Generic MS-based method for the bioanalysis of therapeutic monoclonal antibodies in nonclinical studies

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Aim: A generic bioanalytical method was developed to quantify therapeutic IgG1 monoclonal antibodies (mAbs) in mouse sera by combining an easy sample preparation method with LC/MS using selected reaction monitoring. **Materials & methods:** Rituximab and trastuzumab were used as model mAbs. A synthetic stable isotope-labeled peptide or a stable isotope-labeled mAb was used as an internal standard. The method feasibility was evaluated by a collaborative study involving six laboratories. **Results:** The calibration curve ranged from 1.0 to 1000.0 µg/ml (correlation coefficient >0.99). The validation parameters including selectivity, linearity of calibration curve, accuracy and precision met the predefined acceptance criteria. **Conclusion:** Our method is a useful bioanalytical method for the quantification of therapeutic IgG mAbs in nonclinical animal studies.

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The therapeutic monoclonal antibody (mAb) is one of the most successful biological therapeutics [1]. As of 2019, >50 mAbs were approved in the USA, the EU and Japan [2,3]. The rapid growth of therapeutic mAb applications has increased demand for the bioanalysis of mAb therapeutics. A common bioanalysis technique for mAbs is the ligand-binding assay (LBA), which includes ELISA [4,5]. LBAs are very sensitive and able to measure protein levels as low as the picogram/milliliter level. However, suitable capture reagents for LBAs needed to be developed, which takes time and resources, and the reagents may not be obtained at an early stage of drug development. In addition, measurements by LBAs may be affected by the presence of antidrug antibodies in matrices [6].

Recently, LC/MS has become an important alternative method to LBA for biological therapeutics [7]. The most popular bioanalytical MS method is LC–MS/MS using SRM mode (LC/MS/MS–SRM) on a triple quadrupole mass spectrometer. The LC/MS/MS–SRM assay is already widely used for the quantitative bioanalysis of small-molecule drugs, and has the advantages of high selectivity, a wide dynamic range, and short method development time. In general, the bioanalysis of therapeutic mAbs by LC/MS/MS–SRM is performed as follows: the target

mAb is purified from blood samples and digested to peptides, and the resulting signature surrogate peptide(s) is quantified by LC/MS/MS–SRM [8,9]. The surrogate peptide sequence must be unique to the analytical target proteins in matrices, for example, tryptic peptides from the complementarity determining regions (CDR-peptides) are often used as mAb surrogates. An LC/MS/MS–SRM format using a CDR-peptide could be a useful quantitative bioanalytical method for both nonclinical and clinical studies, and would therefore have a shortened development period. However, this approach could be challenging for the development of a sample preparation method because of the multiple/complicated processes involving affinity purification. In addition, a new LC/MS/MS–SRM method must be developed for each new candidate. To address these issues, several sample preparation kits for mass spectrometric bioanalysis of human mAbs have been developed, and sample preparation is becoming simpler [10].

The ‘universal surrogate peptide approach’ is another LC/MS/MS–SRM method for mAbs analysis in which a common tryptic peptide from the constant region of the mAb is used as a surrogate peptide and such a method was reported previously [11–17]. Furlong *et al.* demonstrated that LC/MS/MS–SRM using the surrogate peptide ‘VSVLTVLHQDWLNGK’ from the heavy chain of human IgG1, 3 and 4 is suitable for the bioanalysis of diverse human Fc region-containing therapeutic protein candidates in plasma samples in animal studies [12]. They also reported the ‘dual universal peptide approach’ using two surrogate peptides from the heavy and light chains, providing increased insight into structural integrity and increased confidence in the study data [17]. In addition to the conventional SRM assay using a triple quadrupole mass spectrometer, an SRM assay using a high-resolution mass spectrometer was also applied for quantitative bioanalysis using a universal surrogate peptide approach [11]. Previous reports suggest that one optimized universal surrogate peptide approach is applicable for the bioanalysis of several therapeutic proteins containing the human Fc-region in animal studies, leading to rapid evaluation of mAb candidates in nonclinical animal studies. However, the robustness and interlaboratory reproducibility of this widely usable approach remains unclear, which hampers its general use. Therefore, we believed that it would be valuable to verify a generic universal peptide approach in multiple laboratories.

In this study, we aimed to develop a simple, robust and low-cost generic LC/MS/MS–SRM approach for target mAb quantification in nonclinical animal studies. We first optimized the sample preparation for mAbs in spiked mouse serum samples, and then evaluated the feasibility of using an LC/MS/MS–SRM method containing the optimized sample preparation method in a collaborative study involving six laboratories, including those from pharmaceutical companies and CROs. Anti-CD20 mAb (rituximab) and anti-Her2 mAb (trastuzumab) were used as model therapeutic mAbs.

Materials & methods

Therapeutic mAbs & mouse sera

Commercially available rituximab and trastuzumab products were used in this study. Pooled sera from normal mice were purchased from Cedarlane Laboratories (Ontario, Canada), Kohjin Bio (Saitama, Japan), Fitzgerald Industries International (MA, USA), Rockland Immunochemicals (PA, USA), Abcam (Cambridge, UK) and Innovative Research (MI, USA).

Reagents & chemicals

The reagents and chemicals used for sample preparation in Lab A are listed in [Supplementary Table 1](#). Trypsin (Trypsin Gold, Promega, WI, USA), synthetic stable isotope-labeled (SIL) peptide (VSVLTVLHQDWL ($^{13}\text{C}_6$; ^{15}N)NGK, SCRUM, Tokyo, Japan), SIL mAb (catalog number; MSQC3, MilliporeSigma, MO, USA), and Oasis HLB μ Elution plate (Waters, MA, USA) were common materials in the collaborative study.

Preparation of stock solutions, serum calibration standards & quality control samples

Stock solutions of rituximab were prepared using phosphate-buffered saline (PBS) at concentrations of 0.01, 0.05, 0.10, 1.00, 5.00 and 10.00 mg/ml. Calibration standards and quality control (QC) samples were prepared by dilution of the rituximab stock solutions with pooled mouse serum. Final concentrations of the calibration standards were adjusted to concentrations of 1.0, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 $\mu\text{g/ml}$, and the QC samples for the collaborative study were prepared at a concentration of 1.0 $\mu\text{g/ml}$. Similarly, the stock solutions, serum calibration standards and QC samples of trastuzumab were prepared, and the QC concentrations were 1.0, 3.0, 50.0 and 800.0 $\mu\text{g/ml}$.

Table 1. The optimized sample preparation method (internal standard: stable isotope-labeled peptide).

Reduction/carbamidomethylation	Tryptic digestion	Peptide purification
Sample solution (5 µl, equivalent to 1 µl serum)[†] ↓←50 mM ABC [‡] (110 µl) ↓←Reducing agent [§] (4 µl) ↓←Incubation at 60°C for 30 min ↓←Alkylation agent [¶] (5 µl) ↓←Incubation at 37°C for 30 min (in dark) RCM sample solution (124 µl)	RCM sample solution ↓←Digesting solution [#] (4 µl) ↓←Incubation at 37°C for 4 h ↓←IS-VVS-pep solution (0.2 µg/ml, 5 µl) Tryptic digest (133 µl)	Tryptic digest ↓←4% Phosphoric acid (300 µl) ↓←Sample apply to Oasis HLB µElution plate ↓←Washing with 5% methanol solution (200 µl) ↓←Eluting with ACN/H ₂ O/acetic acid (100 µl) LC/MS sample (100 µl)

[†]Serum sample was diluted to five volumes using phosphate-buffered saline.
[‡]50 mM ammonium bicarbonate (pH9.0). The pH of ammonium bicarbonate was adjusted to be between pH 7.5 and 8.5 after adding of the digesting solution.
[§]250 mM tris(2-carboxyethyl)phosphine hydrochloride in 50 mM ammonium bicarbonate solution.
[¶]500 mM iodoacetamide in 50 mM ammonium bicarbonate.
[#]1 mg/ml trypsin solution.
 IS-VVS-pep: Internal standard peptide; RCM: Reduced and carbamidomethylated protein.

Internal standard solution

The synthetic SIL peptide (VVSVLTVLHQDWL [¹³C₆,¹⁵N] NGK) was used as an internal standard peptide (IS-VVS-pep). The IS-VVS-pep stock solution was prepared at 1.0 mg/ml using DMSO. An internal standard peptide solution at 0.2 µg/ml (IS-VVS-pep solution) was prepared by diluting the stock solution with DMSO solution.

The SIL mAb was used as an internal standard for the whole mAb (IS-whole mAb). The IS-whole mAb stock solution was prepared at a concentration of 200.0 µg/ml using 0.1% formic acid solution. An IS-whole mAb solution at 10.0 µg/ml was prepared by diluting the stock solution with a 0.1% formic acid solution.

The IS-VVS-pep solution was added to the sample mixture after tryptic digestion, and the IS-whole mAb solution was added before reduction/carbamidomethylation.

Sample preparation

Sample preparation protocols

Serum samples were diluted with four volumes of PBS, and 5 µl of a diluted sample solution (equivalent to 1 µl serum) was used as the starting serum sample for sample preparation. The optimized sample preparation methods are summarized in Table 1 and Supplementary Table 2. The methods consisted of the following three processes: carbamidomethylation, tryptic digestion and peptide purification.

Optimization of the tryptic digestion time

The pooled mouse serum spiked with 1.0 µg/ml rituximab was used as the rituximab sample solution (S). The pooled mouse serum alone was used as the matrix sample solution (M). The S and M solutions were treated according to Table 1. As an additional process, after digestions for 1, 2, 4, 6 and 16 h, synthetic nonlabeled VVS-pep (SCRUM, Tokyo, Japan) was spiked into M (now named as M1) at a molar concentration equal to that of the VVS-pep from rituximab in S (called S1). The samples were analysed with the analytical conditions of Lab A (Supplementary Table 3). The digestion efficiency (%) was calculated by the following formula. The analyses of S1 and M1 were performed in triplicate.

$$\text{Digestion efficacy (\%)} = \frac{\text{average peak area of S1}}{\text{average peak area of M1}} \times 100$$

Peptide recovery of the peptide purification process

Two kinds of matrix sample solutions (S and M) were treated according to Table 1. Additionally, after digestion, S (now called S2) before purification and M (now called M2) after purification were spiked with synthetic nonlabeled VVS-pep at a molar concentration equal to 1.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 µg/ml of rituximab. The sample solutions were analysed by Lab A. The peptide recovery (%) of the peptide purification process was calculated by the following formula. The analyses of S2 and M2 were performed in triplicate.

$$\text{Peptide recovery (\%)} \text{ of the purification process} = \frac{\text{average peak area of S2}}{\text{average peak area of M2}} \times 100$$

Table 2. Parameter and acceptance criteria of analytical method validation used in this study.

Selectivity	Six lots of blanks: <20% of LLOQ for analyte, <5% of IS
Linearity of calibration curve	At least 75% of the calibration standards, with a minimum of six points, including the LLOQ and the highest points, meet the criteria $r > 0.98$ Mean accuracy of each point: within 20% (LLOQ: within ±25%) Precision of each point: ≤20% (LLOQ: ≤25%)
LLOQ	Minimum 3 runs Mean accuracy: within ±25% Precision: ≤25%
Accuracy and precision	Mean accuracy of each QC sample: within ±20% (LLOQ: within ±25%) Precision of each QC sample: ≤20% (LLOQ: ≤25%)
Matrix effect	Six lots Evaluation using QC samples Precision: ≤20%
Carryover	<20% of LLOQ for analyte
Dilution integrity	Mean accuracy: within ±20%, precision: ≤20%
Processed sample stability (stability of MS sample in auto sampler)	Mean accuracy: within ±20%
IS: Internal standard; QC: Quality control; <i>r</i> : Correlation coefficient.	

Collaborative study

The analytical performance of the method was evaluated in terms of selectivity, linearity, accuracy and precision by LSI Medience Corp., Sumika Chemical Analysis Service, Ltd, Sekisui Medical Co. Ltd, Daiichi Sankyo Co., Ltd, Towa Pharmaceutical Co. Ltd, and the National Institute of Health Sciences. The accuracy and precision were calculated based on the measurement results of the QC sample (1.0 µg/ml, *n* = 3) by the following formulas:

$$\text{Accuracy (\%)} = \frac{\text{measurmental}}{\text{theoretical}} \times 100$$

$$\text{Precision CV (\%)} = \frac{\text{standard deviation}}{\text{average}} \times 100$$

The criteria for selectivity, linearity, precision and accuracy were set referring to previous reports (Table 2) [18].

Applicability evaluation

To evaluate the applicability of our method for other mAbs, method validation (selectivity, linearity, LLOQ, accuracy and precision, matrix effect, carryover and stability of the processed sample in the auto sampler) was performed using trastuzumab as a model mAb and IS-VVS-pep as an internal standard by Lab A. The criteria of the method validation are compiled in Table 2.

Results & discussion

Selection of the surrogate peptide

Rituximab and trastuzumab contain the constant regions of the γ1-chain and κ-chain of human antibodies. Supplementary Table 4 shows typical surrogate peptide candidates derived from the γ1-chain and κ-chain in previous reports [11,12,17]. First, we confirmed the ion intensities of these candidates from rituximab. As shown in Figure 1A, the triply charged ion at *m/z* 603.4 of the peptide VSVLTVLHQDWLNGK (VVS-pep) from the human γ1-chain indicated the highest intensity. In addition, its MS/MS spectrum showed preferential generation of the γ_{14}^{2+} fragment ion at *m/z* 805.7 (Figure 1B). The VVS-pep underwent less intensity loss during MS/MS fragmentation, therefore, we decided to use this peptide as surrogate peptide in this study. The Q1 and Q3 transitions were set to *m/z* 603–604 (3⁺) and *m/z* 805–806 (2⁺), respectively. The transitions of the two internal standards, IS-VVS-pep and IS-whole mAb, were set to *m/z* 606 (Q1) and *m/z* 809 (Q3), and *m/z* 606 (Q1) and *m/z* 809–810 (Q3), respectively. Meanwhile, the γ_{11}^{2+} , γ_{12}^{2+} and γ_{15}^{2+} fragment ions were also good candidates for the Q3 ion. When any interferences are problems, using these multiple fragment ions would be more suitable for quantification.

Figure 1. Selection of surrogate peptide.

(A) The ion intensities of precursor ions from the surrogate peptide candidates. (B) The fragmentation pattern of a surrogate peptide, VVSVLTVLHQDWLNGK, from human γ 1-chain obtained by LC/MS/MS.

Optimization of sample preparation

We designed the conditions of the reduction/carbamidomethylation, tryptic digestion and peptide purification processes by referring to previous reports [11–16]. We first evaluated the efficiencies (%) of the tryptic digestion process shown in Table 1 while varying the digestion time at 1, 2, 4, 6 and 16 h (Figure 2A); the most effective digestion time was between 2 and 4 h (78–79%). Interestingly, longer digestion times (6 and 16 h) decreased the efficiency. Although the detailed reason for this decreased efficiency was unclear, ion suppression with an increase in tryptic peptide generations, peptide stability in the reaction solution and/or nonspecific digestion may be involved. Based on the results, the tryptic digestion time was set to 4 h in our method.

Next, we verified the recovery (%) of the surrogate peptide after purification (Figure 2B). When mouse sera sample solutions containing nonlabeled VVS-pep corresponding to rituximab at 1.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 μ g/ml were tested, the peptide recoveries (%) of all sample solutions were almost constant at 70–80%

Figure 2. Optimization of sample preparation processes.

(A) The influence of tryptic digestion time on peptide recovery. **(B)** The influence of the concentration of monoclonal antibody in the serum sample on peptide recovery. The values in the panels were calculated using the average values of triplicate experiments ($n = 3$).

recovery. We confirmed that the performance of our peptide purification process was suitable in the range of 1.0 to 1000.0 $\mu\text{g/ml}$ mAb.

Optimization of LC/MS conditions

The optimal LC/MS conditions are dependent on the specific LC/MS system, including the type of analytical column, in each laboratory. Therefore, we optimized the LC/MS conditions in each laboratory (Supplementary Table 3).

Evaluation of method feasibility through a collaborative study on rituximab

As shown in the previous sections, we successfully designed an LC/MS/MS–SRM method with sample preparation using IS-VVS-pep or IS-whole mAb (Table 1 & Supplementary Table 2). To confirm the feasibility of the methods, its analytical performance was evaluated in terms of selectivity, linearity, accuracy and precision through a collaborative study involving six laboratories. Commercially available therapeutic rituximab was used as a model mAb. The same lots of rituximab, normal mouse sera, trypsin and internal standards (IS-VVS-pep and IS-whole mAb) were used as common materials.

Selectivity

Selectivity was evaluated using double and single blank samples obtained from six individual mouse serum samples. Single blank samples were prepared by spiking appropriate amounts of the internal standards (IS-VVS-pep or IS-whole mAb) into each mouse serum sample in accordance with Table 1 and Supplementary Table 2. As shown in Table 3, the interfering peaks $>20\%$ of the LLOQ (1.0 $\mu\text{g/ml}$) responses of the analyte (VVS-pep), and $>5\%$ of the internal standard responses were not observed in any double blank or single blank samples from any of the laboratories, indicating acceptable selectivity of our method.

Table 3. Selectivity evaluation using six individual mouse sera.

Lab	Double or single blank	Interference peak area against analyte LLOQ peak area (%)						Interference peak area against IS peak area (%)					
		M1	M2	M3	M4	M5	M6	M1	M2	M3	M4	M5	M6
A	Double	0.0	3.7	0.0	0.0	0.0	0.0	0.1/0.0 [†]	0.1/0.0	0.2/0.0	0.2/0.0	0.2/0.0	0.2/0.0
	Single (IS-VVS-pep)	2.4	1.0	0.0	0.0	2.8	0.0	N/A [‡]	N/A	N/A	N/A	N/A	N/A
	Single (IS-whole mAb)	3.0	9.1	4.9	9.0	0.0	5.7	N/A	N/A	N/A	N/A	N/A	N/A
B	Double	0.0	0.0	0.0	0.0	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	Single (IS-VVS-pep)	0.0	0.0	8.3	0.0	0.0	6.2	N/A	N/A	N/A	N/A	N/A	N/A
	Single (IS-whole mAb)	0.0	8.5	0.0	0.0	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A
C	Double	0.0	8.7	0.0	5.5	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	Single (IS-VVS-pep)	0.0	0.0	0.0	0.0	0.0	5.6	N/A	N/A	N/A	N/A	N/A	N/A
	Single (IS-whole mAb)	7.3	2.9	0.0	0.0	0.0	2.3	N/A	N/A	N/A	N/A	N/A	N/A
D	Double	0.0	0.0	0.0	8.0	7.4	8.4	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	Single (IS-VVS-pep)	0.0	0.0	13.8	10.5	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A
	Single (IS-whole mAb)	0.0	0.0	0.0	0.0	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A
E	Double	0.0	0.0	0.0	0.0	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	Single (IS-VVS-pep)	0.0	0.0	0.0	0.0	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A
	Single (IS-whole mAb)	0.0	0.0	0.0	0.0	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A
F	Double	0.0	0.0	0.0	0.0	0.0	0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
	Single (IS-VVS-pep)	0.0	0.0	0.0	0.0	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A
	Single (IS-whole mAb)	0.0	0.0	0.0	0.0	0.0	0.0	N/A	N/A	N/A	N/A	N/A	N/A

[†] The values indicate the interference peak area for IS-VVS-pep and IS-whole mAb (IS-VVS-pep/IS-whole mAb).

[‡] Not applicable.

IS: Internal standard; IS-VVS-pep: Internal standard peptide; IS-whole mAb: Internal standard for the whole monoclonal antibody; Lab: Laboratory.

Linearity of the calibration curve

The linearity of the calibration curve of our method was evaluated using a linear regression model after measuring seven calibration standards spiked with rituximab (1.0, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 µg/ml; $n = 1$). The weighted calibration plot was generated using the $1/x^2$ method in all the laboratories. The calibration curve obtained by Lab A is shown in Figure 3. The calibration fit formulas were $Y = 0.030X - 0.0066$ (correlation coefficient $[r] = 0.9988$) for IS-VVS-pep and $Y = 0.037X - 0.0070$ ($r = 0.9989$) for IS-whole mAb. The accuracy obtained for the calibration standards ranged from 90.7 to 105.5%. Table 4 shows the parameters of the calibration curves from all the laboratories. The calibration curves obtained using IS-VVS-pep and IS-whole mAb showed good linearity from 1.0 to 1000.0 µg/ml (r values from 0.9939 to 0.9992), with good accuracy (83.0–114.7%) for the calibration standards.

Accuracy & precision

Accuracy and precision were determined by analysis of the 1.0 µg/ml QC sample. The measurement was performed in triplicate. When IS-VVS-pep was used as an internal standard, the mean accuracy and precision were 102.3–114.3% and 3.5–11.4%, respectively, and when IS-whole was used as an internal standard, these parameters were 82.4–111.0% and 2.5–8.7%, respectively (Table 5). When IS-VVS-pep was used as internal standard, the accuracy was above 100%, suggesting the low recovery of the standard peptide. However, the same trend was not observed in QC assays using trastuzumab (Supplementary Table 7). These results suggested that the increased trend was not specific to the standard peptide. In addition, the difference between internal standard peptide (IS-pep) and IS-whole mAb did not influence the variability of our SRM assay. In the case of SRM assay without affinity purification process, IS-pep might be enough as internal standard.

Collectively, the feasibility of using LC/MS/MS–SRM with our sample preparation method was demonstrated in all laboratories regardless of the different analytical conditions of the instrumentation and LC/MS systems in each laboratory. Therefore, our method is sufficiently robust.

Figure 3. Calibration curves for the quantification of rituximab in mouse serum obtained by laboratory A. (A) The calibration curve using IS-VVS-pep (internal standard peptide) and (B) the calibration curve using internal standard-whole monoclonal antibody.

Applicability evaluation with trastuzumab

The applicability of our method was evaluated by method validation using calibration standards and QC samples spiked with another mAb, trastuzumab. The evaluation was performed by only Lab. A and the results are summarized in Supplementary Tables 5–11.

Selectivity

Selectivity was evaluated using double blank samples obtained from six individual mouse serum samples. Interfering peaks for the analyte (VVS-pep) and internal standard (IS-VVS-pep) were detected in the SRM chromatograms

Table 4. Standard curve parameters and accuracy of calibration standards.

Lab	IS	Slope	Intercept	r	Nominal concentration of monoclonal antibody (µg/ml), accuracy (%)						
					1000.0	500.0	100.0	50.0	10.0	5.0	1.0
A	IS-VVS-pep	0.030	-0.0066	0.9988	100.0	101.9	105.5	101.2	98.7	90.7	101.9
	IS-whole mAb	0.037	-0.0070	0.9989	103.3	103.6	97.6	104.4	93.1	96.7	101.3
B	IS-VVS-pep	0.075	-0.0174	0.9980	95.5	103.4	111.0	98.4	95.8	94.0	102.0
	IS-whole mAb	0.147	-0.0483	0.9967	99.7	102.2	112.0	95.8	102.0	86.4	102.0
C	IS-VVS-pep	0.089	-0.0031	0.9992	94.9	105.6	96.4	102.2	99.1	102.2	99.6
	IS-whole mAb	0.146	0.0110	0.9988	95.7	103.8	101.0	94.4	107.0	97.6	99.8
D	IS-VVS-pep	0.021	0.0010	0.9939	112.0	108.4	107.0	92.2	93.8	83.0	104.0
	IS-whole mAb	0.074	-0.0213	0.9983	98.9	101.2	104.0	105.0	101.0	88.2	102.0
E	IS-VVS-pep	0.013	-0.0025	0.9943	107.0	111.0	107.0	97.8	87.9	85.0	104.0
	IS-whole mAb	0.078	-0.0028	0.9962	103.0	102.4	110.0	92.4	104.0	85.6	103.0
F	IS-VVS-pep	0.303	-0.0750	0.9981	92.3	98.4	103.4	108.0	103.9	93.1	100.8
	IS-whole mAb	0.272	-0.0780	0.9952	87.9	91.2	104.6	98.0	114.7	106.3	97.3

IS-VVS-pep: Internal standard peptide; IS-whole mAb: Internal standard for the whole monoclonal antibody; Lab: Laboratory; r: Correlation coefficient.

Table 5. Accuracy and precision (1.0 µg/ml, n = 3).

Laboratory	Internal standard			
	IS-VVS-pep		IS-whole mAb	
	Mean accuracy (%)	Precision (CV%)	Mean accuracy (%)	Precision (CV%)
A	105.9	5.5	91.8	6.8
B	112.1	11.0	111.0	6.8
C	107.3	3.5	110.7	8.7
D	114.3	8.7	98.5	8.4
E	102.3	5.5	82.4	4.7
F	102.9	11.4	92.6	2.5

IS-VVS-pep: Internal standard peptide; IS-whole mAb: Internal standard for the whole monoclonal antibody.

of M1, M4 and M6, and of M4 and M5, respectively (Supplementary Table 5). However, interfering peaks with responses >20% of the response of the LLOQ (1.0 µg/ml) of VVS-pep, and >5% of that of IS-VVS-pep were not observed in any samples. These data confirm the acceptable selectivity of our method.

Linearity of the calibration curve

The linearity of the calibration curve was evaluated using a linear regression model after measuring seven calibration standards spiked with trastuzumab (1.0, 5.0, 10.0, 50.0, 100.0, 500.0 and 1000.0 µg/ml; n = 2) (Supplementary Table 6 & Supplementary Figure 1). The weighted calibration plot was generated using the $1/x^2$ method as the plot for rituximab was. The calibration fit formula was $Y = 0.025X - 0.0069$ ($r = 0.9977$). The accuracy of the calibration standards ranged from 85.2 to 109.4%, satisfying the predefined acceptance criteria.

LLOQ

The LLOQ was defined as the lowest concentration of the calibration curve with acceptable accuracy, which was determined to be 1.0 µg/ml (Supplementary Table 6). The mean accuracy at the LLOQ was within $\pm 11\%$ of the theoretical concentration, and the precision at the LLOQ was not >11% (Supplementary Table 7).

Accuracy & precision

The accuracy and precision were evaluated by analysing three replicates of pooled mouse serum samples spiked with trastuzumab at four concentrations: the LLOQ, low and intermediate concentrations, and the ULOQ (i.e., 1.0, 3.0, 50.0 and 800.0 µg/ml, respectively). As shown in [Supplementary Table 7](#), the mean accuracy and precision at the four level concentrations were 90.6–107.7% and 2.1–10.9%, respectively.

Matrix effect

Six mouse serum samples were analysed to determine the matrix effect at the low (3.0 µg/ml) and ULOQ (800.0 µg/ml) concentrations. The mean concentrations determined at the low and ULOQ were 2.7 and 825.3 µg/ml, respectively, and the precision was within the accepted criteria ($\leq 20\%$) ([Supplementary Table 8](#)).

Carryover

Carryover, which is defined as residual analyte in the autosampler and column, was investigated by analyzing a double blank sample for the analyte (VVS-pep) and internal standard (IS-VVS-pep) after analyzing the highest-concentration calibration standard (1000.0 µg/ml). Carryover was calculated as the percent response in the double blank sample relative to that in the LLOQ sample. No carryover peaks were observed for VVS-pep and IS-VVS-pep ([Supplementary Table 9](#)).

Dilution integrity

The effect of the dilution steps in our bioanalytical method on the rituximab concentration was evaluated by preparing a mouse serum sample spiked with 1500.0 µg/ml rituximab, and analyzing five tenfold dilutions of this sample. The mean accuracy and precision of the diluted samples were 105.2 and 4.4%, respectively. These values met the performance criteria, and therefore, suggested no analyte interference from dilution ([Supplementary Table 10](#)).

Processed sample stability

The processed sample stability at the low (3 µg/ml) and ULOQ (800.0 µg/ml) concentrations was evaluated at 5°C for 48 h. The mean accuracy (%) of the low concentration and ULOQ was 90.5 and 108.3%, respectively ([Supplementary Table 11](#)).

Our group believes that the universal surrogate peptide approach is an important and useful bioanalytical method for mAb analysis in nonclinical animal studies due to its high versatility and applicability. To increase the generalization of this noteworthy approach, we developed a universal surrogate peptide approach by combining the original sample preparation method and a conventional SRM assay and demonstrated that this method is a useful general and robust approach for mAb analysis in a collaborative study. The most common method used in pharmacokinetic studies of rituximab and trastuzumab is ELISA. In a nonclinical pharmacokinetic study, the mean values of maximum concentration (C_{max}) in a single- and multiple-dose study of 20 mg/kg rituximab were 726.0 ± 138.0 and 1230 ± 313.0 µg/ml, respectively [19]. Additionally, it was reported that the mean value of minimum concentration (C_{min}) in a multiple-dose study of 10 mg/kg rituximab was 59.5 ± 64.9 µg/ml [20]. In another nonclinical pharmacokinetic study, the mean values of C_{max} in a single-dose study of 10 mg/kg trastuzumab were 269 ± 52.7 and 281 ± 49.9 µg/ml [21]. Our method showed a broad dynamic range from 1.0 to 1000.0 µg/ml, and the accuracy and precision of all calibration standards and QC samples met the predefined performance criteria. Therefore, our method can be applied to serum concentration assays of therapeutic IgG1 (and IgG4) mAbs in nonclinical studies.

Our group also discussed the acceptance criteria of accuracy and precision for method validation, based on the recommendations in previous reports [18,22,23]. The variability in the SRM assay results for large molecules is potentially higher than that for small molecules because the complexity of the sample preparation process is greater for the former than for the latter. Moreover, the bioanalysis of rituximab and trastuzumab have been generally performed with the LBA method; for these reasons, the criteria ($\leq 20\%$ [LLOQ: $\leq 25\%$]) in the guideline on bioanalysis by the LBA method were adopted as the criteria of our method validation [24]. However, in our collaborative study, four laboratories achieved accuracy and precision values below the acceptance criteria (accuracy, $100 \pm 15\%$ [LLOQ: $\pm 20\%$]; precision, $\leq 15\%$ [LLOQ: $\leq 20\%$]) for the bioanalysis of small molecules using a chromatographic method [25]. Discussion on the acceptance criteria for MS-based bioanalysis of large

molecules is still ongoing in the bioanalytical community, and the criteria have not been clearly established in any guideline/guidance. Our current data could accelerate such discussions for mAbs.

Conclusion

In this study, we developed a simple, robust and low-cost general LC/MS/MS–SRM bioanalytical method for therapeutic IgG1 mAb candidates in mouse serum by combining a developed sample preparation method and a conventional LC/MS/MS–SRM. We also demonstrated the feasibility of the method through a collaborative study involving six laboratories. This method has a sufficient calibration range (1.0–1000.0 µg/ml) and LLOQ (1.0 µg/ml) for use in animal studies and will be a useful bioanalytical method for the quantification of therapeutic IgG mAbs in nonclinical animal studies.

Future perspective

The universal surrogate peptide approach using LC/MS/MS–SRM is useful for the quantification of therapeutic mAb candidates in nonclinical animal studies. However, the robustness and interlaboratory reproducibility of the approach remain unclear. In this study, we developed a generic MS method for the quantification of therapeutic mAb candidates in mouse serum. In the future, we hope that our method will be widely used as a template method for MS-based bioanalysis of mAb candidates in nonclinical animal studies.

Summary points

Background

- To increase the generalization of a universal surrogate peptide approach, a generic bioanalytical method for quantification of therapeutic monoclonal antibodies (mAbs) in mouse sera was developed.

Experimental

- Rituximab and trastuzumab were used as model mAbs.
- The analytical performance of the LC/MS/MS–SRM method with the optimized sample preparation method was evaluated through a collaborative study involving six laboratories.

Results & discussion

- A simple and easy sample preparation method consisting of carbamidomethylation, tryptic digestion and peptide purification processes was established.
- The LC/MS/MS–SRM method showed a broad dynamic range from 1.0 to 1000.0 µg/ml.
- The selectivity, linearity of calibration curve, accuracy and precision determined by all laboratories in the collaborative study met the predefined acceptance criteria.

Conclusion

- Our method is a useful bioanalytical method for the quantification of therapeutic IgG mAbs in nonclinical animal studies.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2019-0253

Author contributions

N Hashii designed the study and wrote the initial draft of the manuscript. N Hashii and Y Tousaka contributed to the optimization of sample preparation method, and N Hashii and Y Tousaka, K Arai, R Goda, N Inoue, K Murata, T Okuzono, S Sasahara, T Shigeyama, H Tachiki and S Yamane contributed to the verification and validation of our LC/MS/MS–SRM method. A Ishii-Watabe and Y Saito supervised the study. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript for submission.

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