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Determination of a dipeptidyl peptidase IV agonist, β-aminoacyl containing thiazolidine derivatives (KR-66223) in rat plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed for a novel dipeptidyl peptidase IV agonist (DDP-IV) agonist, KR-66223, in rat plasma. It involves liquid-liquid extraction (LLE) followed by HPLC separation and electrospray ionization tandem mass spectrometry. KR-66223 and imipramine (IS) was separated on Gemini-NX C18 column with mixture of acetonitrile–ammonium formate (10 mM) (90:10, v/v) as mobile phase. The ion transitions monitored were m/z 553.2 → 206.2 for KR-66223, m/z 281.3 → 86.1 for imipramine in multiple reaction monitoring (MRM) mode. The linear ranges of the assay were 0.003–10 μg/ml with a correlation coefficient (R^2) greater than 0.99 and the lower limit of quantification was 3 ng/ml. The average recovery was 78.9% and 87.1% from rat plasma for KR-66223 and imipramine, respectively. The coefficients of variation of intra- and inter-assay were 3.9–14.4% and the relative error was 0.8–11.5%. The method was validated and successfully applied to the pharmacokinetic study of KR-66223 in rat.

1. Introduction

Diabetes mellitus is one of the main threats to human health worldwide, with an estimated 23.6 million affected children and adults (7.8% of the population) in the United States [1]. Type 2 diabetes, which accounts for over 90% of cases globally, is usually caused by resistance to insulin's action in the setting of an inadequate compensatory insulin secretory response [2]. Current treatment of type 2 diabetes includes carefully managing the diet, taking oral medication, and using some form of insulin [3]. Such therapies have limited effectiveness, are unsustainable, and may result in significant side effects [4].

Incretin hormones are candidates for the treatment of type 2 diabetes. They act through a variety of complementary mechanisms, including secretion of insulin and release of glucagon-like peptide 1 (GLP-1) from the gut in response to the digestion of food, which inhibits the release of glucagon [5]. Other known effects of GLP-1 are slowed gastric emptying, reduced appetite, and increased insulin-sensitivity in β-cells [6,7]. GLP-1 has a short lifetime due to rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4), which cleaves the two N-terminal amino acids to give the inactive GLP-1 amide [8]. Thus, inhibition of DPP-4, which leads to increases in the circulating level of GLP-1, is an alternative approach for the treatment of type 2 diabetes.

KR-66223, a β-aminoacyl containing thiazolidine derivatives, is a newly synthesized small-molecule inhibitor of DPP-4, licensed from the Korea Research Institute of Chemical Technology [9]. This compound showed excellent selectivity toward DPP4 inhibition with an IC_{50} value of 1 nM *in vitro* compared with another DDP isozyme, sitagliptin, which has an IC_{50} value of 20 nM. KR-66223 is chemically and metabolically stable, and showed no CYP inhibition, hERG binding, or cytotoxicity [10,11].

Selective and sensitive analytical methods for the quantitative estimation of drugs are critical for preclinical biopharmaceutical and clinical pharmacology studies [12–14]. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has been widely used to determine drug levels in biological fluids, because it can provide better sensitivity and selectivity than other traditional methods [15–17]. Multiple reaction monitoring (MRM) can be programmed to select certain ions chosen by the operator. This experiment is the ability to detect a specific precursor ion, to isolate that ion for collision-induced fragmentation. It is possible to detect a specific product ion following fragmentation [18].

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The purpose of this study is to validate an LC/MS/MS method using simple liquid–liquid extraction for the quantitative analysis of KR-66223 in rat plasma. The method is evaluated with regard to its accuracy, precision, selectivity, sensitivity, reproducibility, and stability. This is the first method for the biological quantitation of KR-66223 and preliminary pharmacokinetic application as a new drug candidate of DPP-4 inhibitor for the treatment of type 2 diabetes using LC/MS/MS method.

2. Experimental

2.1. Chemicals

KR-66223 was synthesized by the Medicinal Science Division at the Korea Research Institute of Chemical Technology (Daejeon, Korea). Imipramine was purchased from Sigma–Aldrich (St. Louis, MO, USA). Organic solvents of HPLC grade (ethyl acetate, methanol, acetonitrile) were from J.T. Baker (Phillipsburg, NJ, USA). Distilled water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of the highest analytical grade available.

2.2. Calibration standard and quality control samples

Stock solutions were prepared by separately dissolving either KR-66223 or imipramine (internal standard; IS) in methanol at 1 mg/ml. A series of KR-66223 working standard solutions were prepared by further diluting the stock solution (1 mg/ml) in methanol: water (50:50, v/v) mixture to obtain the following KR-66223 concentrations. Internal standard working solution (3 μg/ml) was prepared from an internal standard (IS) stock solution (1 mg/ml) with methanol: water (50:50, v/v). All solutions were stored at −20 °C when not in use.

A calibration curve for KR-66223 was prepared by spiking working standard solution equivalent to levels of 0.003, 0.01, 0.5, 2, 8, and 10 μg/ml in blank plasma. Quality control (QC) samples were also prepared for KR-66223 concentrations of 0.01, 0.5 and 8 μg/ml in rat plasma.

2.3. Instrumentation and chromatographic conditions

Sample analyses were carried out with an Agilent 1200 series HPLC system coupled to an API 4000 Q trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo electrospray interface in positive ionization mode for the LC/MS/MS analysis. The spectrometer was used in multiple reaction monitoring (MRM) mode. The optimized instrument conditions were as follows: source temperature, 400 °C; curtain gas, 20 psi; nebulizing (GS1), 50 psi; heating (GS2), 50 psi; collision energy (CE), 39 V for KR-66223 and 25 V for imipramine, respectively. The most abundant product ions of compounds were at m/z 553.2 from the parent m/z 206.2 of KR-66223, and m/z 86.1 from the m/z 281.3 of IS. Analyst software (ver. 1.4) was used for instrument control and data collection.

The LC chromatograph was equipped with a Gemini-NX C18 column (50 mm × 2.0 mm i.d., 3 μm; Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile-ammonium formate (10 mM; 90:10, v/v), and was filtered and degassed before use. The flow rate was set at 0.3 ml/min for sample analysis. The method used isocratic elution with a total run time of 2 min. The temperatures of the autosampler and column oven were 4 °C and 40 °C, respectively.

2.4. Sample preparation

A rat plasma sample (50 μl) was placed in a 1.5 ml microfuge tube. The concentrations of the KR-66223 in the initial timed samples collected after intravenous administration frequently exceeded the highest calibration standard. To minimize reassays, the certain samples (drawn at 0.033 and 0.167 h post-dose) were chosen for ten-fold dilution with blank plasma.

The samples with 20 μl of IS solution (3 μg/ml imipramine) was vortex mixed for 1 min and 1 ml of cold ethyl acetate was added. After vortexing for another 5 min, the extract was centrifuged (13,000 rpm, 5 min, 4 °C). Next, 1 ml of the supernatant was transferred to another tube and dried by centrifugal evaporator (EYELA, Tokyo, Japan; 1000 rpm, 40 °C). The sample was reconditioned with 200 μl mobile phase and vortexed for 1 min. Finally, 2 μl of the supernatant was injected onto the analytical column.

2.5. Validation

The analytical method was validated with regard to selectivity, linearity, accuracy, and precision. The selectivity of the method was assessed by analysing extract from six different sources for the presence of analytical interferences. Calibration curves were constructed by linear regression of the peak area–ratios (y) of KR-66223 to internal standard, versus the concentration (x) in μg/ml. The lower limit of quantitation (LLOQ) was defined as the lowest concentration that could be accurately quantified above the noise level, with acceptable precision (within 20%). Precision and accuracy of the method were estimated using replicate samples (n = 5). The intra- and inter-day precisions were estimated by analysing the spiked samples at five different concentrations (0.01, 0.03, 0.5, 8, 80 μg/ml) in a single day and for five days, respectively. For the accuracy of the dilution step, the 80 μg/ml sample that exceeded the upper limit of quantitation (ULOQ) was prepared. The percentage of deviation of the mean from estimated concentrations was expressed as the relative error (RE). Precision was expressed as the relative standard deviation (RSD).

2.6. Matrix effect and recovery

The matrix effect, recovery, and process efficiency for KR-66223 were assessed by analysing three sets of standards at three concentrations (0.01, 0.5, 8 μg/ml). The recovery was determined by comparing the peak areas of analyte spiked before extraction (set 1) with those of analyte spiked post-extraction matrix (set 2). To determine the matrix effect, set 2 was compared with reference standards prepared by spiking with the same concentration of KR-66223 in mobile phase (set 3). The process efficiency was calculated by comparing the peak area of set 3 with that of set 1. Each sample set was analysed in triplicate.

2.7. Stability

The stability of KR-66223 was analysed to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability was estimated at −20 °C for 3 weeks. The study of KR-66223 stability in rat plasma included short- and long-term tests at the QC level. The short-term stability included: (a) freeze–thaw cycle stability; (b) exposure of samples to room temperature for 1 day; (c) exposure to 4 °C for 1 day; (d) exposure to −20 °C for 1 day; (e) exposure to −80 °C for 1 day; (f) exposure in mobile phase at 4 °C for 1 day and 1 week after preparation, and (g) exposure in mobile phase at room temperature for 1 day after preparation. Long-term stability included: (a) exposure

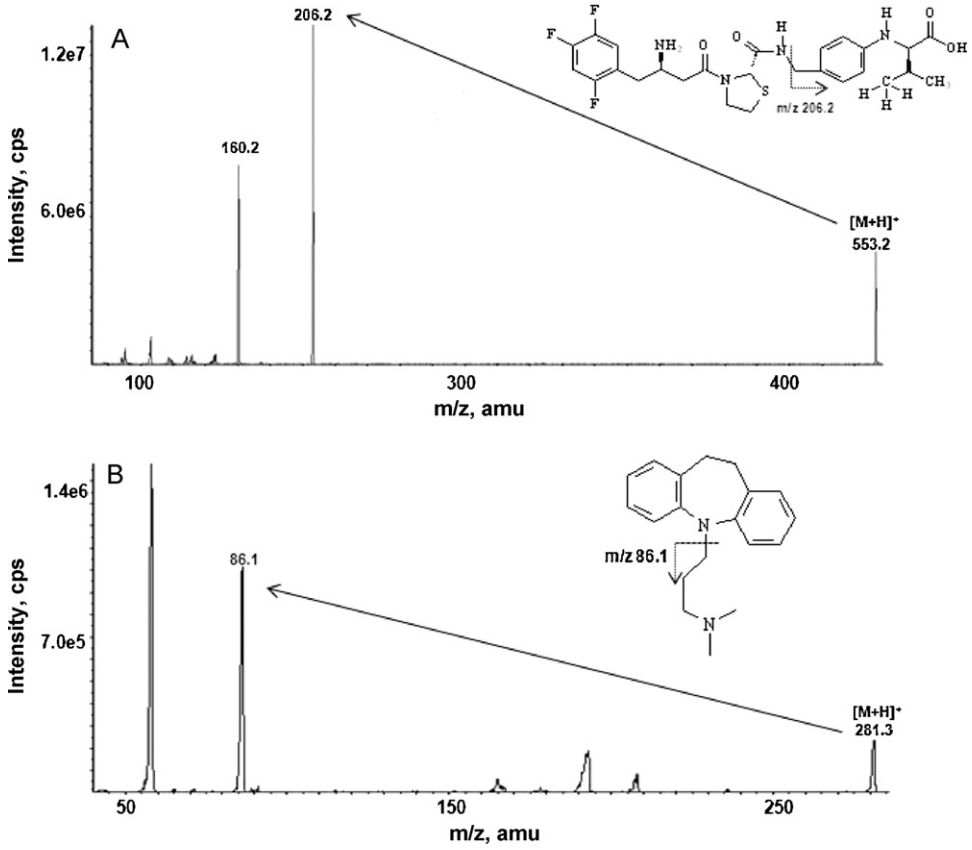

Fig. 1. The structures and product-ion scan spectra of: (A) KR-66223 and (B) imipramine (IS).

of samples to −80 °C for 30 days, and (b) exposure to −20 °C for 30 days.

2.8. Pharmacokinetic study

Four male Sprague-Dawley rats, aged 8 weeks old and weighing 252.5 ± 22.3 g (NARA-Bio Company, Pyungtaek, Kyunggido, Korea), were used for the pharmacokinetic disposition study. The room was maintained at a temperature of 23 ± 3 °C, relative humidity of 50 ± 10%, and an approximately 12/12-h light/dark cycle. Food and water were supplied *ad libitum*. Animals were fasted for 12 h before experiments except water. The jugular and femoral veins of male SD rats under ketamine-administered anesthesia were cannulated with polyethylene tubing (PE-50; Intramedic, USA). Animals were kept in plastic cages and allowed to recover from anesthesia for 1 day prior to the study.

The intravenous dose solutions (10 mg/kg) were formulated in distilled water. Blood (about 0.2 ml) was collected at predose, 0.033, 0.167, 0.5, 1, 2, 4, and 8 h after intravenous administration. Blood samples were centrifuged immediately and stored at −80 °C until analysis.

A non-compartmental method using the nonlinear least squares regression program WinNonlin (Pharsight, Mountain View, CA) was used to calculate the pharmacokinetic parameters. The area under the plasma concentration–time curve from time zero to the last measured concentration ($AUC_{0\rightarrow last}$) and to infinite time ($AUC_{0\rightarrow\infty}$) by adding extrapolated area were calculated. The terminal elimination half-life ($t_{1/2}$), total body clearance (CL), volume of distribution at steady state (Vss), and mean residence time (MRT) for KR-66223 were obtained using individual plasma concentration–time profiles.

3. Results and discussion

3.1. Mass spectra and chromatography

The chemical structure and product ion mass spectra of KR-66223 and the internal standard (imipramine) are presented in Fig. 1. KR-66223 and IS were investigated for the abundant precursor ions $[M+H]^+$ at *m/z* 553.2 and 281.3, respectively. The quantification of analytes was performed using MRM mode for high selectivity and sensitivity of acquisition data. To ensure the correct identification and to prevent false positives, two or more different ions were selected for each analyte, and the peak area ratio of two selected ions (quantitative ion and confirmative ion) was compared with that of the standard compound. A positive identification yielded a peak area ratio within 15–20% of that of the standard compound. The fragmentation ion at *m/z* 206.2 (C–N bond cleavage) and at *m/z* 160.2 ($[206.2–COOH_2]^+$) were the prominent products ions for KR-66223. The more sensitive ion at *m/z* 206.2 was chosen as the quantitative ion, and that at *m/z* 160.2 was used as the confirmative ion for KR-66223. Imipramine (IS) was fragmented to produce intense product ion signals at *m/z* 281.3 → 86.1.

The optimization of chromatographic conditions was based on peak selectivity and retention time and was performed using a mixed mobile phase consisting of acetonitrile and methanol with various aqueous buffer solutions. As a result, KR-66223 and IS eluted at 0.71 min and 0.94 min with apparently symmetric peaks, respectively. Increasing the amount of organic solvent in the mobile phase, the peak shape of the analyte and IS was sharp, but the retention time tended to decline. Typical peak shapes and retention times of MRM chromatograms are shown in Fig. 2.

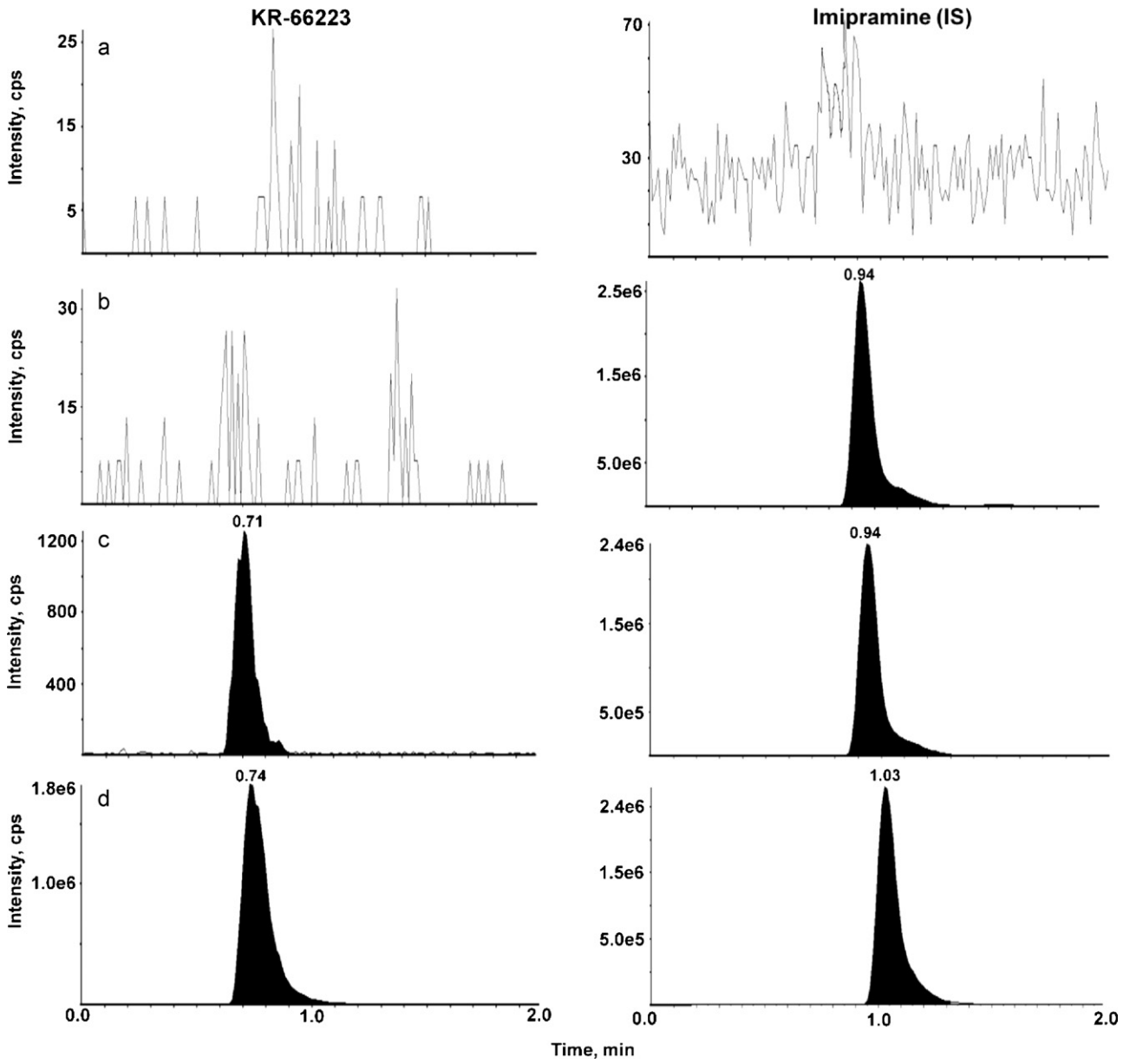

Fig. 2. Representative MRM chromatograms of (a) double blank rat plasma, (b) blank rat plasma spiked with IS, (c) blank rat plasma spiked 0.003 μg/ml (LLOQ) of KR-66223 and IS, (d) a plasma sample obtained at 10 min after intravenous administration of KR-66223 at 10 mg/kg to rats.

3.2. Sample preparation

The sample was extracted with a suitable solvent to remove the sample matrix and extract KR-66223. Ethyl acetate, methylene chloride, and methyl-*t*-butyl ether (MTBE) were used as LLE solvent to extract KR-66223 from spiked plasma. Methylene chloride and MTBE, a relatively non-polar solvent, exhibit a low extraction efficiency for KR-66223 (recoveries 5–11%), but ethyl acetate is the most effective solvents for the recovery of spiked KR-66223 (recoveries > 70%). Ethyl acetate, a relatively polar solvent, extracted both target compounds and polar impurities, and no interference peaks and a stable baseline appeared in MRM mode.

Extraction was considered to be satisfactory if it required no more than 5 min and consumed about 1 ml of solvent for a 50 μl plasma sample extracted once.

3.3. Validation and matrix effect

Six different lots of rat plasma, double blank plasma, blank plasma, and the lower limit of quantification (LLOQ = 3 ng/ml) samples (Fig. 2) were analysed. No significant interference from constituents of drug-free rat plasma was coincident with the retention times of the analyte or IS. Calibration standards consisted of 50 μl samples of blank plasma spiked with 0.003, 0.01, 0.5, 2, 8, and 10 μg/ml KR-66223. The calibration curves were generated by a linear least squares regression analysis of the KR-66223/IS peak area ratio versus the amount of spiked KR-66223. The correlation coefficient (R^2) for KR-66223 was greater than 0.99, indicating excellent linearity. The intra-day accuracy ranged from −11.49 to 4.96% (defined as RE), with RSD values ranging from 11.16 to 14.35%, and the inter-day accuracy ranged from −2.62 to 6.48%, with RSD values ranging from 3.92 to 6.39%, indicating excellent accuracy (Table 1). The accuracy and precision was maintained in the 80 μg/ml samples that underwent 10-fold dilution with blank plasma prior to extraction.

Recovery, matrix effect, and process efficiency results are presented in Table 2. The mean matrix effects at KR-66223 concentrations of 0.01, 0.5, and 8 μg/ml were 82.5, 94.4, and 85.4%, respectively, which are well within acceptable limits (<20%); the

Table 1
Reproducibility and accuracy for KR-66223 in rat plasma ($n = 5$).

Theoretical concentration (μg/ml)	Intra-day			Inter-day		
	Concentration found (μg/ml)	RSD[a] (%)	RE[b] (%)	Concentration found (μg/ml)	RSD (%)	RE (%)
0.003	0.003	14.35	−11.49	0.003	6.39	−2.62
0.01	0.01	12.39	4.96	0.01	5.84	6.48
0.5	0.53	12.36	−1.71	0.49	3.92	5.14
8	8.23	11.16	−1.94	7.85	4.49	2.82
80[c]	80.67	12.17	4.10	83.28	4.29	0.83

[a] RSD (%) = standard deviation of the concentration/mean concentration × 100.
[b] RE (%) = (calculated concentration − theoretical concentration)/theoretical concentration × 100.
[c] Sample were diluted 10-fold with blank plasma prior to analysis.

Table 2
Recovery, matrix effect and process efficiency ($n = 5$).

	Concentration (μg/ml)	Matrix effect[a] (%)	Recovery[b] (%)	Process efficiency[c] (%)
KR-66223	0.01	82.5	77.1	63.6
	0.5	94.4	75.3	71.1
	8	85.4	84.4	72.1
	Mean	87.4	78.9	68.9
IS (Imipramine)	3	98.1	87.1	85.3

[a] 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 3) multiplied by 100.
[b] Recovery calculated as the ratio of the mean peak area of an analyte added before extraction (set 1) to the mean peak area of an analyte spiked post-extraction (set 2) multiplied by 100.
[c] Process efficiency calculated as the ratio of the mean peak area of an analyte added before extraction (set 1) to the mean peak area of the same analyte standards (set 3) multiplied by 100.

Table 3
Stability of KR-66223 in rat plasma ($n = 5$).

Condition tested	QCL (0.01 μg/ml)			QCM (0.5 μg/ml)			QCH (8 μg/ml)		
	Mean	RSD[a] (%)	RE[b] (%)	Mean	RSD (%)	RE (%)	Mean	RSD (%)	RE (%)
Short-term stability									
Control samples (Freshly prepared)	–	8.7	–	–	2.9	–	–	2.4	–
Freeze–thaw (−80 °C, 3 cycle)	0.009	14.1	5.4	0.456	5.4	8.7	7.506	5.2	6.2
Bench (room temperature, 1 day)	0.010	6.1	2.4	0.508	3.4	1.6	7.889	9.9	1.4
Refrigerator (4 °C, 1 day)	0.010	9.2	1.6	0.455	10.8	9.0	8.656	2.2	8.2
Freezer (−20 °C, 1 day)	0.009	10.4	7.3	0.487	7.4	2.7	7.363	7.7	8.0
Freezer (−80 °C, 1 day)	0.009	9.1	5.4	0.492	2.8	1.7	7.734	10.2	3.3
Post-preparative stability (4 °C, 1 day)	0.009	7.6	10.3	0.445	9.8	11.0	7.820	11.3	2.2
Post-preparative stability (4 °C, 1 week)	0.009	5.4	9.7	0.509	4.6	1.8	7.723	3.3	3.5
Post-preparative stability (room temperature, 1 day)	0.009	7.4	5.5	0.450	14.4	10.0	7.883	12.1	1.5
Long-term stability									
Freezer (−80 °C, 30 days)	0.009	11.1	13.4	0.457	3.7	8.5	7.259	1.5	9.3
Freezer (−20 °C, 30 days)	0.009	4.1	13.7	0.461	3.8	7.8	7.046	2.0	11.9

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

mean percentage recoveries at the three concentrations were 77.1, 75.3, and 84.4%, respectively. These results indicate that the current analytical method is sufficiently reliable and has a minimal matrix effect. Process efficiency was evaluated by a combination of standards spiked before extraction and standards injected directly in the mobile phase. The results obtained by this method showed adequate efficiency for KR-66223 in plasma matrix (63.6–72.1%).

3.4. Stability

The stability of KR-66223 was investigated under a variety of conditions used for sample handling, and the stability of processed samples was evaluated. The stock solution of KR-66223 (1 mg/ml) was investigated for 3 weeks at −20 °C; KR-66223 was stable, ranging from 93.7 to 98.8%. The short-term and long-term stability of KR-66223 in plasma are shown in Table 3. There was no significant change when fresh plasma were kept −20 °C, −80 °C for short- and long-term after spiking with KR-66223 in QC samples (0.01, 0.5, 8 μg/ml). Three freeze–thaw cycles and post-preparative stability at low and high concentrations had little effect on the quantification. The stabilities of KR-66223 at room temperature for 1 day were 102.4%, 101.6% and 98.6%, respectively, and at 4 °C for 1 day they were 101.6%, 91.0% and 91.8%, respectively. These results indicate that KR-66223 is considered stable under sample preparation and storage conditions in the study.

3.5. Application to clinical testing

The proposed method was applied to the analysis of KR-66223 in plasma samples collected from male rats that had received intravenous KR-66223 at a dose of 10 mg/kg. The mean plasma concentration profiles of KR-66223 in rats are illustrated in Fig. 3. The concentration of KR-66223 was readily measurable in plasma samples collected up to 8 h post-dose. The pharmacokinetic parameters derived from these profiles are presented in Table 4.

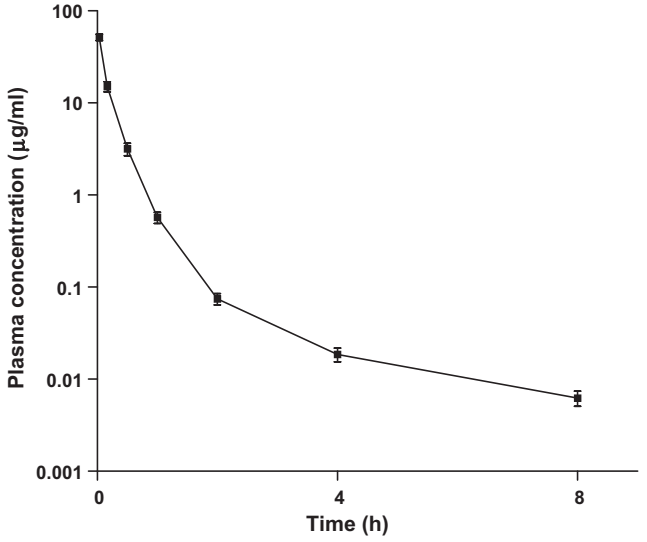

Fig. 3. Mean plasma concentration–time plot of KR-66223 after intravenous administration of KR-66223 at 10 mg/kg to rats (mean ± standard deviation, $n = 4$ rats).

Table 4
Pharmacokinetic parameters of KR-66223 following an intravenous administration at a dose of 10 mg/kg in rats ($n = 4$).

Pharmacokinetic parameter	Mean ± SD
$AUC_{0\to\infty}$ (μg h/ml)	10.93 ± 1.79
$AUC_{0\to 8\,h}$ (μg h/ml)	10.91 ± 1.78
$t_{1/2}$ (h)	1.75 ± 0.06
CL (l/h/kg)	0.93 ± 0.14
Vss (l/kg)	0.21 ± 0.02
MRT (h)	0.21 ± 0.02

4. Conclusions

The current analytical method provides a simple, sensitive, and selective method for the determination of KR-66223 in rat plasma. The method consists of liquid–liquid extraction followed by LC/MS/MS analysis. The method was validated in terms of selectivity, linearity, accuracy, precision and stability. The linearity of the calibration curve was greater than 0.99, and the recovery of spiked plasma samples was 78.9%. The intra-day and inter-day accuracy and precision for the analyte were less than 11.5 (defined as RE). In the stability test of KR-66223, no significant problems were observed under any of the examined conditions. The method was successfully used in a pharmacokinetic study of KR-66223 in rats. This result provides beneficial information for the preclinical study of KR-66223.

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