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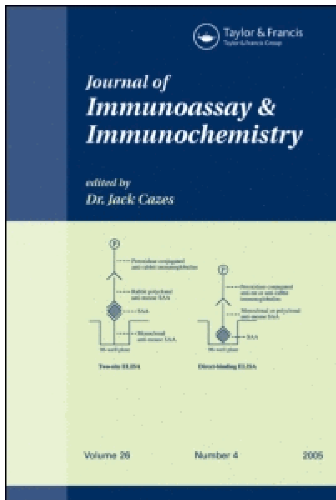

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Determination of KRN321 in Rat Serum by ELISA and Correlation Between ELISA and *in vitro* Bioassay

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Abstract: KRN321, darbepoetin alfa, is a hyperglycosylated analog of recombinant human erythropoietin (rHuEPO, epoetin alfa). We carried out the validation study by using a commercially available ELISA assay kit to establish the ELISA quantitation method for KRN321 in rat serum for the implementation of the pharmacokinetic studies with a lower limit of quantitation at 100 pg/mL and quantitation range from 100 to 4,000 pg/mL. We also established the *in vitro* bioassay method as an index of biological activity using UT-7/Epo, derived from a human leukemia cell line with a lower limit of quantitation, at 10 ng/mL. Furthermore, good correlation was observed between the two methods; it indicated that KRN321 concentration determined by the ELISA maintained biological activity.

Keywords: KRN321, Darbepoetin alfa, ELISA, Bioassay, Serum, Validation

INTRODUCTION

Recombinant human erythropoietin (epoetin alfa) has been used for the treatment of chronic kidney disease,[1] anemia of cancer, and anemia

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associated with chemotherapy.[2] KRN321 is a hyperglycosylated analog of epoetin alfa that stimulates erythropoiesis by the same mechanism as the endogenous hormone, but affinity of KRN321 to the EPO receptor is less than for the natural ligand.[3] KRN321 possesses a structure in which five amino acid residues are mutated in the peptide moiety and two *N*-binding type sugar chains are incorporated into the sugar-chain moiety. In other words, native EPO has three *N*-linked oligosaccharide chains, whereas darbepoetin alfa has five such chains.[4–6] It has been expected that the elimination half-life of KRN321 is longer than that of rHuEPO; increase of the *N*-linked sugar chains contributes to the prolonged period of pharmacological activity.[6]

So far, there are many assay methods which have been evaluated or established with EIA and RIA for determination of rHuEPO (EPO) concentration in animal and human matrices by many investigators. For example, some reports have described sensitive ELISA in murine serum[7] and canine plasma,[8] respectively. Regarding the human matrix, Egrie has primarily developed an RIA for determination of human EPO.[9] Then, some ELISA methods have been reported for measuring erythropoietin in human serum.[10,11] Furthermore, there are a few reports on evaluation of the correlation between EIA and bioassay with murine serum[12] and human serum or plasma.[13,14] Recently, Benson evaluated chemiluminescence immunoassay as an alternative for the analysis of EPO.[15] ELISA methods to determine KRN321 concentrations in the serum of various animal species after administration of KRN321 in non-clinical studies have already been established by Amgen Inc. For the determination of the serum concentration of KRN321, a commercially available kit has been used. However, it was not clear whether the serum concentration determined by ELISA reflected the biological activity. In the present study, we established an analytical method for the determination of the concentration of KRN321 in rat serum by ELISA, by using the commercially available EPO assay kit prior to implementing non-clinical pharmacokinetic studies in Japan. Additionally, we established the *in vitro* bioassay method by using UT-7/Epo, which is an EPO-dependent cell line in which biological activity of KRN321 is used as an index. Furthermore, we studied the correlation between the ELISA and the *in vitro* bioassay to verify that the observed serum concentration determined by the ELISA actually reflects the biological activity.

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EXPERIMENTAL

Test Substance

KRN321 drug substance, manufactured by Amgen Inc. and supplied from the Pharmaceutical Development Laboratories of Kirin Brewery Co., Ltd., was used as the test substance. The test substance was confirmed to be stable at

2 to 8°C for 12 months from the date manufactured. Each drug substance used in this study had approximately 100% activity by *in vitro* bioassay relative to the standard.

Assay Kit

The ELISA kit for measurement of EPO (Quantikine® IVD® Erythropoietin, Cat # DEP00, R&D Systems Inc., USA) was used for determination of KRN321.

UT-7/Epo Cell Line for *in vitro* Bioassay

UT-7/Epo cells,[16] EPO-dependent human leukemia cell line, were kindly supplied by Dr. Komatsu, Jichi Medical School, Tochigi, Japan, and were maintained by subculture at our laboratory.

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ELISA Procedure

The serum samples were assayed by sandwich ELISA with Quantikine® IVD® Erythropoietin (R&D Systems Inc., USA) according to the following procedure, which was modified to prevent serum interference. This kit was adapted to the determination of the concentration of KRN321 in serum samples by first checking the cross-reactivity of KRN321 against the antibody for rHuEPO. Each sample was assayed with analytical runs in duplicate. Briefly, 150 μL of Assay Diluent was applied onto the 96-well microplate, which was pre-coated with an anti rHuEPO murine monoclonal antibody, and 50 μL of the serum sample were added to each well. After incubation at room temperature for about 2-hr and washing the wells with Wash Buffer, 4 times, an anti rHuEPO rabbit polyclonal antibody conjugated to HRP, horseradish peroxidase, was applied to each well as a second antibody. After incubation at room temperature for about 2 hr and washing the wells with Wash Buffer, 4 times, the mixture was colored by the color reagent, substrate solution, i.e., TMBZ (tetramethylbenzidine). After incubation at room temperature for 30 min and addition of a 100-μL portion of a stop solution to each well to terminate the reaction, the absorbance at 450 nm was measured with a well-reader (SK601, Seikagaku Corporation).

In vitro Bioassay Procedure

The standard samples and QC samples, which were prepared at a concentration of 10 ng/mL, were 1.5-fold diluted with an assay medium (RPMI

Medium 1640 containing 3.8% fetal bovine serum, FCS, GIBCOBRL® (Lot #AJB9765: Hyclone)) and serum samples were diluted with the blank serum to a concentration of about 10 ng/mL by referring to the results of ELISA.

A 200 μL of UT-7/Epo cell suspension, prepared at 5×10^4 cells/mL, was applied to each well of the 96-well plate (FALCON®353072: Becton Dickinson). Here, each sample was composed of 8 points of diluting series and 15 μL of the dilution solution was added to a well. The analytical run was performed in triplicate. To the wells surrounding the wells to which the measurement samples were added, 200 μL of sterilized PBS (−) was added. After the samples were incubated in a CO_2 incubator (37°C, 5% CO_2) for 4 days, 20 μL of AlamarBlue™ (Trek Diagnostic Systems, Inc.) was added to the respective wells. The cells were further continuously incubated for 24-hr, followed by measurement of fluorescence intensity at an excitation wavelength of 530 nm and a measurement wavelength of 590 nm using a plate reader (1420 multilabel counter, ARVOsx, Wallac).

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Calibration Curve and Calculation for Determination of KRN321 by ELISA

By use of prepared concentrations of the standard samples for a calibration curve, ranging from 62.5 to 4,000 pg/mL of KRN321, the absorbance, logistic regression (4 parameters) was done within the examined concentration range. The absorbance values used in the preparation of calibration curves were mean values of two analytical runs (duplicate). A SOFTmax® PRO (Nihon Molecular Devices) was used as analytical software. The concentrations in the samples were determined from the absorbances of measured samples by use of the calibration curve. The concentration data were the means of two analytical runs (duplicate). The concentrations of KRN321 in the samples were calculated by subtracting KRN321 concentration corresponding to the endogenous EPO in the blank serum.

Calibration Curve and Calculation for Determination of KRN321 by *in vitro* Bioassay

The concentrations of KRN321 in the samples by *in vitro* bioassay were determined by calculating from a common ratio against the standard samples after verification of the parallelism and linearity by parallel line assay. The acceptable criterion was that 3 points or more of the concentration points that were in the linear region could be adopted.

Validation Items and Evaluation Criteria for ELISA and *in vitro* Bioassay

The assays of the replicate samples were validated for specificity, and intra- and inter-assay reproducibility. With respect to the specificity, serum samples by spiking to the blank sera at a concentration of 200 pg/mL of 10 individual sera and at a concentration of 10 ng/mL of 6 individual serum randomly selected for ELISA and *in vitro* bioassay, respectively, were analyzed. Acceptance criterion was that if the variations of observed concentrations in all the samples examined were within ±20% of against the nominal concentration, specificity for determination of KRN321 of ELISA was accepted. Meanwhile, the acceptance criteria for *in vitro* bioassay were that fluorescence intensity by individual blank serum did not affect the quantitation and the variations of observed concentrations against the prepared concentrations were within ±25%. Additionally, five samples on a single plate and one sample by 3 different assay runs at each concentration were analyzed to evaluate respective intra-assay reproducibility and inter-assay reproducibility. When both the precision (C.V. %), and accuracy (deviation %) of the determined concentrations against the prepared concentrations were 20% or less and within ±20% (at the lower limit of quantitation (LLOQ): 25% or less and within ±25%, respectively), intra-assay and inter-assay reproducibility were acceptable. On the other hand, for *in vitro* bioassay, three samples and one sample at the same time and one sample by 3 different assay runs were analyzed with the standard samples to evaluate respective intra-assay reproducibility and inter-assay reproducibility. The acceptance criteria were defined to be that the precision was 25% or less, and the accuracies of mean values were within ±25% (at the LLOQ: 30% or less and within ±30%, respectively).

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Animal Study

All animal experiments were reviewed and approved by the Ethics Committee for Animal Experiments of our institute.

Male Sprague-Dawley rats, 8 weeks old, were purchased from Charles River Inc., Japan, and housed in a metal wire cage with 2 animals, in an animal breeding facility. After one week or longer of acclamation period, under conditions of allowable range of temperature and humidity (from 20 to 25°C and 40 to 70%, respectively), a 12-hr cycle of lighting, animals were allowed free access to a solid diet (CE-2, Clea Japan Inc.) and tap water filtered through a 5 μm aqua filter, automatically supplied, four animals were assigned to two groups. A single dose of 2.5 μg/kg KRN321 was administered intravenously, via the tail vein, to each animal, using a 1 mL disposable syringe fitted with a 27 gauge needle (Terumo Corporation). Sampling time points were predefined as at time of pre-dosing and 10 min, 1,

4, and 24-hr, and 30-min, 2 and 8-hr after administration of KRN321 for groups 1 and 2, respectively. The tail vein was cut, and approximately 800 μL of blood was collected with a glass capillary tube. The volume of blood collected before administration, i.e., pre-dosing, was to be approximately 300 μL because it was only quantitated by ELISA. The collected blood was immediately transferred to a 0.8 mL tube containing agent of serum separation (Microtainer®, Nippon Becton Dickinson Company, Ltd.), and allowed to stand at room temperature for 10-min or longer. After coagulation, the blood samples were centrifuged at 12,000 rpm for 5 min at room temperature (model 1120, Kubota Corporation) to separate the serum. The serum samples thus obtained was transferred to two polypropylene (PP) tubes with a capacity of 0.5 mL and stored under frozen conditions at −80°C until measurement.

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Correlation Between ELISA and *in vitro* Bioassay

ELISA and *in vitro* bioassay were implemented by using the same samples from the animal study and concentrations of KRN321 in serum samples were determined. To analyze the correlation between the two methods, a scatter graph was prepared in which the measured values of ELISA and those of the *in vitro* bioassay were plotted on the x-axis and y-axis, respectively, by the Passing-Bablok method using the software, Analyse-it™. Then, the linear-regression correlation equation was calculated by using a least squares method. In addition, the 95% confidence intervals were obtained to evaluate the correlation criteria.

RESULTS

ELISA

Over the concentration range from 62.5 to 4,000 pg/mL, the desirable calibration curve was observed to be approximate a 4th order regression (4 parameter) equation with good correlation coefficient values, i.e., more than 0.998 (data not shown).

The specificity was examined for the rat serum obtained from 10 individual animals after spiking with KRN321 at a nominal concentration of 200 pg/mL (Table 1); in all of the individual specimens, the endogenous concentrations compared to those of KRN321 were observed, and were det5ermined to be in the range from 46.9 to 133.2 pg/mL (91.1 ± 35.2 pg/mL). The results of the concentrations of KRN321, after subtraction of endogenously observed concentrations from the actually measured ones, ranged from 189.2 to 232.3 pg/mL (211.4 ± 14.1 pg/mL). The range of accuracy was -5.4% to 16.2% (mean 5.7%). These values satisfied the

Table 1. Specificity for KRN321 determination in rat serum by ELISA

Animal	Blank (pg/mL)	Observed (pg/mL)	KRN321 concentration (pg/mL)	Deviation (%)
1	46.9	279.2	232.3	16.2
2	81.0	284.0	203.0	1.5
3	130.3	352.0	221.7	10.9
4	51.9	263.4	211.5	5.7
5	52.7	258.6	205.9	3.0
6	87.6	276.8	189.2	−5.4
7	74.4	289.5	215.1	7.6
8	133.2	324.4	191.2	−4.4
9	129.3	348.6	219.3	9.7
10	123.3	347.7	224.4	12.2
Mean	91.1	–	211.4	5.7
S.D.	35.2	–	14.1	7.1
C.V. (%)	–	–	6.7	–

–: Not calculated.

KRN321 concentrations were obtained by subtracting endogenous EPO concentration determined with blank serum measured as KRN321 from observed concentration.

A deviation (%) was calculated to compare with nominal concentration.

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Table 2. Intra- and inter-assay reproducibility of KRN321 determination in rat serum by ELISA

KRN321 concentration (pg/mL)			
Nominal	Mean ± S.D.	C.V. (%)	Deviation (%)
Intra-assay (n = 5)			
100	90.2 ± 12.4	13.7	−9.8
800	821.8 ± 17.3	2.1	2.7
4000	4034.6 ± 62.2	1.5	0.9
Inter-assay (n = 3)			
100	109.2 ± 7.6	6.9	9.2
800	742.0 ± 49.3	6.6	−7.3
3000	2556.0 ± 157.8	6.2	−14.8

KRN321 concentrations were obtained by subtracting endogenous EPO concentration determined with blank serum measured as KRN321 that was 143.5 pg/mL in the intra-assay reproducibility and 101.7 to 116.8 pg/mL in the inter-assay reproducibility.

A deviation (%) was calculated to compare with nominal concentration using mean observed concentration value.

criteria for specificity that the accuracy of recovered concentrations were to be within ±20%.

Intra-assay reproducibility (Table 2) was examined by use of samples prepared at concentrations of 100, 800, and 4,000 pg/mL. The precision and accuracy at 100 pg/mL set at the LLOQ were 13.7% and −9.8%, respectively. Since the acceptance criteria defined for the LLOQ were 25% or less for the precision and within ±25% for the accuracy, the values satisfied these criteria. The respective precision and accuracy were 2.1% and 2.7% at 800 pg/mL, and 1.5% and 0.9% at 4,000 pg/mL. These values satisfied the acceptance criteria for ELISA, i.e., that the precision and accuracy at the concentrations, except for the LLOQ, were to be 20% or less and within ±20%. Inter-assay reproducibility was examined by use of samples prepared at concentrations of 100, 800, and 3,000 pg/mL. The precision and accuracy were from 6.2% to 6.9% and from −14.8% to 9.2%, respectively. These values met the acceptance criteria for ELISA that was the same ranges of the precision and accuracy of the intra-assay reproducibility.

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In vitro Bioassay

Before determination of the reproducibility, the specificity of this method by using six individual serum samples of rats at 10 ng/mL was confirmed (Table 3). The determined concentrations of KRN321 were from 9.3 to

Table 3. Specificity for KRN321 determination in rat serum by *in vitro* bioassay

Animal	Blank (ng/mL)	KRN321 concentration (ng/mL)	Deviation (%)
1	N.A.	9.3	−7.0
2	N.A.	9.5	−5.0
3	N.A.	10.5	5.0
4	N.A.	10.1	1.0
5	N.A.	10.0	0.0
6	N.A.	9.8	−2.0
Mean	–	9.9	−1.3
S.D.	–	0.4	4.3
C.V. (%)	–	0.04	−3.2

N.A.: Not applicable (Individual blank serum did not give a KRN321 concentration because of denial of parallelism between standard and sample curves.).

–: Not calculated.

Each specificity sample was prepared to be 10 ng/mL in rat serum.

Table 4. Intra- and inter-assay reproducibility of KRN321 determination in rat serum by *in vitro* bioassay

KRN321 concentration (ng/mL)			
Nominal	Mean ± S.D.	C.V. (%)	Deviation (%)
Intra-assay (n = 3)			
10	10.6 ± 0.5	4.7	6.0
20	19.7 ± 0.3	1.5	−1.5
200	207.3 ± 8.1	3.9	3.7
Inter-assay (n = 3)			
10	10.1 ± 0.7	6.9	1.0
20	18.8 ± 0.9	4.8	−6.0
200	202.7 ± 8.3	4.1	1.3

A deviation (%) was calculated to compare with nominal concentration using mean observed concentration value.

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10.5 ng/mL, and the deviation against the prepared concentration ranged from -7.0% to 5.0%. The results obtained for all the samples met the acceptance criterion of which the deviation against the preparation concentration was within ±25%. Table 4 represents the results of the intra- and inter-assay reproducibility of *in vitro* bioassay. The precision and accuracy were, respectively, 4.7% and 6.0% at 10 ng/mL, 1.5% and −1.5% at 20 ng/mL, and 3.9% and 3.7% at 200 ng/mL in the intra-assay reproducibility. Regarding the inter-assay reproducibility, the precision and accuracy were, respectively, from 4.1% to 6.9% and from −6.0% to 1.3% for the samples of concentrations ranging from 10 to 200 ng/mL. These values satisfied the acceptance criteria for *in vitro* bioassay, i.e., that the precision and accuracy were 25% or less and within ±25%, respectively.

Correlation Between ELISA and *in vitro* Bioassay

The serum concentrations of KRN321, determined by both ELISA and *in vitro* bioassay, of the total 28 serum samples up to 24-hr after a single intravenous administration of KRN321 to 8 rats, are plotted in Fig. 1. The measured concentrations generated by the ELISA and the *in vitro* bioassay were from 9.1 to 82.7 ng/mL and from 7.7 to 89.6 ng/mL, respectively (individual values were not shown). A linear regression line in this figure represents the correlation between ELISA and *in vitro* bioassay, and the correlation coefficient and 95% confidence intervals are also shown in the figure. The correlation equation that was obtained by Passing-Bablok linear regression was $y = 1.0392x - 1.612$.

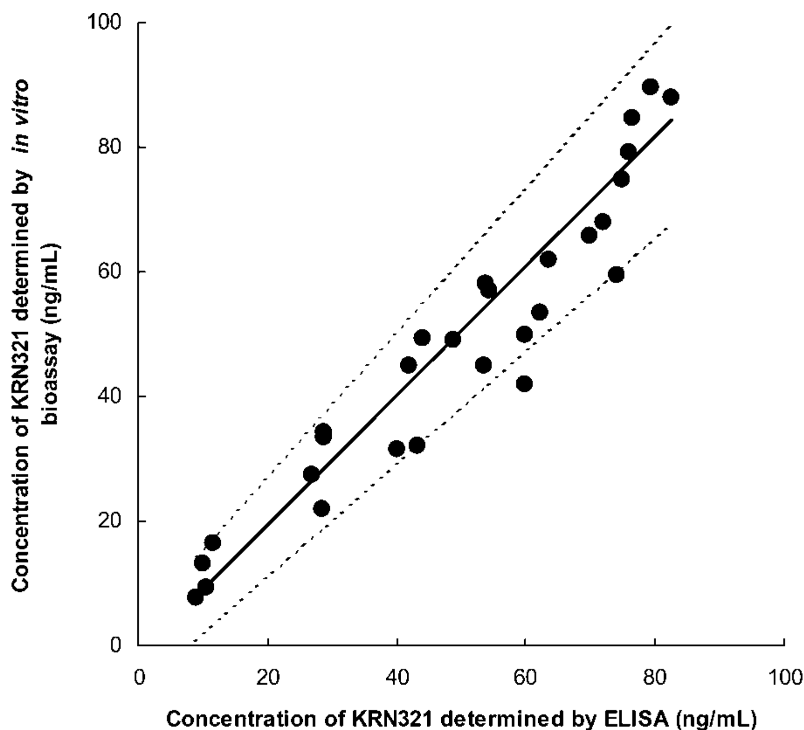

Figure 1. Correlation between ELISA and *in vitro* bioassay in determination of KRN321 in rat serum. Each point expresses a concentration of KRN321 in rat serum obtained from rats administered intravenously at a dose of 2.5 μg/kg. Passing-Bablok regression analysis gave a slope of 1.0392, an intercept of −1.612 over the range tested. A solid line and dotted lines express a linear regression line and upper/lower confident interval lines, respectively.

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Additionally, we confirmed freeze-thaw stability and stability under freezing condition at −80°C by both methods (data not shown). As results of the freeze-thaw stability, we confirmed that the treatment did not affect the observed values. It indicated that KRN321 was not affected by 3 cycles of freeze-thaw treatment and maintained biological activity during the treatments. Furthermore, we confirmed that KRN321 was stable in rat serum under freezing condition for 12 weeks and maintained biological activity for 8 weeks.

DISCUSSION

Prior to the implementation of the pharmacokinetic studies in rats, we carried out a quantitation validation study to establish the determination method for

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KRN321 in rat serum by using a commercially available ELISA assay kit, Quantikine® IVD® Erythropoietin. Since KRN321 is a hyperglycosylated analog of epoetin alfa, it was confirmed in a preliminary study that KRN321 formed a conjugate with the rHuEPO antibody adapted the kit to ELISA. Thus, when KRN321 is used as standard sample for a calibration curve, it was thought that the endogenous EPO concentrations, in terms of KRN321, might be calculated to be higher than the actual concentrations because the affinity of KRN321 to the antibody was supposed to be lower than that of rHuEPO. Furthermore, since cross-reactivity was observed between rHuEPO antibody and the endogenous EPO or KRN321, KRN321 could not be specifically determined by the present method. Actually, in the study of the specificity, the mean endogenous EPO levels, in terms of KRN321 concentration, was determined to be 91.1 pg/mL; the value was thought to be the level to have an influence on the measured concentrations of KRN321. It was, however, confirmed that KRN321 per se could be quantitated by subtracting the concentrations of the endogenous EPO from the total serum concentration, because KRN321 concentrations determined by such a means satisfied the criteria of the specificity within $\pm 20\%$ of the spiked concentration.

In studies of the intra- and inter-assay reproducibility by ELISA, the accuracy and precision were confirmed at concentration ranges from 100 to 4,000 pg/mL and from 100 to 3,000 pg/mL, respectively. The criteria for the accuracy and precision, in both the intra-assay and the inter-assay reproducibility, were, respectively, within $\pm 20\%$ and below 20%, while within $\pm 25\%$ and below 25% at the LLOQ. As a result, the LLOQ was defined to be 100 pg/mL, and the quantitation range was fixed to be 100 to 4,000 pg/mL.

Meanwhile, serum concentration of KRN321 has been quantitated by ELISA; the method for analyzing biological activity in the serum sample has not yet been established. Hence, it was not clear whether the serum concentration determined by ELISA actually reflect the biological activity. So, in order to establish the analytical method for concentration of KRN321 in rat serum by using biological activity as an index, the *in vitro* bioassay method using UT-7/Epo, human leukemia cell line, was validated. The specificity was examined for the sample that was prepared by adding KRN321 to the individual blank sera at a concentration of 10 ng/mL. Regarding the blank serum, it did not influence the fluorescence intensity to determine the concentration of KRN321 (Table 3). This result indicated that the determined KRN321 was not affected in the present quantitation method in which biological activity was used as an index. On the other hand, the measured values of 9.3 to 10.5 ng/mL were obtained in the spiking samples. Since all of these values satisfied the acceptance criteria, the specificity of the method for determination, at a concentration of 10 ng/mL, of KRN321 in serum samples was confirmed. In regard to the study of intra-assay and inter-assay reproducibility by *in vitro* bioassay, the precision and accuracy at all concentrations examined were verified to satisfy the acceptance criteria. The LLOQ was fixed to be 10 ng/

mL in the present method because it was clarified that analytical specificity was not satisfied at 5 ng/mL (data not shown).

To verify whether the measured values of KRN321 in serum by the ELISA actually reflected the biological activity, the correlation between the ELISA and the *in vitro* bioassay was evaluated; good correlation was observed between the two methods, based on the correlation equation by the Passing-Bablok linear regression. In addition, the result that the correlation equation and the origin of the graph were included within the 95% confidence intervals also indicated the good correlation of the two measurement methods. The present result indicates that the ELISA and the *in vitro* bioassay were precisely correlated. In other words, the measured values of KRN321 in rat serum obtained by the ELISA actually reflect the concentrations of KRN321 that maintain biological activity determined by the *in vitro* bioassay.

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CONCLUSIONS

Prior to the implementation of the pharmacokinetic studies in rats, the validation study by using a commercially available ELISA assay kit (Quantikine® IVD® Erythropoietin) and *in vitro* bioassay by using UT-7/Epo, human leukemia cell line, was carried out to assess the quantitation method for KRN321 in rat serum. On the basis of results of reproducibility, it was confirmed that both determination methods of KRN321 in rat serum were established within the concentration range from 100 to 4,000 pg/mL for ELISA and diluting the concentration to about 10 ng/mL for *in vitro* bioassay.

In addition, the correlation between the two methods was conducted, and good correlation was observed between the ELISA and the *in vitro* bioassay by the Passing-Bablok linear regression. This indicates that the concentrations of KRN321 in rat serum obtained by the ELISA actually reflect the concentrations of KRN321 that maintain biological activity determined by the *in vitro* bioassay.

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