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Development of the Complement C5 Assay by LC−MS/MS in Monkey Serum and Comparison with Enzyme-Linked Immunosorbent Assay

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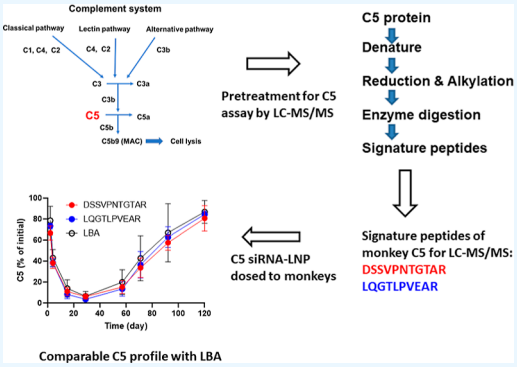
ABSTRACT: Complement C5 (C5) is the key component for the complement activation pathway, which is important for innate immunity, and inhibition of C5 is considered to be effective in antibody-mediated rejection in organ transplantation. Thus determination of C5 levels in systemic circulation is a simple way to understand efficacy of drugs that aim to inhibit C5 production. We have developed a simple liquid chromatography with tandem mass spectrometry (LC−MS/MS) assay for C5 in cynomolgus monkey serum. C5 in monkey serum was subjected to tryptic digestion, and two signature peptides, DSSVPNTGTAR and LQGTLPVEAR, were assayed by LC−MS/MS with electrospray ionization in the positive ion mode. Assay reproducibility in serum samples was evaluated, and the assay was applied to the C5 assay in monkey serum after administration of C5 siRNA encapsulated in lipid nanoparticles to monkeys. The time profiles of C5 after administration of C5 siRNA were comparable between the two signature peptides by LC−MS/MS and were also similar to those by an enzyme-linked immunosorbent assay using an assay kit. These findings suggest that the established LC−MS/MS assay of C5 is reliable to determine C5 levels in monkey serum.



1. INTRODUCTION

Complements are involved in immune responses, and activation of complements results in various defenses against infection. Complement C5 (C5) is a key component since C5 is the common component in all the pathways for activation of complements. Inhibition of C5 is thus considered to be effective in attenuating complement-oriented immune responses, and thus various modalities of anti C5 therapeutics are currently under development.[1] Among them, small interfering RNA (siRNA) against C5 (C5 siRNA) is under development as a therapeutic oligonucleotide[2,3] and the determination of C5 levels in the blood is important to understand whether C5 siRNA therapeutics are efficacious. Assays of complements in the systemic circulation (i.e., blood, plasma, or serum) have been traditionally conducted by ligand binding assays (LBA) including enzyme-linked immunosorbent assay (ELISA).[4−6] Indeed, various assay kits for the quantification of complements, including C5, are commercially available. In clinical settings, these assay kits are frequently used for research purposes.

Liquid chromatography with tandem mass spectrometry (LC−MS/MS) is another powerful assay platform for selective determination of drugs and/or biomarkers, and it is considered that a higher number of researchers can operate the LC−MS/MS than LBA. Indeed, C5 has been also used as a biomarker.[7,8] In addition, comparing results from the two assay platforms is essential to ensuring that the obtained results are reliable. However, the assay of complements by LC−MS/MS is very limited. Cao et al. developed a C5 assay in monkey serum.[9] They established a total C5 assay from 3 to 250 μg/mL using 25 μL of monkey serum samples and applied the method to determine C5 levels in monkeys that received a therapeutic antibody against C5. A typical assay scheme of proteins by LC−MS/MS (i.e., denature, alkylation, and digestion) was employed for the C5 assay, and LQGTLPVEAR was finally selected as a signature peptide of C5.[9] It is important to monitor multiple signature peptides to ensure whether the assay, by using a selected surrogate signature peptide, represents that of the protein. In this sense, we searched for other signature peptides that can be useful for the assay of C5 and successfully identified another signature peptide for accurate determination of C5 in monkey serum. Using multiple

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Table 1. Mass Spectrometric Conditions for the Complement C5 Assay

signature peptides	precursor ion (m/z)	product ion (m/z)	collision energy (V)	declustering potential (V)	collision cell exit potential (V)
DSSVPNTGTAR	552.768	716.369	28.8	71.4	6
LQGTLPVEAR	542.311	571.320	28.4	70.7	6

signature peptides, we ensured that the identified signature peptide is useful for quantifying C5 levels.

This study presents the development of a simple assay for the determination of C5 levels in cynomolgus monkeys by LC−MS/MS and compares the results with those obtained by ELISA using a commercially available assay kit. Comparable assay results between the two platforms ensure that the assay results are reliable and that LC−MS/MS is a powerful platform for the assay of C5.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents. C5-siRNA encapsulated in LNP was prepared at Eisai Co., Ltd. (Ibaraki, Japan). Blank dog serum was prepared by centrifugation of whole blood collected from several dogs by Sunplanet Co., Ltd. (Ibaraki, Japan). Individual blank monkey serum was purchased from Eve Biosciences (Wakayama, Japan). Cynomolgus Complement C5 Protein His Tag was purchased from AcroBiosystems, Inc. (Newark, DE). A human C5 assay kit, which was used for monkey C5 assays, was purchased from Abnova (Taipei, Taiwan). Sodium deoxycholate was purchased from Tokyo Chemical Co., Ltd. (Tokyo, Japan). Triethylammonium bicarbonate (TEAB) and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid was purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan).

2.2. Assays. *2.2.1. LC−MS/MS Assay.* Monkey C5 protein in purified water (2 mg/mL) was diluted 3-fold by dog serum, and the following calibration samples were prepared by a mixture of dog serum-purified water (2:1, v/v): 1, 3, 10, 50, 100, 400, and 500 μg/mL in serum. Quality control (QC) samples were prepared in the same manner as calibration samples at 3 (low concentration QC, LQC), 50 (mid concentration QC, MQC), and 400 (high concentration QC, HQC) μg/mL. It is not possible to use monkey serum in preparing the calibration and QC samples since endogenous monkey complement C5, which hinders the assay of spiked complement C5, is present. As long as no interferences by endogenous complement C5 are observed, blank serum from other animal species can be used. Dog blank serum was used in this study since no interfering peaks derived from endogenous dog complement C5 were observed. In addition, dogs are typical large animal species used for toxicological or pharmacological studies in drug development, as are monkeys. Signature peptides for monkey C5 were searched using the Skyline software, with their mass transitions by multiple reaction monitoring via the mass spectrometer, Triple Quad 6500+ (Sciex, Framingham, MA). Suitable signature peptides were selected using dog serum samples spiked with monkey C5 authentic standard at low and high concentrations, along with nonspiked blank samples. The samples were subjected to tryptic digestion and an assay using the mass transition designated by the Skyline software. In the pretreatment for the C5 assay, 5 μL of water was added to 10 μL of serum samples containing C5. To the mixture, 20 μL of 1% sodium deoxycholate in 50 mM TEAB and 5 μL of 40 mM DTT in 50 mM TEAB were added and incubated for 1 h at 60 °C. After centrifugation (200g, 5 s), 5 μL of 40 mM iodoacetamide in 50 mM TEAB was added and incubated at room temperature for 10 min with protection from light. After incubation, 40 μL of 50 mM TEAB was added, followed by 10 μL of 2 mg/mL trypsin in 50 mM TEAB. The mixture was incubated at 37 °C for 4 h, 5 μL of formic acid was added, and the final mixture was centrifuged (10,000g, 30 min, 4 °C). A 5 μL aliquot was injected into the LC−MS/MS system. The signature peptides were assayed by a Nexera MP HPLC system (Shimadzu, Kyoto, Japan) with a triple quadrupole mass spectrometer, Triple Quad 6500+. The signature peptides were separated on an analytical column, Peptide (5 μm, 2.1 mm inner diameter ×50 mm, Sciex), at 40 °C. The mobile phase consisting of (A) 0.02% formic acid in water and (B) 0.02% formic acid in acetonitrile, at a flow rate of 0.7 mL/min, was used for separation under the following gradient conditions: 2% (B) was maintained up to 0.5 min, and % (B) was increased to 25% until 8 min. The % (B) was increased linearly to 90% until 8.5 min and maintained for 1 min. The % (B) was decreased linearly to 2% up to 10 min and maintained for 2.5 min. The total assay time was 12.5 min per sample. The signature peptides were detected by the following analyte-independent mass spectrometry conditions: ion spray voltage, 4000 V; source temperature, 600 °C; curtain gas flow, 30 psi; collision gas, 10; ion source gas 1 and 2, 50, and 70 psi, respectively. The analyte-specific conditions are represented in Table 1.

The signature peptides were selected by taking the following into consideration: (1) minimal selectivity issues (C5-derived peaks were separated from interferences derived from endogenous proteins), (2) a high signal-to-noise ratio at the lower limit of quantification, and (3) relatively high intensities with proportional signals depending on the spiked C5 concentrations. Multiple concentrations of monkey C5 authentic standard were spiked in blank dog serum samples for preparing calibration curves. The C5 concentrations of the calibration samples and the corresponding peak area of the signature peptides were plotted, which were subjected to linear regression. The signature peptides with acceptable linearity and a relative error (RE) $\leq$ 20% across the tested calibration concentrations were finally selected for the C5 assay in serum.

2.2.2. LBA. The commercially available human C5 assay kit was used in this study, and the assay kit utilized ELISA. The standard solution (10 ng/mL) was diluted by diluent included in the assay kit to prepare the following standard solutions: 0.313, 0.625, 1.25, 2.5, 5.0, and 10.0 ng/mL. These calibration standard solutions were prepared just prior to use. Concentrations of QC samples were 0.313 (lower limit of quantification, LLOQ), 0.625 (LQC), 2.5 (MQC), 8.0 (HQC), and 10.0 (upper limit of quantification, ULOQ) ng/mL, respectively. The concentrations of calibration samples and QC samples were represented without considering the minimum required dilution (MRD). C5 in monkey serum was assayed by a sandwich ELISA using the assay kit for human C5 per instruction. C5 was captured by precoated anti-human C5 polyclonal antibody and detected by biotinylated anti-human C5 polyclonal antibody, which is recognized by streptavidin-

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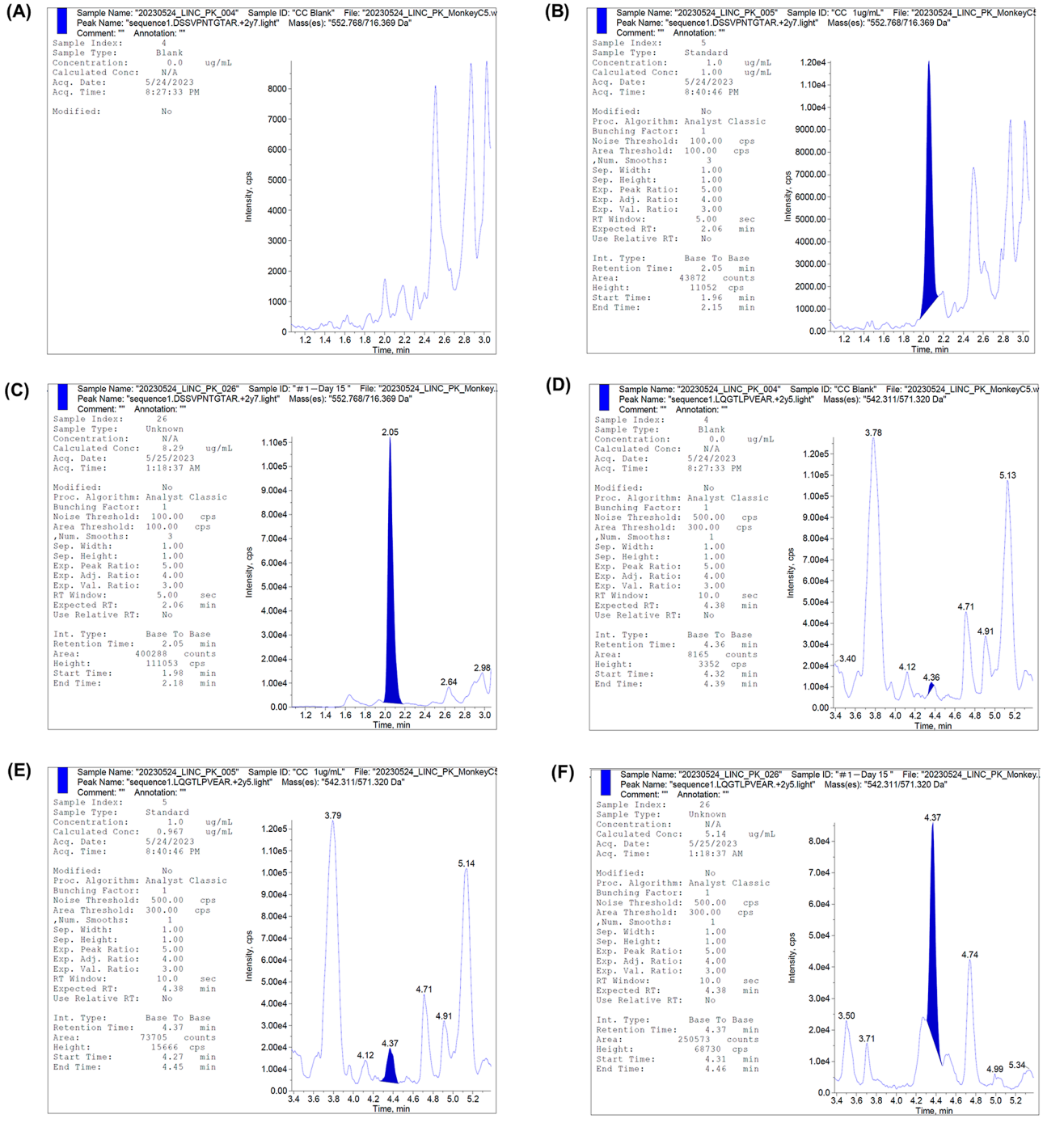

Figure 1. Typical chromatograms of signature peptides of complement C5 by LC−MS/MS. Chromatograms of blank serum, the lower limit of quantification sample, and the postdose sample on Day 15 for the two signature peptides, DSSVPNTGTAR and LQGTLPVEAR. (A and D) chromatograms of blank samples for DSSVPNTGTAR and LQGTLPVEAR, respectively. (B and E) Chromatograms of the LLOQ samples for DSSVPNTGTAR and LQGTLPVEAR, respectively. (C and F) Chromatograms of postdose samples for DSSVPNTGTAR and LQGTLPVEAR, respectively.

peroxidase conjugate with chromogen as a substrate. Diluted serum samples with MRD of 2000 (50 μL) were added to 96 wells of assay plates in which the capture reagent was immobilized and was incubated for 2 h at room temperature. Wells were washed 6 times with 300 μL of wash buffer, and the detection antibody (50 μL) was added. After 1 h of incubation at room temperature, the wells were washed 6 times with 300 μL of wash buffer. The streptavidin-peroxidase conjugate (50 μL) was added to the wells and incubated for 0.5 h at room temperature. The wells were washed 6 times with 300 μL of wash buffer, and then chromogen (50 μL) was added to the wells as a substrate and incubated for 0.2 h at room temperature. The reaction was terminated by adding stop solution (50 μL) and the absorbance was measured at 450 and 570 nm. Nominal C5 concentrations of the calibration samples and the corresponding mean absorbance (450−570 nm) of duplicate determination were plotted as a calibration curve and was subjected to four-parameter regression as in the following

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ACS Omega 2024, 9, 6797−6802

equation. Mean absorbance = $[(A - D)/[1 + (\text{concentration}/C)^B] + D$, where A, B, C, and D represent the signal when concentration is zero, the slope of the linear part of the calibration curve, concentration corresponding to the midpoint between A and D, and signals corresponding to the asymptote at high values on the x-axis, respectively. C5 concentrations were calculated from the corresponding signals.

2.3. Method Validation. *2.3.1. LC−MS/MS Assay.* The C5 assay by LC−MS/MS was validated by evaluating intra-batch reproducibility and processed sample stability. As C5 deficient monkey serum is not available, it was not possible to assess selectivity in monkey serum, and dog blank serum was used to prepare calibration samples and QC samples. The calibration samples of monkey C5 ranging 1−500 μg/mL and the QC samples (1, 3, 50, and 400 μg/mL) were subjected to the assay. In the intra-batch reproducibility test, QC samples (five replicates per concentration) were assayed with calibration samples, and C5 concentrations were determined. The RE and relative standard deviation (RSD) were calculated based on the following equations.

$$\text{RE (\%)} = (\text{mean determined concentration} - \text{nominal concentration})/\text{nominal concentration} \times 100$$

$$\text{RSD (\%)} = \text{standard deviation of determined concentrations}/\text{mean determined concentration} \times 100$$

The RE and RSD should be within ±20% and 20%, respectively (±25% and 25% were allowed at the LLOQ). In the processed sample stability test, percentage of bias (% of bias) was calculated for samples stored under the designated conditions against the time = 0 samples in the following equation.

$$\text{\% of bias} = \text{Mean of } [(\text{peak area of the stored samples} - \text{peak area of the time} = 0 \text{ samples})/(\text{peak area of the time} = 0 \text{ samples} \times 100)]$$

% of bias should be within ±20% when judged stable.

2.3.2. LBA. Intra-batch reproducibility, dilution integrity, and stability were evaluated. In the intra-batch reproducibility test, the QC samples (0.313, 0.625, 2.5, 8.0, and 10.0 ng/mL, six replicates per concentration) were assayed with calibration samples with varying C5 levels (0.313−10 ng/mL). The RE and RSD of the QC samples at the five levels were calculated. The RE and RSD should be within ±20% and 20%, respectively (±25% and 25% were allowed at the LLOQ). Dilution integrity was assessed by diluting monkey serum by 5000-, 10,000-, 20,000-, and 40,000-fold (triplicates per dilution fold), and C5 concentrations by each dilution fold were determined. Percentage of bias (% of bias) of determined C5 concentrations by 5000-, 10,000-, and 40,000-fold dilution was calculated against the one at 20,000-fold dilution. The % of bias should be within ±20%. The dilution integrity was also evaluated using spiked serum samples. To monkey serum diluted by 2000- and 5000-fold, 5 ng/mL C5 was spiked, and RE of the resulting samples was calculated. The RE should be within ±20%. In the stability assessments, freeze−thaw and frozen stability were evaluated using naive monkey serum without spiking C5 standard (triplicates per concentration). The % of bias in concentrations of the stored samples was calculated against the initial concentrations (time = 0). The % of bias in the stability tests should be within ±20% when judged stable.

2.4. In Vivo Study in Monkeys. Animal care and experimental procedures were performed in an animal facility accredited by the Health Science Center for Accreditation of Laboratory Animal Care and Use of the Japan Health Sciences Foundation. All protocols were approved by the Institutional Animal Care and Use Committee at Eisai Co., Ltd. and Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan) and carried out in accordance with the Animal Experimentation Regulations.

C5 siRNA encapsulated in lipid nanoparticle (LNP) was intravenously administered to four male cynomolgus monkeys, and blood samples were serially collected up to 127 days postdose. Predose samples were also collected 1 day before the administration. C5 siRNA encapsulated in LNP was used in this study to assess changes in complement C5 in monkeys. C5 siRNA encapsulated in LNP is currently under development at Eisai Co., Ltd. and it is critical to ensure C5 knockdown in monkeys for translation of pharmacological outcomes in clinical trials. Among the samples collected, all the samples were subjected to the LBA while a portion of samples were subjected to the LC−MS/MS assay. The samples for the LC−MS/MS assay were as follows: −1, 2, 4, 15, 29, 57, 71, 92, and 120 days postdose. The serum samples were stored frozen until the assay of C5 levels and % of initial was calculated against the predose C5 level (on day −1) in each individual monkey.

3. RESULTS AND DISCUSSION

3.1. Method Validation. *3.1.1. LC−MS/MS Assay.* Calibration curves showed that C5 was quantifiable from 1 to 500 μg/mL by using the two signature peptides. Selectivity was assessed in blank serum to check whether any interfering peaks are eluted at the retention times of the signature peptides. In blank serum, no interfering peaks were observed at the elution of the two signature peptides (Figure 1). The peak area at the LLOQ showed sufficiently high signal-to-noise ratio (>5), which allows reproducible detection of the LLOQ samples (Figure 1). To ensure reproducibility of the developed LC−MS/MS assay, accuracy and precision of the assay was estimated at four concentrations (1, 3, 50, and 400 μg/mL) in the intra-batch reproducibility (Table 2). The RE at the LLOQ (1 μg/mL) was 12.0 and 5.0%, for the signature peptides, DSSVPNTGTAR and LQGTLPVEAR, respectively. The RSD at the LLOQ was 9.4 and 8.0% for DSSVPNTGTAR and LQGTLPVEAR, respectively. The RE and RSD were within ±9.3% and 5.0%, respectively, at the other three concentrations for the signature peptide, DSSVPNTGTAR. The RE and RSD were equal to or less than ±11.6% and 3.8%, respectively, at the other three concentrations for the signature peptide, LQGTLPVEAR. These data indicated that the accuracy and precision were within the predefined acceptance criteria, which ensured the reproducibility of the assay. It is ideal to use a stable isotope labeled signature peptide as the IS, however, this assay without the IS is justified given that accuracy and precision were within the acceptance criteria. Once the assay reproducibility is ensured, even without the IS, assays without the IS are considered to be more cost-effective. The processed sample stability was assessed by injecting the stored processed samples (1, 3, 50, and 400 μg/mL) in the autosampler stored at 4 °C for 20 h. The % of bias of the

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Table 2. Intra-Batch Accuracy and Precision of the Complement C5 Assay by LC−MS/MS With Multiple Signature Peptides[a]

peptides	concentration (μg/mL)	accuracy (% RE)	precision (% RSD)
	DSSVPNTGTAR		
LLOQ	1	12.0	9.4
LQC	3	9.3	5.0
MQC	50	9.0	2.6
HQC	400	6.8	3.0
	LQGTLPVEAR		
LLOQ	1	5.0	8.0
LQC	3	9.7	3.8
MQC	50	11.6	0.7
HQC	400	7.8	2.2

[a]HQC, high concentration QC; LC−MS/MS: liquid chromatography with tandem mass spectrometry; LLOQ, lower limit of quantification; LQC, low QC; MQC, mid QC; QC, quality control; RE, relative error; and RSD, relative standard deviation. Five replicates per concentration (LLOQ, LQC, MQC, and HQC) in a batch were assayed to estimate accuracy and precision.

stored samples was within ±14.9% and ±19.2% of the initial injection samples (time = 0), for the signature peptides, DSSVPNTGTAR and LQGTLPVEAR, respectively, suggesting that the signature peptides were stable under the designated condition. Given the results of the intra-batch reproducibility test and stability assessment of the processed samples, the signature peptide, DSSVPNTGTAR, was found to be most suitable.

The established LC−MS/MS assay had a wide dynamic quantification range from 1 to 500 μg/mL, which is much wider than LBA. The wider dynamic range achieved minimized the chance of dilution steps, which led to higher assay throughput.

When compared to the reported C5 assay by LC−MS/MS,[9] a higher sensitivity (1 μg/mL vs 3 μg/mL) despite a lower volume of serum (10 μL vs 25 μL) was achieved. A lower sample volume used for the assay is useful in real sample assays when the sample volume is limited by multiple repeated blood samplings in monkeys.

3.1.2. LBA. The intra-batch reproducibility was tested to estimate the accuracy and precision of C5 assay by LBA. Six replicates at the five concentrations (LLOQ, LQC, MQC, HQC, and ULOQ) were assayed, and accuracy and precision were within ±20% and 20%, respectively, which met the acceptance criteria (Table 3). As the C5 assay in monkey serum was performed by multiple dilution factors, dilution integrity was tested by 5000-, 10,000-, 20,000-, and 40,000-fold dilutions. The percentage of bias by 5000-, 10,000-, and 40,000-fold compared to the results by 20,000-fold dilution was within ±20%, and its RSD was within 20%. In addition, the RE of the spiked (5 ng/mL) serum samples diluted by 2000- and 5000-fold was within ±20%. These findings indicated that dilution integrity was ensured up to 40,000-fold. Stability of C5 in monkey serum (freeze−thaw and frozen stabilities) was assessed by the LBA using naive monkey serum. The % of bias of the samples after three freeze−thaw cycles was within ±14.6%. The RE of the frozen stored samples was within ±9.4%. The RE was within ±20%, which met the acceptance criteria, and the findings thus indicate that C5 is stable after three freeze−thaw cycles and for 367 days when stored at −80 °C in monkey serum.

Table 3. Intra-Batch Accuracy and Precision of the Complement C5 Assay by LBA[a]

QC samples	concentration (ng/mL)	accuracy (% RE)	precision (% RSD)
LLOQ	0.313	13.4	4.7
LQC	0.625	4.2	2.5
MQC	2.50	−1.1	1.6
HQC	8.00	−5.9	2.3
ULOQ	10.0	−3.6	1.8

[a]HQC, high concentration QC; LBA, ligand binding assay; LLOQ, lower limit of quantification; LQC, low QC; MQC, mid QC; ULOQ, upper limit of quantification; QC, quality control; RE, relative error; and RSD, relative standard deviation. Six replicates per concentration (LLOQ, LQC, MQC, HQC, and ULOQ) were assayed to estimate accuracy and precision.

3.2. In Vivo Study in Monkeys. C5 concentrations in serum samples obtained from monkeys given C5 siRNA encapsulated in LNP were determined by LC−MS/MS and LBA. By LC−MS/MS, C5 levels were determined using the two signature peptides, DSSVPNTGTAR and LQGTLPVEAR. Each peak of the two signature peptides was eluted at the same retention times as the LLOQ samples (Figure 1).

The time profiles of relative C5 levels (% of initial) in monkey serum samples are represented in Figure 2. The two

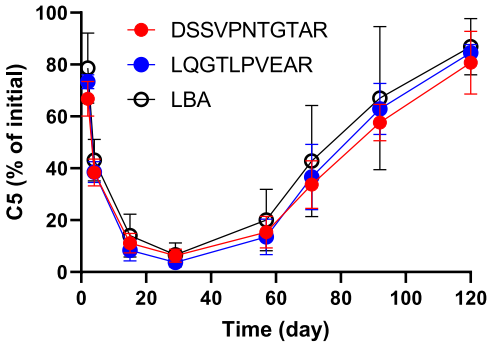

Figure 2. Comparison of complement C5 levels in monkey serum between LC−MS/MS and LBA. The percentage of initial (predose) complement C5 in serum is represented. C5 siRNA encapsulated in the lipid nanoparticle was intravenously administered at a dose of 1 mg/kg, and blood samples were obtained serially up to 120 days postdose. The data represents the mean ± standard deviation of four monkeys. C5 in monkey serum samples was quantifiable at 0.626 μg/mL by the LBA, while it was quantified at 1 μg/mL by LC−MS/MS with monitoring of the two signature peptides, DSSVPNTGTAR and LQGTLPVEAR.

signature peptides showed comparable results; the % of initial C5 levels was 11.1 ± 3.8 (%) and 8.4 ± 4.1 (%) on day 15 postdose by the signature peptides DSSVPNTGTAR and LQGTLPVEAR, respectively. C5 levels were also determined by the LBA, and the time profile of relative C5 levels is also shown in Figure 2. The time profile of C5 in monkey serum samples was in good agreement with that by LC−MS/MS. The % of initial C5 levels on day 15 postdose was 14.0 ± 8.3 (%), which was also similar to that observed by LC−MS/MS with the two signature peptides. As stated, there are some limitations in the LC−MS/MS assay since determination of absolute C5 levels in monkey serum might be different using dog serum since tryptic digestion efficiency and ionization efficiency might be different in serum samples between the two

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animal species. It is ideal to use C5-deficient blank monkey serum to prepare calibration samples for more accurate absolute determination of C5 levels in monkey serum, but it is not available. However, if the readout is % of the initial to assess the efficacy of C5 knockdown, the established LC−MS/MS assay with the multiple signature peptides is useful. In addition, in the LBA, C5 levels in monkey serum were determined using the human C5 assay kit. It is evident that monkey C5 assay kits which use monkey C5 as a standard provide more accurate C5 levels in monkeys, but it is possible to assay monkey C5 using a human C5 assay kit due to the similarity in the amino acid sequence of C5 between monkeys and humans. Although this study used the commercially available human C5 assay kit, if the study objective is to understand the residual % (i.e., % of initial) of C5 levels, the human C5 assay kit used in this study also fits for this purpose.

LBA, ligand binding assay; LC−MS/MS, liquid chromatography with tandem mass spectrometry.

4. CONCLUSIONS

A simple C5 assay in monkey serum was developed and validated by LC−MS/MS using the two signature peptides DSSVPNTGTAR and LQGTLPVEAR after tryptic digestion of monkey C5 protein. The higher signal-to-noise ratio of the peak at the LLOQ and sufficient stability in processed samples led us to select DSSVPNTGTAR as the most suitable signature peptide for the C5 assay in this assay condition. The accuracy and precision of the intra-batch reproducibility test indicate that the developed LC−MS/MS assay is reproducible. The established assay was applied for the determination of C5 in serum samples from monkeys and confirmed that the time profiles of relative C5 levels in monkey serum were similar to those by the LBA. These findings suggest that the established LC−MS/MS assay with the two signature peptides for C5 in monkey serum is reliable and useful for the determination of C5 levels in monkey serum.

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Author Contributions

K.K. conducted the assay validation and sample assay of C5 in monkey serum by LC-MS/MS. Y.I. and T.Y. monitored the validation study and sample assay by LBA at the contract research organization in Japan. Y.M. conducted data interpretation and wrote the manuscript, preparing the figures and tables. All authors contributed to the edition and approved the final submitted manuscript.

Notes

The authors declare no competing financial interest.

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