Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 872–877

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantification of CKD-501, lobeglitazone, in rat plasma using a liquid-chromatography/tandem mass spectrometry method and its applications to pharmacokinetic studies

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ARTICLE INFO

Article history:
Received 22 December 2008
Received in revised form 29 May 2009
Accepted 3 June 2009
Available online 12 June 2009

Keywords:
CKD-501
Rosiglitazone
Pharmacokinetics
LC–MS/MS
FF-PPT

ABSTRACT

CKD-501 (i.e., lobeglitazone), a potent agonist for both PPARα/γ, is a new drug that has potential clinical applications in the management of type-2 diabetes. The objective of this study was to develop a rapid and sensitive method for the determination of CKD-501 in rat plasma and to assess the applicability of the assay to pharmacokinetic studies. Rat plasma samples were processed using a fast flow protein precipitation (FF-PPT) method and then introduced onto an LC–MS/MS system for quantification. The analyte and rosiglitazone, an internal standard, were analyzed by multiple reactions monitoring (MRM) at *m*/*z* transitions of 482.0 → 258.0 for CKD-501 and 358.0 → 135.0 for the internal standard. The lower limit of quantification (LLOQ) was determined at 50 ng/mL, with an acceptable linearity in the range from 50 to 10,000 ng/mL ($R > 0.999$). Validation parameters such as accuracy, precision, dilution, recovery, matrix effect and stability were found to be within the acceptance criteria of the assay validation guidelines, indicating that the assay is applicable to estimating the concentration in the range studied. The concentration of CKD-501 was readily quantifiable in plasma samples up to 24 h post-dose in rats that had received an oral dose of 1 mg/kg. These observations suggest, therefore, that the validated assay can be used in pharmacokinetic studies of CKD-501 in small animals such as the rat.

1. Introduction

Recent findings on the progression of type-2 diabetes mellitus indicate that the insulin resistance in peripheral tissues induces compensatory hyperinsulinemia, followed by β-islet cell failure, initially leading to prandial pain and subsequently to obvious fasting hyperglycemia [1,2]. Peroxisome proliferator-activated receptors (PPARs), which are largely expressed in adipose tissue in comparison to skeletal muscle and liver tissue, are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily [3,4]. Thiazolidinediones (TZDs)-based PPAR activators, such as CKD-501, rosiglitazone, and pioglitazone, are believed to intensify the action of insulin, thus promoting the utilization of glucose in peripheral tissues, and, as a result, are therapeutically useful in the management of type-2 diabetes [5,6].

CKD-501 (i.e., lobeglitazone) is a new drug that is under development for therapeutic applications in the treatment of diabetes. Since the compound is reported to have a higher affinity towards the receptor [7–11], the effective dose may be lowered, along with cardiovascular side effects (i.e., common for TZD PPAR activators), compared to those of other TZD analogues [7–11]. CKD-501 was reported to have adequate pharmacokinetic properties and to have more efficacious in vivo effects in an animal model of type-2 diabetes compared to rosiglitazone and pioglitazone [7,11]. Thus, CKD-501 would be expected to be an useful addition to the management of the hyperglycemic condition caused by the type-2 diabetes. The compound is currently under phase II trials in Korea.

The preparation of complex biological matrices (e.g., rat plasma) for a chromatographic assay is often involved and, as a result, the complicated processing protocol is frequently a limiting factor in the throughput of the assay. A fast flow protein precipitation (FF-PPT) method [12–14] was recently reported to facilitate the processing of complex biological matrices such as plasma samples in analytical procedures. Accelerated processing has the potential to enhance throughput as well as the stability of the analyte during an analytical procedure. The objective of this study, therefore, was to develop and validate an analytical methodology involving the use of FF-PPT for the quantification CKD-501 in rat plasma that is

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doi:10.1016/j.jpba.2009.06.003

consistent with the bioanalytical method validation guidelines of the FDA [15]. Since the assay is intended for the study of the pharmacokinetics of CKD-501, its applicability to its quantification was also assessed.

2. Experimental

2.1. Chemicals and reagents

CKD-501 (98.5% purity) and rosiglitazone [99.0% purity, an internal standard (IS) of this study] were provided by Chong Kun Dang Pharmaceuticals (Seoul, Korea). Acetonitrile (HPLC grade) and formic acid were obtained from J.T. Baker (Phillipsburg, NJ, USA) and from Fluka (Cambridge, MA, USA), respectively, and used without further purification. Blank rat plasma samples containing heparin (an anticoagulant) were obtained from the Korea Institute of Toxicology (Daejeon, Korea).

2.2. LC conditions

A HP 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of a binary pump, an online degasser, an autosampler, and a column heater, equipped a reversed phase HPLC column [Zorbax C18 column (2.1 mm × 100 mm, 3.5 μm internal diameter; Agilent Technologies)] for the chromatographic separation of CKD-501 and the IS was used in the study. The mobile phase was composed of acetonitrile–water–formic acid (60:40:0.25, v/v/v) and eluted at a flow rate of 0.2 mL/min. In this study, the sample volume was set at 5 μL, and the analytical column and samples were maintained at 30 °C and 20 °C, respectively.

2.3. Mass spectrometer conditions

Mass spectrometric detection was performed with a Quattro Micro™ quadrupole mass spectrometer (Waters, Milford, MA, USA), equipped with an electro-spray ionization (ESI) source operating in the positive-ion mode. In this study, the spectrometer was set in the multiple reaction monitoring (MRM) mode. The settings of the mass spectrometer for CKD-501 were 3.5 kV for the capillary voltage, 40 V for the cone voltage, 200 ms for the dwell time, 40 V for the collision energy, 120 °C for the source temperature and 300 °C for the desolvation temperature. The analytical conditions for the IS were identical to those for CKD-501, except for the collision energy of 30 V. In this study, MRM m/z transitions at 482.0 → 258.0 for CKD-501 and 358.0 → 135.0 for the IS were simultaneously monitored. Data acquisition and processing were performed with the MassLynx software (version 4.0; Waters).

2.4. Sample preparation by FF-PPT

An aliquot (i.e., 45 μL) of thawed plasma sample was transferred to a Whatman Protein Precipitation Unifilter™ Fast Flow (Whatman, Florham Park, NJ, USA). An aliquot (i.e., 5 μL) of the working internal standard solution (concentration of 1000 ng/mL, see Section 2.5) was then added to the sample using an eight-channel pipette, and the plate vortexed for a few seconds. Approximately 200 μL of acetonitrile was dispensed to each well of the filter plate and mixed for 2 min using a plate vortexer. The Whatman Unifilter™ was placed on top of a vacuum manifold and a 96-well collection plate was placed at the bottom of the manifold. A reduced pressure of 18 in. of Hg was applied until all of the wells were cleared. The collection plate was then placed on an autosampler rack held at 4 °C. A 5 μL aliquot from each well was injected on to the HPLC system.

2.5. Standards and quality control (QC) samples

Stock solutions of CKD-501 and the IS were prepared in acetonitrile at concentrations of 1000 μg/mL and 1000 ng/mL, respectively. A set of CKD-501 standard solutions and QC solutions were obtained by a successive dilution of the stock solutions with acetonitrile. The IS working solution was prepared daily in acetonitrile. A 5 μL aliquot of CKD-501 standard solution was spiked to 45 μL of blank rat plasma, resulting in eight nonzero calibration standards, to give concentrations of CKD-501 at 50, 100, 250, 500, 1000, 2500, 5000, or 10,000 ng/mL. Using a similar preparation method, the QC samples were prepared to give concentrations of CKD-501 of 50, 150, 1000, or 8000 ng in mL of rat plasma. The samples were then processed similar to the procedure described in Section 2.4.

2.6. Method validation

2.6.1. Selectivity

The selectivity of the analysis was evaluated, using six lots of blank matrices (i.e., samples without CKD-501 and IS), of zero samples (i.e., blank plasma added with the IS), and of the lower limit of quantification (LLOQ) samples, for the presence of any interfering peak in the chromatograms.

2.6.2. Linearity

Calibration curves were constructed with the ratios of the peak area of CKD-501 to that of the IS against the CKD-501 concentration in the plasma standards. A series of linear regression analyses were carried out assuming with or without the intercept, and weighing factor ($1/x$, $1/x^2$ or none). A preliminary experiment indicated that the model having a weighing of $1/x$ with an intercept provided the best-fit (i.e., the smallest sums of square value) for the data. In subsequent studies, the best-fit model was used throughout the study.

2.6.3. Precision, accuracy and dilution

Three batches were used to assess the precision and accuracy of the assay. In the validation study, each batch, consisting of a set of six replicates of QC samples in a single run, was processed on separate days. The precision of the assay was estimated by the relative standard deviation at each concentration level. The accuracy of the assay was determined by calculating the difference between the calculated and theoretical concentrations. In addition, another batch with six replicates of the plasma samples containing 80,000 ng/mL was prepared. The samples were then diluted tenfold with blank rat plasma to obtain an expected concentration at 8000 ng/mL. The diluted samples were then processed and analyzed to assess the concentration of the original sample adequately.

2.6.4. Matrix effect and recovery

The recovery and matrix effect were also determined in this study. The absolute/relative matrix effect and recoveries of CKD-501 and rosiglitazone were assessed by analyzing three sets of standards at three concentrations (i.e., 150, 1000 and 8000 ng/mL). To determine the absolute matrix effect for CKD-501 and rosiglitazone, blank plasma, obtained from six different rats, were extracted as described previously, and CKD-501 and rosiglitazone were added to the post-extraction sample to have the three concentration levels (set 2). The mean peak areas of the analyte were compared with the mean peak areas from the neat solutions of the analyte in acetonitrile (set 1). For the case of the relative matrix effect, the variability, expressed as precision (CV, %), in the peak areas of the analyte added to the post-extraction samples from the blank plasma of six different rats (set 2) was determined and considered as the relative matrix effect [16].

Recoveries of CKD-501 and rosiglitazone were determined by comparing mean peak areas of analytes added before extraction into the same six different sources as set 2 (set 3) with those of the analytes added post-extraction samples from different lots of rat plasma at three concentrations (set 2).

2.6.5. Stability

To evaluate the stability of the stock solution, a set of stock solutions for CKD-501 were freshly prepared and the response from the LC–MS/MS in fresh solutions compared with that from the stored stock solution. In this study, two storage conditions [i.e., a 6 h storage at room temperature (20 °C) and a 3-week storage under refrigeration] were used. In addition, the stability of CKD-501 after three cycles of the freeze–thaw process was evaluated. Thus, QC samples at 150 and 8000 ng/mL were freshly prepared, frozen at −80 °C and, and the samples were then thawed at room temperature. The samples were then subjected to two additional cycles of the freeze–thaw process (i.e., total of three cycle). After the third thaw step, the samples were analyzed. Further, the post-preparative stability of processed samples in the autosampler (i.e., operating at 4 °C) was assessed to determine whether an occasional delay in the analysis could lead to instability of the analyte. Short-term (benchtop) stability was determined by allowing the QC samples to stand on the benchtop for 24 h prior to the analysis. Long-term stability of the analyte in rat plasma at −80 °C was evaluated by analyzing QC samples over a time course of 2 weeks. In the stability assessment studies (i.e., stock solution stability, freeze–thaw stability, post-preparative stability and short-/long-term stability), the analyte was considered stable if the difference is less than 15% in the response from that in the corresponding fresh sample (in the case of the stock solution stability) or in the concentration from the theoretical value (in the case of other stability studies).

2.7. Application of the assay

To determine the applicability of the assay to pharmacokinetic studies involving CKD-501, hydrochloride salt form of CKD-501 in a 5% gum arabic solution was orally administered to rats and the assay used to determine its concentration in plasma samples. Female Sprague–Dawley rats, weighing 125.0–139.8 g (i.e., 5 weeks of age) were used in this study. Experimental protocols involving animals in this study were reviewed by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology, according to National Institutes of Health guidelines (NIH publication number 85–23, revised 1985) "Principles of Laboratory Animal Care". All animals used in this study were cared for in accordance with the principles outlined in the NIH publication of "Guide for the Care and Use of Laboratory Animals".

Hydrochloride salt form of CKD-501 was dispersed in a 5% gum arabic solution and the solution administered orally at a dose of 1 mg/kg. Blood samples (0.25 mL) were collected into heparinized tubes via the tail vein prior to and at 0.5, 1, 2, 3, 4, 8, and 24 h after administration of the drug. Plasma samples, obtained by centrifugation of the blood at 13,200 rpm for 5 min, were stored at −80 °C prior to analysis.

When it was necessary to determine the pharmacokinetic parameters, the standard moment analysis was used. The area under the CKD-501 concentration in the plasma–time curve from time zero to infinity ($AUC_{0-\infty}$) and the area under the respective first moment–time curve from time zero to infinity ($AUMC_{0-\infty}$) were calculated by linear trapezoidal method and appropriate area extrapolation [17]. MRT, the mean residence time, was estimated using by the equation below:

$$MRT = \frac{AUM_{0-\infty}}{AUC_{0-\infty}}$$

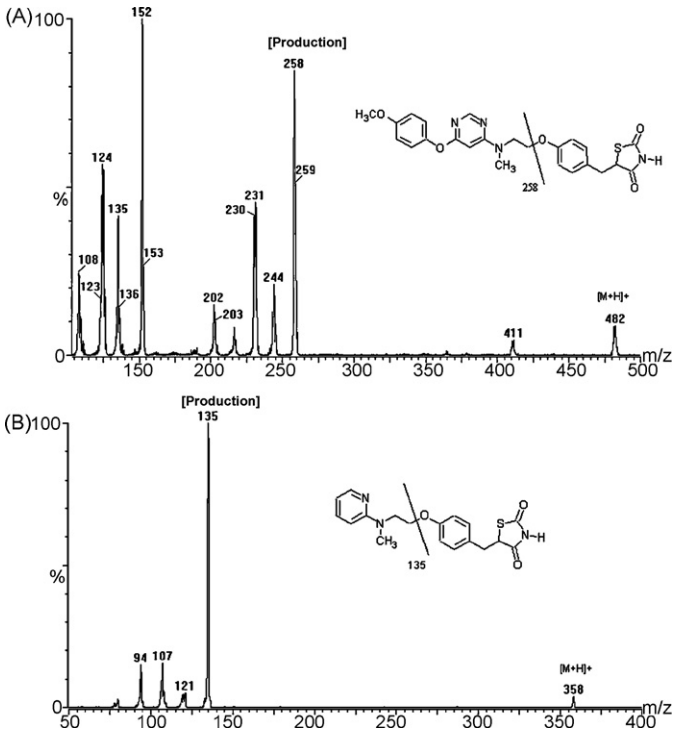

Fig. 1. The structures and product-ion scan spectra of: (A) CKD-501 and (B) rosiglitazone (i.e., the internal standard).

The terminal phase half-life ($T_{1/2}$) was calculated from the slope (λ) of the log–linear portion of the concentration time profile using the equation below:

$$T_{1/2} = \frac{0.693}{\lambda}$$

The maximum CKD-501 concentration (C_{max}) and the time to reach C_{max} (T_{max}) were read directly from the temporal profile of CKD-501 concentration in the plasma.

3. Results and discussion

3.1. Chromatography

The chemical structures of CKD-501 and rosiglitazone (i.e., the IS) are shown in Fig. 1. Preliminary studies, involving the adjustment of the collision energy and cone voltage, indicate that the transition conditions of m/z 482 → 258 (for CKD-501) and m/z 358 → 135 (for IS) are adequate for its detection and the quantification. Therefore, these analytical conditions were used in subsequent studies.

Preliminary studies were also carried out to optimize the selectivity as well as the throughput of the assay by adjusting the chromatographic conditions. As a result, the chromatographic condition with retention times of 1.3 min for CKD-501 and 2.6 min for the IS were found to be adequate with apparently symmetric peaks for both CKD-501 and the IS (Fig. 2). These chromatographic conditions were used in subsequent studies.

3.2. Specificity and lower limit of quantification

Under the LC–MS/MS conditions used, CKD-501 and the IS were clearly separated from endogenous peaks originating from the blank matrix (Fig. 2). Similarly, chromatograms of six lots of blank analyses indicate that the analyte peaks were well separated from interfering peaks (Fig. 2 and Table 1) and that the assay condition

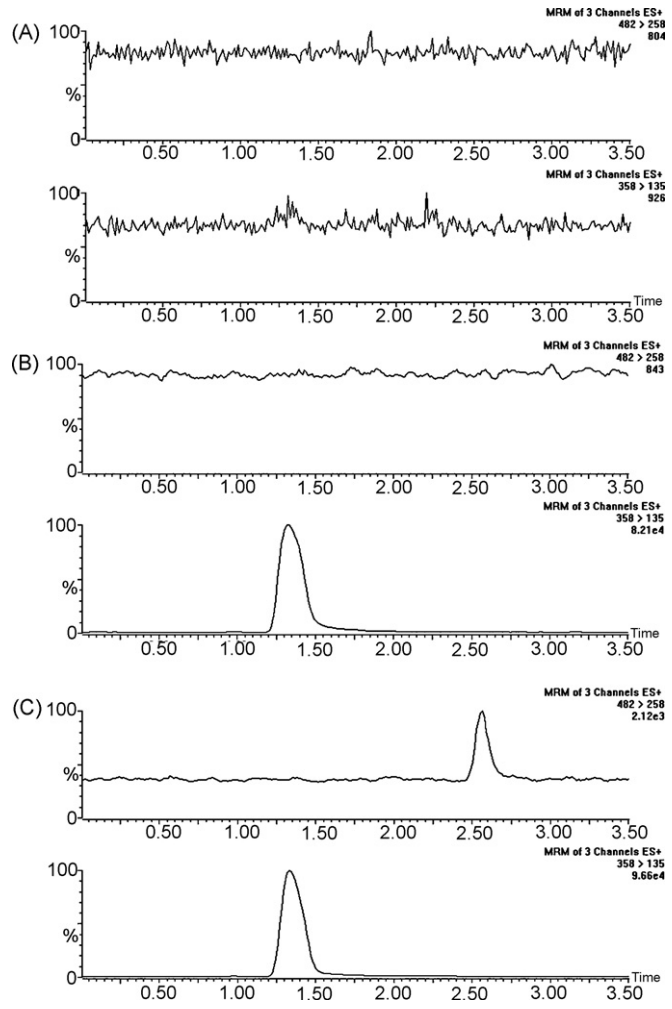

Fig. 2. Multiple reaction monitoring (MRM) chromatograms of: (A) double blank plasma, (B) plasma containing rosiglitazone (IS, 1000 ng/mL), and (C) plasma containing CKD-501 at LLOQ (50 ng/mL) and IS.

has an adequate specificity for CKD-501. Results from our preliminary study, indicates that the concentrations of metabolites, having the *m*/*z* values (i.e., 218, 231, 466), accounted for significantly less (i.e., approximately 12.5%) than that of the parent drug at various times up to 4 h. In addition, these metabolites do not appear to possess pharmacological activities. At the lowest concentration level (i.e., 50 ng/mL) of CKD-501, the accuracy (RE) and precision (CV) for the six replicates were −7.2% and 1.3%, respectively (Table 3). Furthermore, the signal to noise level was at least 33 at this concentration. Taking the above data into consideration, the lower limit of the quantification was determined at 50 ng/mL for this assay.

Table 1
Specificity of CKD-501 measurements in rat plasma.

Matrix lot.	Response (peak area)			
	Blank[a]	Zero blank[b]	LLOQ (50 ng/mL)	HQC (8000 ng/mL)
1	0.0	0.0	152.7	18330.6
2	0.0	0.0	143.5	18938.1
3	0.0	0.0	144.1	18555.7
4	0.0	0.0	144.7	18689.1
5	0.0	0.0	148.2	18209.1
6	0.0	0.0	142.4	18636.8
Mean	0.0	0.0	145.9	18559.9
CV (%)[c]	0.0	0.0	2.6	1.4

[a] Rat plasma, containing no analyte or IS, were extracted and analyzed.
[b] Rat plasma, containing only IS, were extracted and analyzed.
[c] CV (%) = standard deviation/mean × 100.

Table 2
Calibration curves generated for CKD-501 in rat plasma.

Run	Slope	Intercept	*R*
1	0.166	2.982	1.000
2	0.174	1.392	0.999
3	0.153	3.780	0.999
Mean	0.164	2.718	0.999
CV (%)[a]	6.5	–[b]	–

[a] CV (%) = standard deviation/mean × 100.
[b] Not applicable.

3.3. Linearity

The calibration curves for CKD-501 in rat plasma appeared to be linear over the concentration range of 50–10,000 ng/mL. A linear regression analysis of the data indicated that the correlation coefficient was in excess of 0.999 for three batches of calibration curves. Table 2 shows the results of a statistical analysis obtained from three runs of calibration curve for CKD-501.

3.4. Accuracy, precision, and sample dilution

QC samples, having four concentration levels (50, 150, 1000, and 8000 ng/mL), were analyzed in six replicates to determine the intra-day accuracy and precision. The accuracy (RE) for CKD-501 ranged from −7.2% to 5.7% with a precision (CV) of between 1.3% and 5.7% (Table 3). In addition, the inter-day accuracy and precision were estimated using six QC replicates at the four concentration levels on 3 different days. The accuracy (RE) for CKD-501 ranged from −5.3% to 1.7% with a precision (CV) of between 4.0% and 5.0% (Table 3).

In pharmacokinetic studies, the concentrations of analyte in certain plasma samples may exceed the upper limit of quantification set by the assay. Therefore, an adequate assay should be capable of properly estimating the concentration of such samples by appropriate dilution and correction. To demonstrate the versatility of the current assay, a set of plasma samples were prepared so as to contain a CKD-501 concentration of 80,000 ng/mL (viz, the upper limit of quantification to be 10,000 ng/mL in this assay). The samples were then diluted tenfold to give the expected concentration of CKD-501 of 8000 ng/mL and analyzed. As shown in Table 3, the calculated concentration was found to be 7923.4 ng/mL (i.e., the

Table 3
Summary quality control sample runs for of the present assay for CKD-501 in rat plasma.

Batch	Theoretical concentration (ng/mL)				
	LLOQ 50	LQC 150	MQC 1000	HQC 8000	HQC 8000[a]
(A) Intra-day accuracy and precision					
Number of samples	6	6	6	6	6
Mean estimated concentration	46.4	143.4	1056.5	7549.6	7923.4
CV (%)[b]	1.3	5.7	2.3	3.6	1.9
RE (%)[c]	−7.2	−4.4	5.7	−5.6	−1.0
(B) Inter-day accuracy and precision					
Number of samples	18	18	18	18	
Mean estimated concentration	47.3	144.0	1017.0	7745.6	
CV (%)	5.0	5.0	4.3	4.0	
RE (%)	−5.3	−4.0	1.7	−3.2	

[a] Analyzed after a tenfold dilution with blank rat plasma (i.e., 80,000 → 8000 ng/mL).
[b] CV (%) = standard deviation of the concentration/mean concentration × 100.
[c] RE (%) = (calculated concentration − theoretical concentration)/theoretical concentration × 100.

Table 4
Matrix effect, recovery, and precision (CV, %) for CKD-501 and rosiglitazone (internal standard) in six different lots of rat plasma.

Nominal concentration (ng/mL)	Absolute matrix effect[a] (%)		Recovery[b] (%)		Precision[c] (CV, %)					
	CKD-501	IS	CKD-501	IS	CKD-501		IS		CKD-501/IS	
					Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
150	135.2	87.2	79.0	77.2	5.7	3.0	4.2	2.9	7.0	5.8
1000	123.7	87.4	93.3	90.0	2.3	1.6	2.1	4.5	2.2	5.8
8000	111.9	88.1	97.8	86.8	5.1	3.6	7.9	3.8	5.4	7.9

[a] Absolute matrix effect expressed as the ratio of the mean peak area of an analyte added post-extraction (set 2) to the mean peak area of the same analyte standards (set 1) multiplied by 100.
[b] Recovery calculated as the ratio of the mean peak area of an analyte added before extraction to the mean peak area of an analyte spiked post-extraction (set 2) multiplied by 100.
[c] Precision of determination of peak areas of CKD-501 and rosiglitazone, and peak area ratios (CKD-501/rosiglitazone) in set 1 and 2 as the measure of relative matrix effect.

percent deviation from the theoretical value of −1.0%) with a CV of 1.9%, suggesting that the assay is capable of reasonably estimating CKD-501 concentrations in samples that exceed the upper limit by appropriate dilution.

3.5. Matrix effect and recovery

The matrix effect and recovery was estimated in six different rat plasma by comparing the mean area of the analyte peaks from extracted QC samples at concentrations of 150, 1000, and 8000 ng/mL. The absolute matrix effect was ranged from 111.9% to 135.2% and from 87.2% to 88.1% for CKD-501 and rosiglitazone, respectively (Table 4). The relative matrix effect was assessed based on the direct comparison of the peak areas of CKD-501 and rosiglitazone added to post-extraction samples of plasma from six different sources of rat plasma (set 2). The precision of determination of set 2 (i.e., relative matrix effect) ranged from 1.6% to 3.6% for CKD-501 and from 2.9% to 4.5% for rosiglitazone. The variability appeared comparable to the variability data obtained with the standard solution in which the analyte was dissolved in acetonitrile (i.e., set 1, CKD-501; 2.1–5.7%, rosiglitazone; 2.1–7.9%). These data confirm the absence of the relative matrix effect for CKD-501 and rosiglitazone. The precision (CV, %) of the ratio of CKD-501/rosiglitazone was ranged from 5.8% to 7.9% for samples spiked post-extraction into blank extracts and from 2.2% to 7.0% for standards injected directly in acetonitrile, indicating that the absolute and relative matrix effects for ratio of peak areas of CKD-501 and rosiglitazone are insignificant for the determination of CKD-501 added to six different lots of rat plasma.

The overall recovery of CKD-501 was 79.0%, 93.3% and 97.8% for 150, 1000 and 8000 ng/mL concentration levels, respectively and the recovery for rosiglitazone (i.e., the IS) was found to be 77.2%, 90.0% and 86.8% (Table 4). Collectively, these observations indicate that the current sample processing conditions support adequate recoveries for both the analyte and the IS.

3.6. Stability

Analyte stability was examined for a variety of conditions used in the handling and storage of both standards and samples. The results are summarized in Tables 5 and 6. The stock solution stability of CKD-501 at a concentration of 250 ng/mL was investigated over a 6 h period at room temperature (20 °C) and a 3-week period under refrigeration (4 °C) with six replicates each. The concentration of the stored sample was found to be is 99.2% (i.e., difference of 0.8%) at the 20 °C storage conditions and 99.6% (i.e., the difference of 0.4%) at the 4 °C conditions (Table 5) of the concentration found in a corresponding fresh sample (i.e., indicated as 0 h in Table 5). When QC samples, having concentrations of 150 ng/mL and 8000 ng/mL, were allowed to stand at room temperature for 24 h (i.e., indicated as benchtop stability in Table 6), the calculated concentration was close to the theoretical value (i.e., −2.8% and −0.6% for 150 ng/mL and 8000 ng/mL samples, respectively). Similarly, a post-preparative stability assessment indicated that the concentrations found in the samples subjected to the post-preparative stability study conditions were different by −1.4% and −0.9% from the theoretical values for 150 ng/mL and 8000 ng/mL, respectively. Three cycles of freeze–thaw had no effect on the stability of CKD-501 as evidenced by the fact that the estimated concentration was only slightly different from the theoretical values (i.e., −5.7% for 150 ng/mL and 1.3% for 8000 ng/mL in Table 6). Long-term storage of rat plasma samples at −80 °C was found to be adequate in terms of the stability of CKD-501 since the estimated concentration

Table 5
Stability of CKD-501 in stock solutions.

Batch	Response (peak area)[a]		
	0 h (initial)	6 h (room temp.)	3 weeks (refrigerated)[b]
Number of samples	6	6	6
Mean response	1855.4	1840.1	1847.7
CV (%)	1.3	0.7	1.4
Relative conc. (%)[c]	100	99.2	99.6

[a] Stock solutions were diluted to 250 ng/mL for analysis.
[b] Stock solutions were stored at 4 °C.
[c] Relative concentration (%) obtained from the measured value divided by the initial value.

Table 6
Stability of quality control samples.

Batch	Theoretical concentration (ng/mL)	
	150	8000
(A) Benchtop stability at room temperature for 24 h		
Number of samples	3	3
Mean estimated concentration	145.8	7955.4
CV (%)[a]	2.0	2.1
RE (%)[b]	−2.8	−0.6
(B) Post-preparative stability at 4 °C for 3 days		
Number of samples	3	3
Mean estimated concentration	147.9	7925.2
CV (%)	4.7	2.3
RE (%)	−1.4	−0.9
(C) Freeze–thaw stability (3 cycles)		
Number of samples	3	3
Mean estimated concentration	141.4	8107.1
CV (%)	1.7	0.8
RE (%)	−5.7	1.3
(D) Long-term stability for 2 weeks		
Number of samples	3	3
Mean estimated concentration	146.7	8119.1
CV (%)	2.2	2.7
RE (%)	−2.2	1.5

[a] CV (%) = standard deviation of the concentration/mean concentration × 100.
[b] RE (%) = (calculated concentration − theoretical concentration)/theoretical concentration × 100.

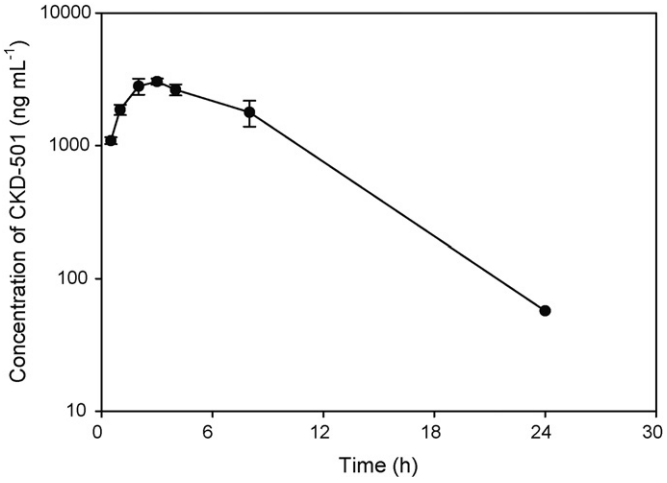

Fig. 3. Temporal profile of plasma concentration of CKD-501 in rats receiving an oral administration of 1 mg/kg CKD-501 (mean ± standard deviation, $n = 3$ rats).

Table 7
Pharmacokinetic parameters of CKD-501 following an oral administration of CKD-501 at a dose of 1 mg/kg in rats ($n = 3$).

Pharmacokinetic parameters	Mean ± SD
T_{max} (h)	2.3 ± 0.5
C_{max} (ng/mL)	3046.7 ± 160.4
$T_{1/2}$ (h)	5.3 ± 2.6
MRT (h)	8.7 ± 3.7
$AUC_{0-\infty}$ (ng h/mL)	34028.6 ± 8726.7

was close to the real values (i.e., −2.2% for 150 ng/mL and 1.5% for 8000 ng/mL in Table 6). Therefore, these observations indicate that CKD-501 is stable under the handling and storage conditions used in the study, and that typical processing and storage conditions do not affect the estimation of CKD-501 concentrations in rat plasma samples.

3.7. Applicability to pharmacokinetic study

To assess the applicability of the present assay to use in pharmacokinetic studies involving CKD-501 administration, the assay was used to determine the plasma concentration of CKD-501 in female rats that has received orally hydrochloride salt form of CKD-501 at a dose of 1 mg/kg. The temporal profile for the mean plasma concentration of CKD-501 is shown in Fig. 3. In all plasma samples collected up to 24 h post-dose, the concentration of CKD-501 was readily measurable, suggesting that the current assay is adequate for determining the pharmacokinetic characteristics of CKD-501 at an oral dose of 1 mg/kg. The calculated pharmacokinetic parameters, including C_{max}, T_{max}, $T_{1/2}$, MRT and $AUC_{0-\infty}$, listed in Table 7.

4. Conclusions

An analytical method for the determination of CKD-501 in rat plasma was developed and validated in terms of selectivity, linearity, accuracy, precision, dilution, recovery, matrix effect and the stability of the assay. The features of the present method include a simple sample preparation procedure, a short LC run-time and an acceptable sensitivity/reliability for pharmaco-/toxico-kinetic studies involving CKD-501. Therefore, the present assay promises to be useful in the further development of CKD-501 as a new drug.

Acknowledgement

This study was supported, in part, by a grant (A030001) of the Ministry for Health, Welfare and Family Affairs, Republic of Korea.

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