding assays (LBAs), including immunoassays, show good sensitivity, selectivity, and throughput and are widely used for quantification. However, method development for the evaluation of antibody drugs is generally time-consuming, and the preparation of specific reagents such as anti-idiotype antibodies is expensive. Further, LBAs can be affected by reagents, matrixes, and the experience of researchers [7–9]. Therefore, the use of LBAs in the drug discovery stage, when large numbers of candidates must be screened, is impractical [10].

Alternatively, the development of electrospray ionization (ESI) as a soft ionization method and to provide an interface between liquid chromatography (LC) and mass spectrometry (MS) has popularized analytical approaches using LC-MS [11]. Generally, direct quantification of large biomacromolecules such as antibodies (approximately 150 kDa) is technically difficult, because of unsolved problems with LC separation, such as insufficient range and resolution of MS [7]. Further, analysis of multiply charged ions decreases sensitivity.

Therefore, analysis of representative peptides (surrogate peptides) after proteolysis has become a practical method to quantify antibody drugs [7–9]. This strategy assumes that the surrogate peptide reflects the amount of intact antibody. Therefore, selection of appropriate surrogate peptides and their stoichiometric production from proteolyzed mAbs is required. Unique peptides from an antibody's heavy-chain variable region (V_H) or light-chain variable region (V_L) are used as surrogate peptides for selectivity. This allows multiple antibody drugs to be quantified simultaneously and in turn allows monitoring samples of cassette dosing [12, 13] of several candidate drugs to increase the throughput of pharmacokinetic experiments. Alternatively, the use of peptides from human-specific sequences of the antibody constant region as universal peptides allows the use of animal models to analyze any human antibody during the drug discovery stage [14–17].

However, the development of LC-MS-based quantification methods, such as selected reaction monitoring (SRM), is also time-consuming, because these require, for example, optimizing cleanup, selecting surrogate peptides, preparing standard peptides, and optimizing MS conditions to enhance sensitivity. Immunoaffinity methods are used for cleanup of target antibodies [18, 19] and surrogate peptides [20]. Peptide mapping is typically used for qualification MS, such as an ion trap-MS [7–9]. Alternatively, an in silico strategy without use of MS has been reported to select surrogate peptides of endogenous proteins for the absolute quantification [21]. However, both strategies require surrogate peptides that serve as standards for preparing stock solutions and optimizing SRM conditions.

Further, LC-MS quantification requires appropriate internal standards (ISs), ideally stable isotope-labeled (SIL)-ISs, for example, that compensate for recovery from biological samples and ion suppression. For quantification of antibody drugs, SIL-surrogate peptides are used rather than SIL-intact mAbs as the ISs, because of the easier preparation [22]. In this case, recovery from biological samples and digestion yield cannot be compensated.

As an alternative approach, analogue of the SIL-surrogate peptides, which have extend amino acids overhanging at both the N- and C-terminus, has been reported to compensate the digestion yield [12, 14–20]; however, the digestion yield between the whole protein and the peptide might be different. As a few examples, SIL-antibodies and mutant antibodies have been reported as ideal ISs to give the best precision and accuracy [13, 23–26], however inappropriate for the drug discovery stage. In addition, use of non-related proteins as universal ISs, such as bovine fetuin, has been also reported for mAb quantification to facilitate method development [27]. Furthermore, an intact murine mAb as a generic IS and the protocol have been provided by an MS company as a kit for mAb quantification [28]. Similarly to the disadvantages of LBAs, the development of LC/MS techniques to characterize antibody drugs is generally time-consuming and labor-intensive, and data are difficult to validate. Given this, the American Association of Pharmaceutical Scientists recommends the use of less stringent validation criteria (e.g., precision <20%, accuracy <±20%, 25% limit of quantification) for the quantification of antibody drugs using LC-MS that are required for LBAs [29].

Accordingly, we demonstrate here a more practical approach to developing LC/ESI-SRM/MS methods for the drug discovery stage as follows (Scheme 1):

1. A homologous mAb is added as an IS to compensate for recovery and digestion yields and to avoid use of an SIL-IS.
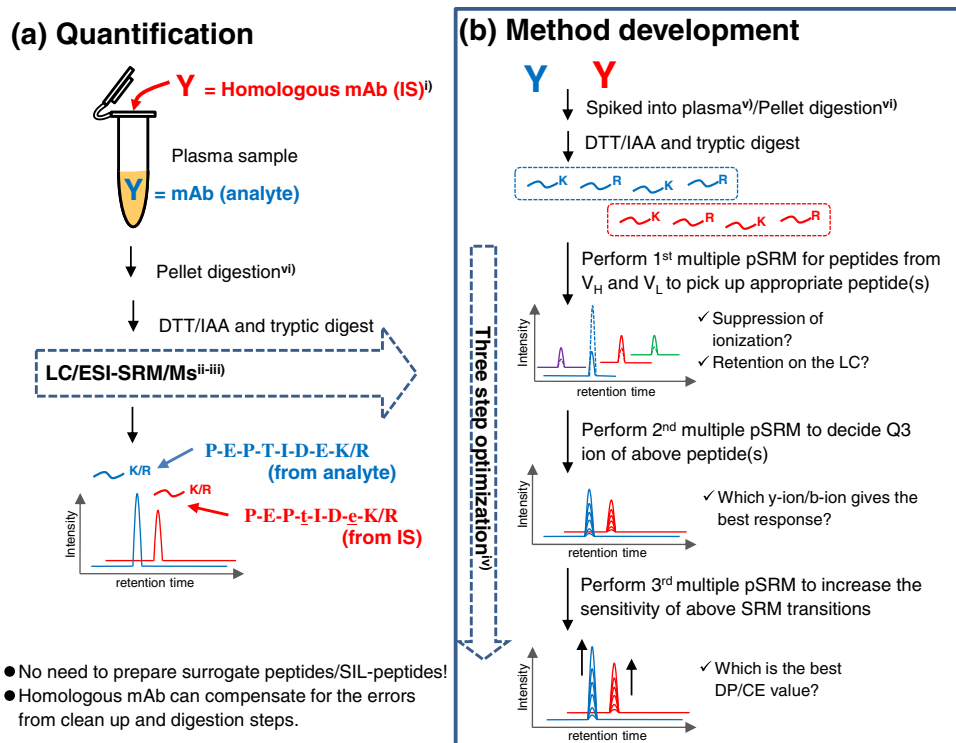
Scheme 1 Workflow of quantification of antibody drugs (**A**) and multiple pSRM-based method development (**B**). (i) A homologous mAb is added as an IS to monitor the recovery and digestion yields and to avoid the use of an SIL-IS. (ii) A constant LC setting is used for the mAbs to avoid the time-consuming requirement to optimize the gradient and solvents. (iii) One triple quadrupole MS is used for the optimizations and following quantification. (iv) Three-step optimization is performed using multiple predicted SRM (pSRM) assays for the selection of surrogate peptides and optimization of quantitative conditions, including the SRM transition, collision energy (CE), and declustering potential (DP). (v) Spiked plasma samples are used to optimize LC-MS to evaluate practical problems associated with the sample matrix. (vi) Pellet digestion is used for the cleanup to facilitate pretreatment of the plasma sample

2. One constant LC setting is used for all mAbs to avoid lengthy gradient and solvent optimizations.
3. One triple quadrupole MS is used for all optimization steps and subsequent quantification.
4. Three-step optimization is performed using a multiple, predicted SRM (pSRM) for selection of surrogate peptides and optimization of quantitative conditions such as SRM transition, collision energy (CE), and declustering potential (DP).
5. Spiked plasma samples are used to optimize LC-MS to evaluate practical problems associated with the sample matrix.
6. Pellet digestion is used for cleanup to facilitate pretreatment of plasma samples.

Materials and methods

Materials

Infliximab (Remicade) was purchased from Janssen Biotech, Inc. (Horsham, PA). Ofatumumab (Arzerra) was purchased from Novartis International AG (Basel, Switzerland). Alemtuzumab (MabCampath) was purchased from Sanofi S.A. (Paris, France). Bevacizumab (Avastin) was purchased from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). These four mAb drugs were used each other as an analyte and the IS after screening appropriate pair of surrogate peptides. Iodoacetamide (IAA) and dithiothreitol (DTT) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ammonium bicarbonate and trypsin from bovine pancreas (TPCK-treated, essentially salt-free, lyophilized powder) were purchased from Merck KGaA (Darmstadt, Germany). Acetic acid, formic acid, ammonia water (28%), methanol (LC-MS grade), acetonitrile (LC-MS grade), and water (LC-MS grade) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Dulbecco's phosphate-buffered saline (D-PBS) was purchased from Life Technologies Co. (Carlsbad, CA). Normal cynomolgus monkey plasma was obtained from Astellas Research Technologies.

LC conditions

The Shimadzu Nexera X2 HPLC System (Shimadzu Kyoto, Japan) consisted of the following components: CBM-20A

controller, LC-30AD pump, and a SIL-30ACMP autosampler. A CTO-20AC column oven was used with the following MS system. Chromatographic conditions were as follows: column, AdvanceBio Peptide Map column (2.7 μm, 120 Å, 250 × 2.1 mm i.d.; Agilent Technologies, Inc., Santa Clara, CA); column temperature, 60 °C; solvent A, 0.1% (v/v) formic acid in water; solvent B, 0.1% (v/v) formic acid in acetonitrile; linear gradient, 20% B at 0 min, 40% B at 4.5 min, 90% B at 4.6 min, 90% B at 5.1 min, 20% B at 5.2 min, and 20% B at 6 min; and flow rate 0.4 mL/min.

MS conditions

A QTRAP 5500 linear ion trap quadrupole mass spectrometer (AB Sciex, Framingham, MA) equipped with an ESI interface was used in positive-ionization mode for the SRM experiments. ESI parameters for peptide selection were as follows: curtain gas, 50 psi; collision gas, 9 psi; ion spray voltage, 4000 V; temperature, 600 °C; ion source gas 1, 50 psi; and ion source gas 2, 70 psi. Typical SRM parameters were as follows: dwell time, 20 ms; DP, 120 V; entrance potential (EP), 10 V; CE, 25 V; and collision cell exit potential (CXP), 9 V. When quantitating mAb, dwell time was changed to 75 ms, and the DP and CE value were changed to the optimized value. Data analysis was performed using Analyst Version 1.6.1 and MultiQuant 2.1.1. Individual standard curves were constructed using the ratios of peak areas of the selected peptide for each analyte mAb instead of that for IS mAb and a 1/concentration^2-weighted linear least-squares regression.

Sample preparation for calibration curves and quality control

The mAbs used in this study were purchased as solutions as follows: infliximab, 5 mg/mL; ofatumumab, 20 mg/mL; alemtuzumab, 30 mg/mL; and bevacizumab, 25 mg/mL. Calibration standards (1, 2, 5, 10, 20, 50, 100, 200, and 500 μg/mL) and quality control (QC) samples (3, 30, and 300 μg/mL) were prepared by serially diluting the mAbs in normal cynomolgus monkey plasma.

Sample preparation for LC-MS analyses

Cleanup was performed using a reported pellet digestion method [30–32] with slight modification as follows. Aliquots (25 μL) of the plasma samples (blank, standard, or QC samples) were transferred into a 96-deep well polypropylene plate. The working solution of the IS mAb (5 μL, 400 μg/mL, in D-PBS) was added to each sample except the blanks (5 μL of D-PBS). Methanol (100 μL) was added into each well to precipitate plasma proteins, followed by vigorous vortex mixing for 2 min and centrifugation at 200×g (rcf) for

2 min. After the supernatant was discarded, the protein pellet was resuspended in digestion buffer (50 μL, 100 mM ammonium bicarbonate) by vigorous vortex mixing for longer than 2 min. The denatured protein suspension was incubated with DTT (10 μL, 100 mM, in water) at 60 °C for 60 min to reduce disulfide bonds. The reduced samples were incubated in darkness with IAA (25 μL, 100 mM, in water) at 30 °C for 30 min to alkylate Cys sulfhydryl groups. After addition of D-PBS 25 (μL), the reduced and alkylated samples were incubated with trypsin (25 μL, 8 mg/mL, 0.1% (v/v) formic acid in water) at 50 °C for 30 min or 1 h. All incubations (reduction, alkylation, and digestion) were conducted in a preheated thermomixer at 1000 rpm. The digestion reaction was quenched by adding 25 μL of 10% (v/v) formic acid in water. The final tryptic digest was centrifuged at 2150×g (rcf) for 10 min, and the supernatant was applied to an Oasis MCX μElution plate (Waters Co., Milford, MA) for cleanup. Briefly, the MCX plate was conditioned with 200 μL of methanol followed by 200 μL of water. The supernatant of the tryptic digest was transferred to the MCX plate that was then washed sequentially with 200 μL of 2% (v/v) formic acid in water and 200 μL of methanol. The peptides were then eluted with 200 μL of 20% (v/v) ammonia water in acetonitrile. After evaporation using a nitrogen stream, the extracts were reconstituted with 100 μL of 10% (v/v) acetic acid in water. An aliquot of each sample (1 μL for peptide selection, 5 μL for mAb quantification) was injected onto the LC-MS/MS system.

Surrogate peptide selection and optimization of quantitative SRM

Blank plasma and mAb-spiked samples (500 μg/mL) were analyzed three times using the multiple pSRM settings described below. Multiple pSRM is a selective and sensitive technique for screening unknown targets using limited information and has been applied to activate drug-mediated protein modifications [33].

The first multiple pSRM for peptide selection was performed as follows: (i) selection of peptides from mAb variable domains (V_H and V_L) using in silico tryptic digestion (ProteinProspector version 5.12.1; http://prospector.ucsf.edu/prospector/mshome.htm) (parameters: missed cleavages, 0 site; minimum length, 5 amino acid residues; maximum length, 30 amino acid residues; structural modifications, carbamidomethylation of Cys; no exclusion of peptides containing specific amino acid residues); (ii) SRM transitions: Q1, doubly and triply charged ions, Q3, b_2 and y_2 ions of each peptide.

The second multiple pSRM of peptides detected by the first multiple pSRM for product-ion selection of Q3 was as follows: (i) Q1, same as the first multiple pSRM, and (ii) Q3, all b and y ion series (except b_1 and y_1).

The third multiple pSRM for optimizing DP and CE was as follows: (i) Q1, same as the first multiple pSRM; Q3, the

highest product ion results of the second multiple pSRM; and (iii) DP, 50–200 V (10 V/step), and CE, 15–50 V (5 V/step).

The amino acid sequences of mAbs were deposited into the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) as follows: infliximab, 4G3Y_H, 4G3Y_L; ofatumumab, PDB: 3GIZ_H, 3GIZ_L; alemtuzumab, 1CE1_H, 1BJ1_L; and bevacizumab, 1BJ1_H, 1BJ1_L. Complementarity-determining regions (CDRs) of mAbs were determined in accordance with the Kabat Numbering.

Results and discussion

Experimental design

For the drug discovery stage, an LC/ESI-SRM/MS method was designed as a practical strategy that added a homologous mAb as a non-SIL-IS that was analyzed as a quantitative surrogate tryptic peptide IS (Scheme 1A). Further, efficient method setup was designed using constant LC conditions for all mAbs to avoid time-consuming optimization of gradients and

solvents. Spiked plasma was used for method development to evaluate problems caused by the matrix effect. Moreover, method development was performed using the same triple quadrupole MS with three-step multiple pSRM to select surrogate peptides and optimize quantitative SRM conditions such as transition, CE, and DP values (Scheme 1B).

Cleanup and LC separation

Several cleanup strategies are used for LC-MS-based mAb quantification. Immunoaffinity extraction is widely used for cleanup and enrichment. However, it is not practical for the drug discovery stage where large numbers of candidate have to be analyzed. We therefore used the pellet digestion method for the initial cleanup before tryptic digestion [30–32]. This method employs protein precipitation, although it was reported to be an efficient cleanup strategy for large proteins to eliminate impurities such as lipids before tryptic digestion. The pellet (protein precipitate) formed using methanol is less dense and easy to resuspend before the next digestion step. Generally, further cleanup after tryptic digestion is necessary and requires SPE to reduce matrix

Table 1 The first multiple pSRM list for screening surrogate peptides of infliximab

Origin	Surrogate peptide candidate	Q1 (m/z)	Q3 (m/z)	
		Charge state	b_2	y_2
V_H	L[4]EESGGGLVQPGGSMK[19]	Double 773.4 Triple 515.9	243.1	278.2
	L[20]SCVASGFIFS<u>NHWMN</u>WVR[38]	Double 1156.0 Triple 771.0	201.1	274.2
	Q[39]SPEK[43]	Double 294.7 Triple 196.8	216.1	276.2
	G[44]LEWVA<u>EIR</u>[52]	Double 536.8 Triple 358.2	171.1	288.2
	<u>S[55]INSATHYAESVK</u>[67]	Double 703.8 Triple 469.6	201.1	246.2
	F[70]TISR[74]	Double 312.2 Triple 208.5	249.1	262.2
	S[79]AVYLQMTDLR[89]	Double 648.8 Triple 432.9	159.1	288.2
	T[90]EDTGVYYCSR[100]	Double 675.8 Triple 450.9	231.1	262.2
	<u>N[101]YYGSTYDYW</u>GQGTTLTVSSASTK[124]	Triple 884.1	278.1	248.2
V_L	D[1]ILLTQSPAILSVSPGER[18]	Double 948.5 Triple 632.7	229.1	304.2
	V[19]SFS<u>CR</u>[24]	Double 378.2 Triple 252.5	187.1	335.1
	<u>A[25]SQFVGSSIH</u>WYQQR[39]	Double 897.4 Triple 598.6	159.1	303.2
	T[40]NGSPR[45]	Double 316.2 Triple 211.1	216.1	272.2
	Y[50]<u>ASESMSG</u>IPSR[61]	Double 642.8 Triple 428.9	235.1	262.2

The underlines indicate complementarity-determining regions (CDRs). Lists of peptides derived from ofatumumab, alemtuzumab, and bevacizumab are shown in ESM, Tables S1–S3, respectively
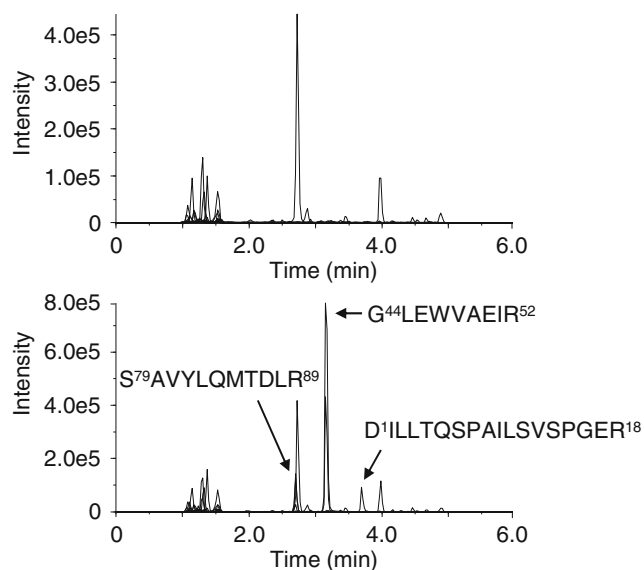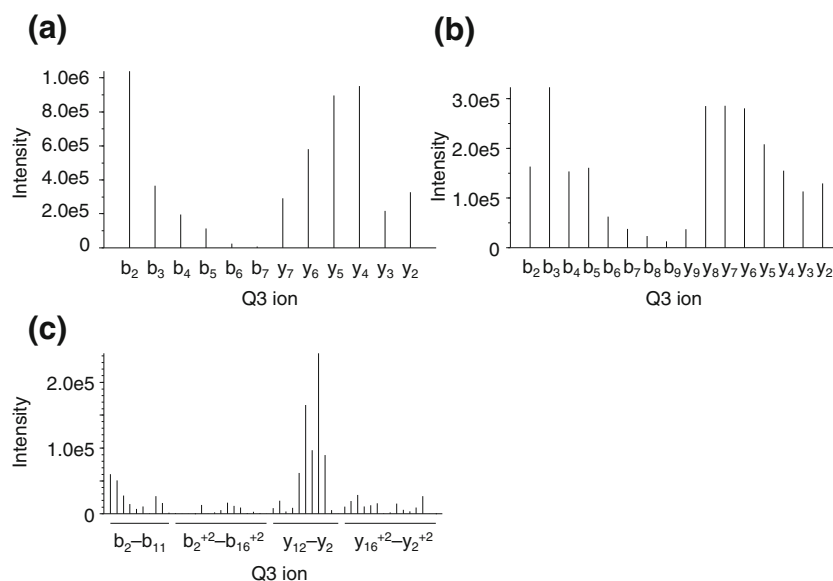
Fig. 1 The first multiple pSRM assay to select surrogate peptide candidates of infliximab, (*upper*) blank plasma, and (*bottom*) mAb-spiked plasma. The data for ofatumumab, alemtuzumab, and bevacizumab are shown in ESM Fig. S1

same gradient was used for all mAbs to avoid the lengthy optimization of individual LC settings.

Surrogate peptide selection and SRM optimization

Surrogate peptides are generally selected from the V_H and V_L domains of the target mAbs, because the peptide sequences can be distinguished among different mAbs. Further, the V_H and V_L domains contain highly similar sequences comprising the framework region that maintains the three-dimensional structure of the CDR. Therefore, different mAbs consist of similar but distinguishable pairs of peptides for use as analytes and as respective ISs. Moreover, the V_H and V_L domains of the four model mAbs were digested in silico to predict candidate peptides (excluding Met-containing peptides, except those comprising the CDR), as summarized in Table 1 and in Electronic Supplementary Material (ESM) Table S1–S3 (infliximab, ofatumumab, alemtuzumab, and bevacizumab, respectively). To select surrogate peptides and optimize the quantitative SRM method, three-step multiple pSRM analyses were performed (Scheme 1) for (a) surrogate peptide selection, (b) Q3 determination, and (c) CE and DP optimization.

We used this strategy to analyze the tryptic digests of each mAb. The results of the first multiple pSRM of infliximab are shown in Fig. 1. Note that the b_2 and y_2 ions are generally sufficient for pSRM-based peptide screening of activated drug-mediated protein modification [33]. Peptides from the V_H and V_L domains were detected in the infliximab-spiked sample as follows: G^{44}LEWVAEIR^{52} (V_H), S^{79}AVYLQMTDLR^{89} (V_H), and D^1ILLTQSPAILSVSPGER^{18} (V_L) (Fig. 1). Similarly, S^{78}LYLQMNSLR^{87} (V_H), E^1IVLTQSPATLSLSPGER^{18} (V_L), L^{46}LIYDASNR^{54} (V_L), and S^{92}NWPITFGQGTR^{103} (V_L) from ofatumumab (ESM, Fig. S1a); G^{44}LEWIGFIR^{52} (V_H), V^{70}TMLVDTSK^{78} (V_H),

effect. However, the reproducibility and the different recovery rates between the analyte peptide and IS peptide can affect the accuracy and the precision, especially when C18 SPE is used. Therefore, MCX µElution plate was employed, because all the tryptic peptides contain Lys or Arg at C-terminus that can be retained on a strong cation exchange SPE.

A core-shell column was employed for LC separation to provide a high-throughput system with a shorter analysis time and improved separation using a higher flow rate. The core-shell column used here was superficially porous (2.7-µm particle, 120 Å pore) to facilitate peptide mapping with shorter runtimes to reduce matrix effects [34]. A standard mobile phase (formic acid/water-acetonitrile) was used here, and the

Fig. 2 Intensities of the second multiple pSRM assay to select product ions (Q3) from surrogate peptide candidates of infliximab, (**a**) G^{44}LEWVAEIR^{52} (V_H) from m/z 536.8 ($[M+2H]^{2+}$), (**b**) S^{79}AVYLQMTDLR^{89} (V_H) from m/z 648.8 ($[M+2H]^{2+}$), and (**c**) D^1ILLTQSPAILSVSPGER^{18} (V_L) from m/z 632.7 ($[M+3H]^{3+}$). The data for ofatumumab, alemtuzumab, and bevacizumab are shown in ESM Figs. S2–S4, respectively
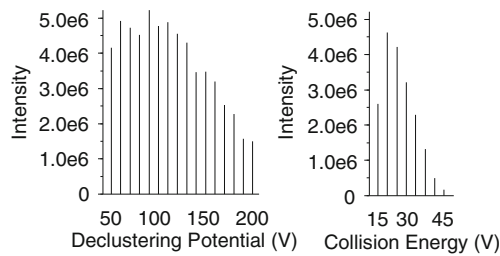
Fig. 3 Intensity of the third multiple pSRM assay to optimize the DP/CE value for a surrogate peptide candidate, G^{44}LEWVAEIR^{52} (V_H) of infliximab with the optimal SRM transition (m/z 536.8 ➔ 488.3). (*Left*) DP and (*right*) CE values. The data for ofatumumab, alemtuzumab, and bevacizumab are shown in ESM Fig. S5

V^{19}TITCK^{24} (V_L), and L^{46} LIYNTNNLQTGVPSR^{61} (V_L) from alemtuzumab (ESM, Fig. S1b); and F^{68}TFSLDTSK^{76} (V_H) and V^{46} LIYFTSSLHSGVPSR^{61} (V_L) from bevacizumab (ESM, Fig. S1c) were detected in the spiked samples. This strategy employed constant LC conditions and mAb-spiked plasma sample. Therefore, highly polar peptides (poorly retained by SPE or an analytical column) and peptide ions suppressed by plasma impurities were practically excluded to develop a robust quantitative method.

The second multiple pSRM performed to select the optimal Q3 ion, which was identified for each peptide in the first multiple pSRM, was set to all b and y ions (excluding b_1/y_1) (ESM, Tables S4–S7). The candidate infliximab peptides identified in the second multiple pSRM are summarized in Fig. 2. For example, among the b and y ions of G^{44}LEWVAEIR^{52} (V_H) from m/z 536.8 ([M+2H]$^{2+}$), the y_4 ion was selected as Q3 for the next experiment because of its intensity and specificity (Fig. 2a). The Q3 ions from S^{79}AVYLQMTDLR^{89} (V_H) (m/z 648.8 [M+2H]$^{2+}$) and

D^{1}ILLTQSPAILSVSPGER^{18} (V_L) (m/z 632.7 [M+3H]$^{3+}$) were optimized (Fig. 2b, c). Data for ofatumumab, alemtuzumab, and bevacizumab are shown in ESM, Figs. S2–S4, respectively. For V^{46}LIYFTSSLHSGVPSR^{61} of bevacizumab, the b_2 ion was the most intense, but we selected the more specific y_{13}^{+2} ion, which contained the CDR.

The third multiple pSRM was performed to optimize the CE and DP values for SRM transitions optimized in the second multiple pSRM to increase signal intensity. For example, CE 20 V and DP 90 V were optimal for G^{44}LEWVAEIR^{52} (V_H, m/z 536.8 ➔ 488.3, y_4) of infliximab (Fig. 3). Data for peptides of ofatumumab, alemtuzumab, and bevacizumab are shown in ESM Fig. S5.

Optimized SRM conditions for surrogate peptides are summarized in Table 2. The typical quantitative SRM chromatograms of each low-quality control (LQC) (a), high-quality control (HQC) (b), and the corresponding ISs (c) are shown in Fig. 4 (infliximab), ESM Fig. S6 (alemtuzumab), and ESM Fig. S7 (bevacizumab). Each surrogate peptide derived from these mAbs appeared as a sharp and symmetrical peak. The LLOQs of infliximab, alemtuzumab, and bevacizumab were 1, 5, and 1 μg/mL, respectively, which are acceptable for pharmacokinetic evaluation during early drug discovery. Ofatumumab was excluded from further investigation, since appropriate IS was not selected from the combination of four mAbs in this study.

Data validation using different combinations of analyte and IS

The V_H and V_L domains of different mAbs commonly contain highly homologous framework regions that maintain three-

Table 2 SRM conditions optimized for mAbs

mAb		Origin surrogate peptide candidate	Retention time (min)	Q1 (m/z)		Q3 (m/z)		DP (V)	CE (V)
				Charge state		Ion			
Infliximab	V_H	G^{44}LEWVAEIR^{52}	3.17	Double	536.8	y_4	488.3	90	20
	V_H	S^{79}AVYLQMTDLR^{89}	2.71	Double	648.8	y_8	1039.5	NT	NT
	V_L	D^{1}ILLTQSPAILSVSPGER^{18}	3.72	Triple	632.7	y_5	545.3	NT	NT
Ofatumumab	V_H	S^{78}LYLQMNSLR^{87}	2.69	Double	612.8	y_6	748.4	NT	NT
	V_L	E^{1}IVLTQSPATLSLSPGER^{18}	2.99	Triple	633.3	y_5	545.3	NT	NT
	V_L	L^{46}LIYDASNR^{54}	1.82	Double	532.8	b_2	227.2	NT	NT
	V_L	S^{92}NWPITFGQGTR^{103}	2.85	Double	682.3	y_9	976.5	110	25
Alemtuzumab	V_H	G^{44}LEWIGFIR^{52}	4.21	Double	545.8	y_4	492.3	70	20
	V_H	V^{70}TMLVDTSK^{78}	1.54	Double	497.3	y_7	793.4	NT	NT
	V_L	V^{19}TITCK^{24}	1.19	Double	361.2	y_2	307.1	NT	NT
	V_L	L^{46}LIYNTNNLQTGVPSR^{61}	2.55	Triple	601.7	y_3	359.2	90	25
Bevacizumab	V_H	F^{68}TFSLDTSK^{76}	2.66	Double	523.3	y_7	797.4	NT	NT
	V_L	V^{46}LIYFTSSLHSGVPSR^{61}	2.80	Triple	588.3	y_{13}^{+2}	719.4	110	25

The underlines indicate complementarity-determining regions (CDRs)

NT not tested

Fig. 4 Representative quantitative SRM chromatograms of surrogate peptides. (**a** and **b**) G^{44}LEWVAEIR^{52} (V_H) from infliximab as the analyte (LQC 3 μg/mL and HQC 300 μg/mL, respectively) and (**c**) G^{44}LEWIGFIR^{52} (V_H) from alemtuzumab as an IS (combination a in Table 3). SRM chromatograms of other analyte and IS combinations are shown in ESM Figs. S6 and S7
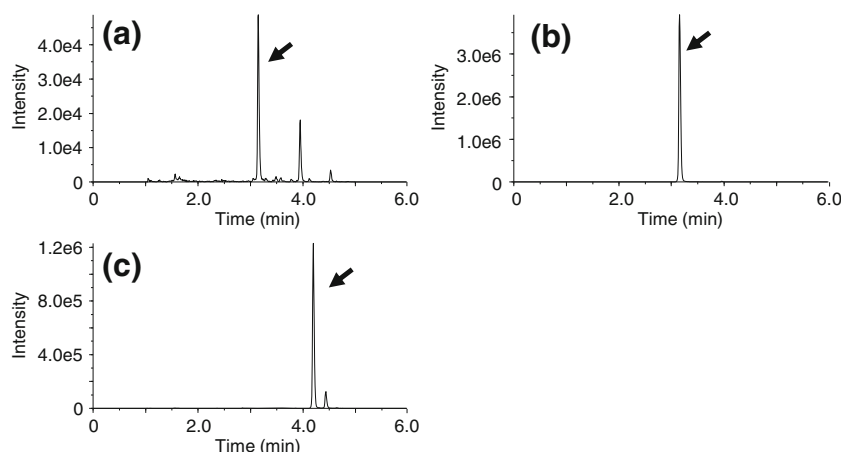
dimensional structure of CDR, and tryptic peptides from two different mAbs may have similar yet distinguishable sequences. Therefore, combinations of two surrogate peptides from two different mAbs were selected as an analyte pair and IS to evaluate the sensitivity, linearity, accuracy, and precision associated with their sequence similarities (Table 3). Sufficient linearity was observed with correlation coefficients (*r*) >0.99. Combination of more similar peptides (>60%) such as G^{44}LEWVAEIR^{52} (V_H of infliximab) vs G^{44}LEWIGFIR^{52} (V_H of alemtuzumab) and L^{46}LIYNTNNLQTGVPSR^{61} (V_L of alemtuzumab) vs V^{46}LIYFTSSLHSGVPSR^{61} (V_L of bevacizumab) achieved sufficient QC precision (<20%) and accuracy (<±20%) within 20% (Table 3, columns a–c), although their retention times were significantly different. In comparison, combination of the dissimilar peptides V^{46}LIYFTSSLHSGVPSR^{61} (V_L of bevacizumab) vs S^{92}NWPITFGQGTR^{103} (V_L of ofatumumab) achieved only

reduced accuracy (>±20%) in the standard and QC samples (Table 3, column d), although their retention times were much closer. Therefore, sequence similarity was more important than retention time through compensation for digestion efficiency. Further, selecting suitable similar peptides from an IS mAb represents an alternative strategy to achieving sufficient accuracy and precision for the drug discovery stage without an SIL-IS.

Conclusion

Here, we describe an efficient and practical strategy for developing a method to quantitate biopharmaceutical antibodies. Quantification, including surrogate peptide selection, was achieved by three-step optimization using multiple pSRM. This approach does not require surrogate peptides as standards

Table 3 Calibration curves and the validation data for mAbs using a homologous peptide from another mAb as the IS

		a		b		c		d	
Analyte	mAb surrogate peptide	Infliximab G^{44}LEWVAEIR^{52}		Alemtuzumab L^{46}LIYNTNNLQTGVPSR^{61}		Bevacizumab V^{46}LIYFTSSLHSGVPSR^{61}		Bevacizumab V^{46}LIYFTSSLHSGVPSR^{61}	
Internal standard	mAb surrogate peptide	Alemtuzumab G^{44}LEWIGFIR^{52}		Bevacizumab V^{46}LIYFTSSLHSGVPSR^{61}		Alemtuzumab L^{46}LIYNTNNLQTGVPSR^{61}		Ofatumumab S^{92}NWPITFGQGTR^{103} [c]	
Homology[a]		67%		63%		63%		0%	
Standard (*n* = 1)		%RE	r	%RE	r	%RE	r	%RE	r
		−16.9 to 19.4	0.994	−15.6 to 7.2	0.996	−14.0 to 15.1	0.993	−21.9 to 15.6	0.990
	Conc.	%RE	%CV	%RE	%CV	%RE	%CV	%RE	%CV
QC (*n* = 6)	3 μg/mL	2.2	7.5	BLOQ[b]	BLOQ	13.9	11.8	13.9	11.9
	30 μg/mL	2.0	6.5	6.1	11.9	8.9	3.2	20.3	2.3
	300 μg/mL	−1.6	8.2	4.4	12.7	16.9	4.7	33.4	10.9

Columns a–c are combinations of highly homologous analyte/IS, and column d is nonhomologous combination of analyte/IS

[a] Based on the number of amino acids in the same region

[b] Below the limit of quantification

[c] Nonhomologous peptides from a different region

and can optimize all conditions using the same triple-stage MS following quantitative SRM. Further, optimization for MS conditions using spiked plasma is a more practical approach that avoids the influence of impurities. Duan et al. have reported similar strategy to select surrogate peptides and SRM optimization in a single injection of spiked sample using much more complicated set of SRM (25 SRM for each peptide = 5 different product ions at Q3 × 5 combinations of tube-lens voltage and CE) using nanoLC and quantitative MS [35, 36]. This sophisticated approach requires that qualitative MS be performed first. Quantification is performed using a mAb standard and synthetic peptide as external standards.

Here, in contrast, we used the homologous peptides of another mAb that provided a more practical IS. Generally, the development of an LC-MS method requires weeks to prepare surrogate peptides and SIL peptides [7–9]. Using our strategy, we developed SRM methods for infliximab, alemtuzumab, and bevacizumab in 2 days with sufficient precision and accuracy for application to the drug discovery stage. This strategy can also be applied to the next generation of antibody biopharmaceuticals such as mAb-drug conjugates [37].

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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