RESEARCH ARTICLE

Application of an electrochemiluminescence assay for quantification of E6011, an antifractalkine monoclonal antibody, to pharmacokinetic studies in monkeys and humans

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Background: E6011, a humanized antifractalkine monoclonal antibody, is under development for the treatment of various inflammatory diseases, such as rheumatoid arthritis. A reproducible assay method has been developed for the determination of E6011 in monkey and human serum by electrochemiluminescence (ECL) assay.

Methods: E6011 in serum was captured by fractalkine and detected by ruthenium-labeled rabbit anti-E6011 Fab polyclonal antibodies for ECL detection. E6011 in serum was quantifiable from 0.02 and 0.1 µg/mL in monkey and human serum, respectively, with minimum required dilution of 500. The method was then validated in accordance with bioanalytical guidelines.

Results: Accuracy and precision of quality control samples at five concentrations in intra- and interbatch reproducibility demonstrated that relative error and relative standard deviation were within acceptable criteria. Recovery of E6011 was 92.9%–121.7% and 85.0%–109.3% in humans and monkeys. Dilution integrity, no prozone effects, and no impacts by antigen were also ensured. Parallelism was also confirmed using incurred clinical sample analysis. Various types of stability were assessed, which confirmed that E6011 in serum was stable for 367 and 735 days in monkey and human sera, respectively, under frozen conditions.

Conclusion: The developed method was successfully applied supporting pharmacokinetic studies in monkeys and humans.

KEYWORDS

E6011, electrochemiluminescence, humans, monkey, validation

1 | INTRODUCTION

Fractalkine (FKN), a member of the CX3C chemokine family, is expressed on vascular endothelial cells and epithelial cells under inflammatory conditions, and binds to its receptor, CX3CR1, which is expressed on macrophages/dendritic cells, natural killer cells, and cytotoxic T cells.^{1,2} Enhancement of FKN/CX3CR1 signaling is involved in various inflammatory responses, and increased FKN

expression was reported in rheumatoid arthritis (RA) patients and an adjuvant-induced arthritis rat model.^{3,4} A previous report demonstrated that anti-mouse FKN monoclonal antibody as surrogate antibody of E6011 ameliorates arthritis by inhibiting infiltration of inflammatory cells into the synovium.⁵ These findings suggest that E6011 is expected to be a promising, therapeutic antibody for the treatment of inflammatory diseases, including RA and inflammatory bowel diseases including Crohn's disease.

Abbreviations: ECL, electrochemiluminescence; FKN, fractalkine; HQC, high-concentration QC; LLOQ, lower limit of quantification; LQC, low-concentration QC; MQC, middle-concentration QC; MRD, minimum required dilution; QC, quality control; RA, rheumatoid arthritis; RE, relative error; RSD, relative standard deviation; ULOQ, upper limit of quantification.

Indeed, a clinical study suggested that E6011 was effective in RA patients.⁶ It is important to determine E6011 concentrations in serum in order to understand the relationship with its efficacy. Therefore, it is required to establish a bioanalytical method for the determination of E6011.

This study presents, for the first time, a robust bioanalytical method for the determination of E6011 levels in serum samples by ligand binding assay platform using electrochemiluminescence (ECL) immunoassay. The developed method was validated according to bioanalytical guidelines by US Food and Drug Administration and European Medicines Agency, and then successfully applied to determine serum E6011 levels in support of pharmacokinetic studies in cynomolgus monkeys and clinical trials. In clinical trials, incurred sample reanalysis was performed to further ensure the robustness of the developed assay method.

2 | MATERIALS AND METHODS

2.1 | Materials

E6011 and fractalkine were produced at KAN Research Institute Inc. (Hyogo, Japan) and Eisai Co., Ltd. (Ibaraki, Japan) using recombinant DNA techniques. A rabbit anti-E6011 Fab polyclonal antibody was produced by Eisai Co., Ltd. Blank human serum was prepared from male and female volunteers, or purchased from KAC Co., Ltd. (Kyoto, Japan). Sulfo-tag NHS-ester was purchased from Meso Scale Discovery (MD, USA). Bovine serum albumin, Tris-buffered saline, and Tween-20 were purchased from Sigma-Aldrich (MO, USA). 2-amino-2-hydroxymethyl-1,3-propanediol, hydrochloric acid, and skim milk were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2 | Preparation of conjugated reagents for detection

Sulfo-tag solution was mixed with anti-E6011 antibody with a molar ratio of 1:18. The mixture was incubated at room temperature for 2 hours with shaking. The mixture was applied to a PD-10 column equilibrated with PBS, and the eluate was fractionated by 0.3 mL each. Four fractions, with high protein concentrations measured by the spectrometer at 280 nm, were mixed. Protein concentrations were then determined with bovine gamma globulin as a standard of calibration. Aliquots of Sulfo-tag labeled anti-E6011 antibody were stored at -80°C .

2.3 | Preparation of samples

A stock solution of E6011 (ca. 10 or 100 mg/mL) was fortified in drug-free, blank sera. For the human assay, calibration samples were prepared at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 $\mu\text{g/mL}$, with 0.025 and 0.05 $\mu\text{g/mL}$ used as anchor points. For the monkey assay, calibration samples were prepared at concentrations of 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, and 200 $\mu\text{g/mL}$ with 0.01 $\mu\text{g/mL}$ as an anchor point.

Quality control (QC) samples, including the lower limit of quantification (LLOQ), low-concentration QC (LQC), middle-concentration QC (MQC), high-concentration QC (HQC), and upper limit of quantification (ULOQ), were prepared by fortifying a standard solution of E6011 to blank sera. The final QC sample concentrations were 0.1 (LLOQ), 0.3 (LQC), 3 (MQC), 75 (HQC), and 100 $\mu\text{g/mL}$ (ULOQ) for human assay, and 0.02 (LLOQ), 0.06 (LQC), 3 (MQC), 150 (HQC), and 200 (ULOQ) $\mu\text{g/mL}$ for monkey assay. QC samples for evaluating potential prozone effects were prepared to have final concentrations of 500 and 2000 $\mu\text{g/mL}$ for human assay, together with 1000 and 5000 $\mu\text{g/mL}$ for monkey assay. QC samples were aliquoted and stored at -20°C or -80°C .

FKN solutions for evaluating impacts of antigen on E6011 assay were prepared by diluting FKN solution with a dilution buffer. Final concentrations of FKN in serum were 1, 5, 10, and 50 ng/mL.

2.4 | Immunoassay procedure

Antigen solution (50 μL) was added to each well of MULTI-ARRAY 96 well plates (L15XA, Meso Scale Discovery, MD, USA) and incubated at 4°C overnight. A blocking buffer (300 μL) was added to each well and incubated at room temperature for 2-3 hours. The plates were washed three times with wash buffer (Tris-buffered saline with Tween 20, pH 8.0). Aliquots (50 μL) of assay samples were added in duplicate to the wells, and incubated at room temperature for 2 hours. After the plates were washed three times with wash buffer, tripropylamine-containing MSD Read buffer T (150 μL) was added to each well. The ruthenium on the Sulfo-tag complex produced chemiluminescent signals, which were triggered when voltage was applied, and were then measured by Sector Imager 6000 (Meso Scale Discovery). Calibration curves were constructed by plotting nominal concentrations of E6011 and ECL signals with 4-parameter regression using the following equation with a weighting of $1/y^2$.

$$y = \text{top} + (\text{bottom} - \text{top}) / (1 + (x/b_1)^{b_2}),$$

where x , y , b_1 , and b_2 are E6011 concentrations, ECL signals, E6011 levels at the midpoint between top and bottom, and slope of the calibration curve, respectively.

2.5 | Method validation

In this study, duplicate wells were assayed per sample, unless otherwise stated. Three replicates were used in stability assessments (total of six wells assayed per sample).

2.5.1 | Calibration curve and prozone effect

Correlation coefficient and inaccuracy as relative error (RE) of back-calculated concentrations were determined using calibration samples across nine and 13 plates for monkey and human assays, respectively. At least 75% of the calibration samples should have RE within $\pm 20\%$ ($\pm 25\%$ was allowed for the LLOQ). The coefficient of determination should be 0.98 or higher. In the assessment of prozone effects, samples at higher concentrations than the ULOQ were assayed to check

whether ECL signals were higher than that of the ULOQ. When the signals were higher, no prozone effects were ensured.

2.5.2 | Selectivity

E6011 at three concentrations (low, mid, and high) was fortified in drug-free blank sera from six individual monkeys and 20 individual humans, and then subjected to immunoassay according to the method described above. At least 80% of tested individual samples should have RE within $\pm 20\%$ of the nominal concentrations. Blank serum from each individual was also assessed to ensure that the determined E6011 levels were below the LLOQ.

2.5.3 | Intra- and interbatch reproducibility and dilution integrity

Accuracy and precision in the intra- and interbatch reproducibility were evaluated using QC samples at five concentrations (LLOQ, LQC, MQC, HQC, and ULOQ) covering the calibration range. Five replicates per concentration were assessed in an assay batch for the intra-batch reproducibility, while the interbatch reproducibility repeated intra batch assay across five separate assay batches. The RE and relative standard deviation (RSD) as imprecision, at each concentration, were calculated. The acceptance criteria for RE and RSD were $\pm 25\%$ and 25% , respectively, for the LLOQ, while $\pm 20\%$ and 20% were acceptable for LQC, MQC, and HQC, and ULOQ. Dilution integrity for assaying samples above the ULOQ was also examined using QC samples diluted 10- and 100-fold with drug-free blank sera. The QC samples for the dilution integrity were assayed to estimate the RE and RSD. The RE and RSD should be within $\pm 20\%$ and 20% , respectively.

2.5.4 | Potential impacts of FKN

Potential impacts of FKN on E6011 assay were evaluated with varying combination of FKN levels and E6011 levels. E6011 concentrations were 0.3, 3, and 75 $\mu\text{g/mL}$ and FKN concentrations were 0, 1, 5, 10, and 50 ng/mL . The RE of E6011 with or without FKN was calculated and checked, verifying whether the RE was within $\pm 20\%$.

2.5.5 | Parallelism

Parallelism was assessed using serum samples from six subjects dosed with E6011. A serum sample was diluted by 2-, 8-, 32, and 128-fold by blank serum and multiple diluted samples along with an undiluted one were assayed to determine E6011 concentrations. Parallelism in the assay was ensured when the RSD of E6011 levels in undiluted and multiple diluted samples was within 20% and % bias of multiple diluted samples was within $\pm 20\%$ of the undiluted one.

2.5.6 | Stability

Various stability assessments including bench-top stability in serum at room temperature, stability in serum after multiple freeze-thaw cycles,

and frozen stability in serum at -20°C for humans and -80°C for monkey and humans were performed at low and high E6011 concentrations using LQC, MQC, and HQC samples. The bench-top stability was assessed for 24 hours, and the freeze/thaw stability was evaluated after five cycles. Frozen stability was checked up to 367 and 735 days for monkey and human sera, respectively. The average RE should be within $\pm 20\%$ of the nominal concentration to be judged stable.

2.6 | Application

The established method was applied to a pharmacokinetic study in monkeys and a phase 1 clinical study. E6011 was intravenously administered at 0.05, 0.3, 1, 3, and 10 mg/kg to monkeys ($n = 3$ per dose) with blood samples obtained at pre-dose, 5 min, 1, 2, 4, 8, 24, 48, 72, and 96 hours, and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 weeks. In humans, E6011 was subcutaneously administered at 50, 100, 200, and 400 mg ($n = 6$ per dose), with blood samples obtained at pre-dose, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 240, 288, and 336 hours, 3, 4, 5, 6, 7, and 8 weeks. Subsequently, serum samples were prepared by centrifugation of blood samples at 6000 g for 10 minutes in monkey while 1300 g for 15 minutes in humans, and then stored at -80°C until assay.

Serum concentration-time profiles were assessed to estimate pharmacokinetic parameters of E6011. Noncompartmental analysis was performed using WinNonlin (Certara, NJ, USA) to estimate pharmacokinetic parameters. The terminal elimination rate constant (k_{el}) was determined from three or more sampling points by the least squares regression analysis of the terminal log-linear portion of the profile, and the elimination half-life ($T_{1/2}$) was calculated by $0.693/k_{el}$. Area under the curve of serum E6011 concentrations-time (AUC) was calculated by trapezoidal rule up to the last quantifiable time point ($\text{AUC}_{0-\text{last}}$). The time to reach the maximum concentration (C_{max}) was represented as T_{max} . Total clearance (CL_{tot}) was calculated by dividing dose by $\text{AUC}_{0-\text{infinity}}$ and volume of distribution at the steady-state ($V_{d,ss}$) was estimated by multiplying CL_{tot} by mean residence time.

An incurred sample reanalysis (ISR) was performed using 48 samples obtained from 24 subjects to further ensure the robustness of the method. Two samples from each subject taken at time points near the T_{max} and during the elimination phase were selected for the ISR assessment. The assay data in the ISR were compared to those in the original assay to assess whether the bias was within $\pm 30\%$ for more than 67% of the samples tested.

3 | RESULTS

3.1 | Method validation

3.1.1 | Calibration curve and prozone effect

A representative calibration curve of E6011 plotting nominal concentrations and corresponding ECL signals in monkey and human sera is shown in Figure 1. The regression was employed using four-parameter logistic model with a weighting of $(1/\text{signal}^2)$, and the RE at each concentration was calculated. Table S1 shows RE and RSD of calibration

samples across all the assay runs. The RE was within $\pm 8.0\%$ and $\pm 1.5\%$ in monkeys and humans, respectively, across concentrations covering the quantification range. The RSD (%) was equal to or less than 4.8% and 2.9% in nine assay runs for monkeys and 13 assay runs for humans, respectively. ECL signals from samples with concentration exceeding ULOQ were higher than those of the ULOQ samples, indicating no prozone effects in both monkey and human assays.

3.1.2 | Selectivity

Selectivity of the assay method was evaluated using QC samples at three concentrations (low, mid, and high), utilizing serum samples from six individual monkeys and 20 individual humans including healthy subjects and subjects with RA or Crohn's disease. The RE of the selectivity samples was within $\pm 20\%$ for 6 individual monkeys and at least 19 individual subjects. Selectivity was ensured as the results were within the acceptance criteria.

3.1.3 | Intra- and interbatch reproducibility and dilution integrity

Intra- and interbatch reproducibility for E6011 assay were evaluated at five E6011 concentrations using LLOQ, LQC, MQC, HQC, and ULOQ in monkey and human sera. The results are presented in Table 1. In the intra batch reproducibility, RE and RSD were within $\pm 19.0\%$ and less than 17.4%, respectively, in monkey assay. In human assay, the RE and RSD were within $\pm 2.8\%$ and 6.0%, respectively. In the interbatch reproducibility, the RE and RSD were equal to or less than $\pm 11.7\%$ and 16.6%, respectively, in monkey assay, while they were $\pm 6.0\%$ and 13.8% in human assay, respectively. These results were within the acceptance criteria recommended by the

FIGURE 1 A typical calibration curve of E6011 in monkey and human sera. E6011 concentrations in monkey serum (●) or human serum (○) and relative mean electrochemiluminescence units are plotted for 4-parametric logistic regression. E6011 was quantifiable from 0.02 and 0.1 $\mu\text{g/mL}$ in neat monkey and human sera, respectively. Although anchoring points (0 and 0.010 $\mu\text{g/mL}$ in monkeys and 0, 0.025, and 0.050 $\mu\text{g/mL}$ in humans) were included in 4-parametric logistic regression, and relative error at those points was not determined

bioanalytical guidelines from US Food and Drug Administration and European Medicines Agency.^{7,8}

For the dilution integrity, QC samples, diluted 10- and 100-fold with drug-free blank serum, were assayed in monkeys and humans. The RE and RSD were within $\pm 9.7\%$ and 1.7%, respectively, in monkeys and were within $\pm 2.5\%$ and 2.4%, respectively, in humans, ensuring the dilution integrity up to 100-fold (Table S2).

3.1.4 | Potential impacts of FKN

Potential impacts of FKN on E6011 assay were evaluated in human serum. In the presence of FKN up to 50 ng/mL , the RE at 0.3, 3, and 75 $\mu\text{g/mL}$ was within $\pm 11.2\%$. This result demonstrated that FKN did not impact the E6011 assay, at least up to 50 ng/mL . As clinically relevant levels of soluble FKN were elevated up to 25 ng/mL in RA subjects,⁹ it is highly likely that FKN would not impact the E6011 assay in clinical settings.

3.1.5 | Parallelism

Parallelism was assessed using 6 serum samples from each subject dosed with E6011. Serum samples diluted by 2-, 8-, 32-, and 128-fold, along with a serum sample without dilution, were assayed to determine E6011 concentrations. The undiluted serum E6011 levels in six subjects were 61.5, 70.8, 71.8, 71.9, 75.8, and 83.2 $\mu\text{g/mL}$ (Figure 2). The measured E6011 levels with multiple dilutions in 6 subjects were within $\pm 12.7\%$ of the undiluted ones. The RSD of undiluted and

TABLE 1 Inaccuracy and imprecision for the assay of E6011 in monkey and human sera

Quality control	Monkey serum		Human serum	
	RE (%)	RSD (%)	RE (%)	RSD (%)
Intrabatch reproducibility				
LLOQ	-17.0	9.1	2.8	5.8
LQC	-19.0	17.4	1.1	6.0
MQC	-5.3	1.9	-2.3	4.2
HQC	-8.3	0.8	-2.6	5.7
ULOQ	-10.2	5.4	-1.3	5.7
Interbatch reproducibility				
LLOQ	-6.0	16.6	-6.0	13.8
LQC	11.7	15.3	-2.1	6.5
MQC	1.5	8.2	-3.7	6.9
HQC	0.5	8.8	-3.5	8.9
ULOQ	-8.7	7.3	1.4	9.0

HQC, high-concentration quality control; LLOQ, lower limit of quantification; LQC, low-concentration quality control; MQC, middle-concentration quality control; RE, relative error; RSD, relative standard deviation; ULOQ, upper limit of quantification.

Intra- and interbatch reproducibility was evaluated using five replicates per concentration.

TABLE 2 Stability of E6011 in monkey and human sera

Stability test	Condition	Serum	Quality control	RE (%)	RSD (%)
Bench-top	24 h at room temperature	Monkey	LQC	-1.4	5.8
			MQC	9.5	0.9
			HQC	-2.5	4.5
		Human	LQC	11.3	0.8
			MQC	8.1	1.2
			HQC	4.9	1.4
Freeze/Thaw	After five cycles at -80°C	Monkey	LQC	-8.1	6.3
			MQC	-6.2	4.5
			HQC	-7.7	2.9
		Human	LQC	13.0	2.2
			MQC	5.4	0.7
			HQC	2.8	0.8
Freeze/Thaw	After 5 cycles at -20°C	Human	LQC	7.1	2.0
			MQC	6.1	1.9
			HQC	2.9	0.8
Frozen	735 days at -20°C	Human	LQC	15.9	1.6
			MQC	8.0	6.1
			HQC	1.2	5.5
Frozen	367 days at -80°C	Monkey	LQC	-0.5	9.4
			MQC	13.4	2.1
			HQC	-3.6	0.3
	735 days at -80°C	Human	LQC	15.0	3.8
			MQC	6.1	4.6
			HQC	-5.0	6.9

HQC, high-concentration quality control; LQC, low-concentration quality control; MQC, middle-concentration quality control; RE, relative error; RSD, relative standard deviation.

Stability was assessed by three replicates per concentration.

FIGURE 2 Parallelism of E6011 assay in human serum. E6011 concentrations in serum were determined with and without dilution. A serum sample from six subjects dosed with E6011 ($n = 1/\text{subject}$) was subjected to multiple dilutions (twofold, eightfold, 32-, and 128-fold). A nondiluted sample and multiple-diluted samples were assayed by the validated electrochemiluminescence assay. Multiple dilutions and determined concentrations were plotted

multiple diluted samples ($n = 5/\text{sample}$) was within $\pm 5.8\%$. These results suggest that the parallelism in the assay was ensured.

3.1.6 | Stability

Results of the stability assessment are represented in Table 2. E6011 was stable in monkey and human sera for at least 367 and 735 days at -80°C , respectively. Stability in serum was also ensured at -20°C up to 735 days in human serum. In both monkey and human sera, bench-top stability was ensured for 24 hours at room temperature. E6011 in sera was stable even after 5 freeze/thaw cycles.

3.2 | Application

Serum concentrations of E6011 were determined in a monkey pharmacokinetic study and a phase 1 clinical study. Serum samples prepared from obtained blood samples were subjected to the assay according to the validated method. Pharmacokinetic profiles of E6011 in monkeys after a single intravenous administration and those in humans after a single subcutaneous administration are

FIGURE 3 Serum concentration-time profiles of E6011 in monkeys and humans. E6011 was intravenously administered to cynomolgus monkeys ($n = 3/\text{dose}$) at doses of 0.05–10 mg/kg (A) and was subcutaneously administered to humans ($n = 6/\text{dose}$) at 50–400 mg (B). Data represent the mean $\pm$ standard deviation

shown in Figure 3. Nonlinear pharmacokinetic profiles of E6011 were observed in monkeys at doses ranging from 0.05 to 10 mg/kg. The elimination half-life of E6011 in serum ranged from 7.6 to 340.9 hours (Table 3).

In humans, serum E6011 levels elevated with increases in doses ranging from 50 to 400 mg after subcutaneous administration (Figure 3). E6011 levels in serum reached the maximum at 156 hours postdose and then eliminated with $t_{1/2}$ of 349 hours at a dose of 400 mg (Table 4). A total of 48 samples from humans were subjected to the ISR, and serum E6011 levels in the reanalysis were compared with those in the original assay. All the samples tested had a bias

within $\pm 30\%$ which met the acceptance criteria (reanalysis results within 30% of their mean for at least 67% of the repeats).

Successful ISR results, along with the method validation study, further support the robustness of the developed method and its usefulness in supporting pharmacokinetic studies in monkeys and humans.

4 | DISCUSSION

Quantification of therapeutic antibodies is important for their pharmacological and toxicological assessment. Therefore, it is

TABLE 3 Pharmacokinetic parameters of E6011 in monkeys after intravenous administration

Parameters	Unit	0.05 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg
$T_{1/2}$	h	7.6 ± 0.6	57.6 ± 7.5	114.7 ± 17.0	214.2 ± 2.3	340.9 ± 61.7
CL_{tot}	mL/h/kg	3.03 ± 0.30	0.718 ± 0.076	0.356 ± 0.039	0.174 ± 0.009	0.129 ± 0.018
$V_{\text{d,ss}}$	mL/kg	27.3 ± 0.5	39.2 ± 0.9	38.0 ± 1.5	37.9 ± 2.0	46.6 ± 3.6

CL_{tot} , total clearance; $T_{1/2}$, elimination half-life; $V_{\text{d,ss}}$, volume of distribution at the steady state.

E6011 was administered intravenously to cynomolgus monkeys at doses of 0.05, 0.3, 1, 3, and 10 mg/kg, and serum E6011 concentrations were determined. Pharmacokinetic parameters were calculated by noncompartmental analysis. Data represent the mean $\pm$ standard deviation of three animals per dose.

TABLE 4 Pharmacokinetic parameters of E6011 in humans after subcutaneous administration

Parameters	Unit	50 mg	100 mg	200 mg	400 mg
C_{max}	$\mu\text{g/mL}$	3.14 ± 0.788	9.66 ± 1.88	27.3 ± 4.47	43.1 ± 8.88
$T_{\text{max}}^{\text{a}}$	h	120 (48–144)	144 (120–240)	156 (120–192)	156 (72–288)
$T_{1/2}^{\text{b}}$	h	40.8 ± 8.42	70.6 ± 19.5	242 ± 30.5	349 ± 37.2
$AUC_{0-\text{last}}$	$\mu\text{g} \times \text{h/mL}$	602 ± 186	2990 ± 745	$11\,900 \pm 2170$	$26\,600 \pm 6770$

C_{max} , maximum serum concentration; $T_{1/2}$, elimination half-life; T_{max} , time to reach the maximum serum concentration; $AUC_{0-\text{last}}$, area under the curve of serum level-time from time zero to the last quantifiable time point.

E6011 was administered subcutaneously to humans at doses of 50, 100, 200, and 400 mg, and serum E6011 concentrations were determined. Pharmacokinetic parameters were calculated by noncompartmental analysis. Data represent the mean $\pm$ standard deviation of six subjects per dose.

^aMedian and range in parenthesis were represented for T_{max} .

^b $T_{1/2}$ at β (50 and 100 mg) and γ (200 and 400 mg) elimination phases were represented.

critical to establish robust and reproducible bioanalytical methods for therapeutic antibodies. Prior to the method validation study, a quantification method of E6011 in sera was established based on the following method development. In both monkey and human assays, coating antigen concentrations, detection antibody concentrations, and minimum required dilution (MRD) of serum samples were evaluated to optimize the assay conditions. In assay optimization, the following combination of three conditions was assessed: 2, 4, 6, and 8 $\mu\text{g/mL}$ for capture antigen levels; 5, 10, and 20 $\mu\text{g/mL}$ for detection antibody levels; and 100, 250, 500, and 1000 for the MRD. As the inaccuracy of each calibration sample of E6011 in monkey serum ranging from 0.02 to 200 $\mu\text{g/mL}$ was acceptable, concentrations of antigen, detection antibody, and MRD were set at 2 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 500, respectively. In setting the quantification range, the LLOQ in the human assay was increased to 0.1 $\mu\text{g/mL}$ from 0.02 $\mu\text{g/mL}$ in monkey serum. This was due to the finding that blank human sera from some subjects showed detectable E6011 levels ($>0.02 \mu\text{g/mL}$). The ULOQ was set at 100 $\mu\text{g/mL}$ in the human assay method validation study as signals at 200 $\mu\text{g/mL}$ were proximal to plateau levels, which would cause variability in back-calculated concentrations. After optimization of the method, the method validation study was performed to ensure accuracy and precision of the established assay in monkey and human sera.

The method validation study was performed in accordance with the bioanalytical guidelines from European Medicines Agency.⁷ The inaccuracy and imprecision in the intra- and inter-batch reproducibility studies, were within the pre-defined acceptance criteria recommended by the regulatory authorities.^{7,8} Other assay parameters, including selectivity, prozone effects, dilution integrity, effects of the target antigen, and stability evaluations, also supported that the established assay is reproducible. Among the method validation parameters, parallelism is one of the critical parameters to evaluate the effects of dilution on the quantification of analytes using in-study samples and not QC samples. In this study, parallelism was evaluated to ensure that the binding characteristics of antibody are similar even in diluted sera with potential catabolites of E6011. Parallelism was ensured using human sera from 6 individuals, which further supports the validity of the established assay method. Hampson et al.¹⁰ ensured parallelism of rituximab in the validated ELISA assay using human serum from three individuals.

The validated assay method of E6011 was applied to in-study sample assay in both preclinical and clinical studies. In both cynomolgus monkeys at 0.05–10 mg/kg of E6011 and humans at 50–400 mg of E6011, nonlinear pharmacokinetic profiles possibly due to target-mediated drug disposition were noted. The AUC increased more than the proportion of doses and elimination half-life was prolonged as the doses increased. The nonlinear pharmacokinetic profiles were also reported in other therapeutic antibodies including those for the treatment of RA.¹¹ The findings of the ISR assessment and performance of QC samples ensured that the assay of in-study sample was

reliable using the validated assay method of E6011. Data from the present validation study demonstrated that the developed ECL method for the determination of E6011 in monkey and human serum is reproducible and was successfully applied, thereby supporting pharmacokinetic studies of E6011 in monkeys and humans.

ACKNOWLEDGMENTS

The authors acknowledge Hiroko Tabuchi (Eisai Co., Ltd, Tokyo, Japan) and Kathleen Vanderhoof (Eisai Inc. MA, USA) for estimation of pharmacokinetic parameters in humans and language edition, respectively.

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REFERENCES

1. Fong AM, Robinson LA, Steeber DA, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med*. 1998;188:1413–1419.
2. Imai T, Hieshima K, Haskell C, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell*. 1997;91:521–530.
3. Ruth JH, Volin MV, Haines GK 3rd, Woodruff DC, Katschke KJ Jr, Woods JM. Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. *Arthritis Rheum*. 2001;44:1568–1581.
4. Blaschke S, Koziol M, Schwarz A, Benohr P, Middel P, Schwarz G. Proinflammatory role of fractalkine (CX3CL1) in rheumatoid arthritis. *J Rheumatol*. 2003;30:1918–1927.
5. Nanki T, Urasaki Y, Imai T, Nishimura M, Muramoto K, Kubota T. Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *J Immunol*. 2004;173:7010–7016.
6. Tanaka Y, Takeuchi T, Umehara H, et al. Safety, pharmacokinetics, and efficacy of E6011, an anti-fractalkine monoclonal antibody, in a first-in-patient phase 1/2 study on rheumatoid arthritis. *Mod Rheumatol*. 2018;28:58–65.
7. EMA/CHMP/EWP, Committee for Medicinal Products for Human Use, Guideline on bioanalytical method validation, European Medicines Agency, 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf
8. US DHHS, FDA and CDER, Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). 2018. <https://fda.gov/downloads/Drugs/Guidances/ucm070107.pdf>. Accessed June 22, 2018.
9. Matsunawa M, Isozaki T, Odai T, et al. Increased serum levels of soluble fractalkine (CX3CL1) correlate with disease activity in rheumatoid vasculitis. *Arthritis Rheum*. 2006;54:3408–3416.
10. Hampson G, Ward TH, Cummings J, et al. Validation of an ELISA for the determination of rituximab pharmacokinetics in clinical trials subjects. *J Immunol Methods*. 2010;360:30–38.
11. Ternant D, Bejan-Angoulvant T, Passot C, Mulleman D, Paintaud G. Clinical pharmacokinetics and pharmacodynamics of

monoclonal antibodies approved to treat rheumatoid arthritis. *Clin Pharmacokinet.* 2015;54:1107-1123.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Aoyama M, Mano Y. Application of an electrochemiluminescence assay for quantification of E6011, an antifractalkine monoclonal antibody, to pharmacokinetic studies in monkeys and humans. *J Clin Lab Anal.* 2019;33:e22625. <https://doi.org/10.1002/jcla.22625>