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Determination of a peroxisome proliferator-activated receptor γ agonist, 1-(trans-methylimino-*N*-oxy)-6-(2-morpholinoethoxy-3-phenyl-1H-indene-2-carboxylic acid ethyl ester (KR-62980) in rat plasma by liquid chromatography–tandem mass spectrometry

Min-Sun Kim[a], Jin Sook Song[a], Hyeongjin Roh[a], Jong-Shik Park[a], Jin Hee Ahn[b], Sung-Hoon Ahn[a], Myung Ae Bae[a,*]

[a] Drug Discovery Platform Technology Team, Medicinal Science Division, Korea Research Institute of Chemical Technology, Daejon 305-600, Republic of Korea
[b] Center for Metabolic Syndrome Therapeutics, Medicinal Science Division, Korea Research Institute of Chemical Technology, Daejon 305-600, Republic of Korea

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ABSTRACT

A novel peroxisome proliferator-activated receptor γ (PPARγ) agonist, KR-62980, was determined by liquid–liquid extraction with ethyl acetate and liquid chromatography–tandem mass spectrometry (LC/MS/MS) in rat plasma. In order to evaluate the pharmacokinetics of KR-62980, a reliable, selective and sensitive high-performance liquid chromatographic method with electrospray ionization tandem mass spectrometry was developed for the quantification of KR-62980 in rat plasma. KR-62980 and imipramine (IS) were separated on Hypersil GOLD C18 column with a mixture of acetonitrile–ammonium formate (10 mM) (80:20, v/v) as mobile phase. The ion transitions monitored were m/z 437.2 → 114.2 for KR-62980, m/z 281.3 → 86.1 for imipramine in multiple reaction monitoring (MRM) mode. The percent recoveries of KR-62980 and imipramine were 90.1 and 98.4% from rat plasma, respectively. The linear dynamic range extended from 0.01 to 10 μg/ml with a correlation coefficient (R^2) greater than 0.99 and the lower limit of quantification was 0.01 μg/ml. The mean of intra- and inter-assay precisions was 2.1 and 9.3%. The method was validated and successfully applied to the pharmacokinetic study of KR-62980 in rat.

1. Introduction

"Peroxisome proliferator-activated receptor γ" (PPARγ) is a fatty acid-activated member of the PPAR subfamily of the nuclear receptor superfamily of transcription factors [1]. Activation of PPARγ is critical in lipid and glucose metabolism, and has been implicated in obesity-related metabolic diseases, such as hyperlipidemia, insulin resistance, and coronary artery disease [2–7]. Thus, some PPARγ agonists are used clinically as anti-diabetic agents. Recently, novel PPARγ ligands, such as GW0072, *N*-(9-fluorenyl)methoxycarbonyl (FMOC)-L-leucine, PAT5A and nTZDpa, have been demonstrated to be drug candidates. KR-62980, 1-(trans-methylimino-*N*-oxy)-6-(2-morpholinoethoxy-3-phenyl-1H-indene-2-carboxylic acid ethyl ester, is functionally active as a selective PPARγ agonist, with an EC_{50} of 15 nM by a transactivation assay [8]. KR-62980 displays anti-hyperglycemic activity, including *in vivo* glucose lowering activity, with little weight gain [8] and anti-adipogenic function, through the suppression of PPARγ-mediated adipocyte differentiation by activating nuclear TAZ (transcriptional coactivator with PDZ-binding motif) [9]. Thus, these findings suggest that KR-62980 may be a good drug candidate for the treatment of type 2 diabetes.

Selective and sensitive analytical methods for the quantitative estimation of drugs are critical for preclinical biopharmaceutical and clinical pharmacology studies [10–12]. 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 [13–15]. Multiple reaction monitoring (MRM) can be programmed to select certain ions chosen by the operator enabling to detect a specific precursor ion and to isolate that ion for collision-induced fragmentation. It is possible to detect a specific product ion following fragmentation [16].

The purpose of this study was to validate an LC/MS/MS method using simple liquid–liquid extraction for the quantitative analysis of KR-62980 in rat plasma. The method was evaluated with regard to its accuracy, precision, selectivity, sensitivity, reproducibility,

* Corresponding author. Tel.: +82 42 860 7084.
E-mail address: mbae@krict.re.kr (M.A. Bae).

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and stability. It was used successfully in a preliminary pharmacokinetic study of intravenously administered KR-62980.

2. Experimental

2.1. Chemicals

KR-62980 was synthesized by the Medicinal Science Division at the Korea Research Institute of Chemical Technology (Daejeon, Korea). Imipramine, sodium fluoride (NaF) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Organic solvents of HPLC grade (ethyl acetate, methanol, acetonitrile, dimethyl sulfoxide) 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

A stock standard mixture was prepared in methanol at 1 mg/ml. Working standard solutions were obtained by further dilution of the standard stock solution in methanol:water (1:1, v/v). Internal standard working solution (3 μg/ml) was prepared from an internal standard (IS) stock solution (1 mg/ml) with methanol:water (1:1, v/v). All solutions were stored at −20 °C.

A calibration curve for KR-62980 was prepared by spiking working standard solution equivalent to levels of 0.01, 0.03, 1, 5, 8, and 10 μg/ml in blank plasma. Quality control (QC) samples were also prepared for KR-62980 concentrations of 0.03, 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 follow: source temperature, 350 °C; curtain gas, 20 psi; nebulizing (GS1), 50 psi; heating (GS2), 50 psi; collision energy (CE), 59 V for KR-62980 and 25 V for imipramine, respectively. The most abundant product ions of compounds were at *m*/*z* 114.2 from the parent *m*/*z* 437.2 of KR-62980, 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 Hypersil GOLD C18 column (100 mm × 2.1 mm i.d., 3 μm; Thermo, Waltham, MA, USA). The mobile phase consisted of acetonitrile–ammonium formate (10 mM; 80:20, v/v), and was filtered and degassed before use. The flow rate was set at 0.2 ml/min for sample analysis. The method used isocratic elution with a total run time of 4 min. The temperatures of the autosampler and column oven were 4 °C and 20 °C, respectively.

2.4. Sample preparation

A rat plasma sample (50 μl) was placed in a 1.5 ml microfuge tube and was added to 20 μl of IS solution (3 μg/ml imipramine). The sample was vortexed 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). Then, 200 μl of mobile phase was added to the dried residue and vortexed for 1 min. Finally, 2 μl of the supernatant was injected onto the analytical column.

2.5. Validation

Validation of the analytical method for KR-62980 was determined with regard to selectivity, linearity, accuracy, and precision. For selectivity validation, blank plasma samples from six different sources were analyzed. Precision and accuracy of the method were estimated using replicate samples ($n = 5$). The intra- and inter-day precisions were estimated by analyzing the spiked samples at four different concentrations (0.01, 0.03, 0.5, and 8 μg/ml) in a single day and for 5 days, respectively. 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). Calibration curves were constructed by linear regression of the peak area ratios (y) of KR-62980 to internal standard, versus the concentration (x) in μg/ml. The lower limit of detection (LLOD) was calculated as three-times the signal-to-noise ratio (S/N), and 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%).

2.6. Matrix effect and recovery

The matrix effect, recovery, and process efficiency for KR-62980 were assessed by analyzing three sets of standards at three concentrations (0.03, 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-62980 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 analyzed in triplicate.

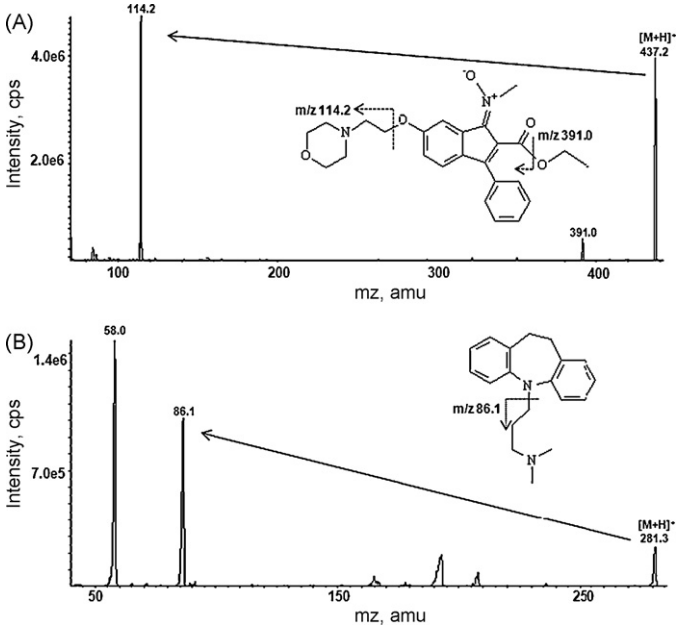

Fig. 1. The structures and product-ion scan spectra of (a) KR-62980 and (b) imipramine (IS).

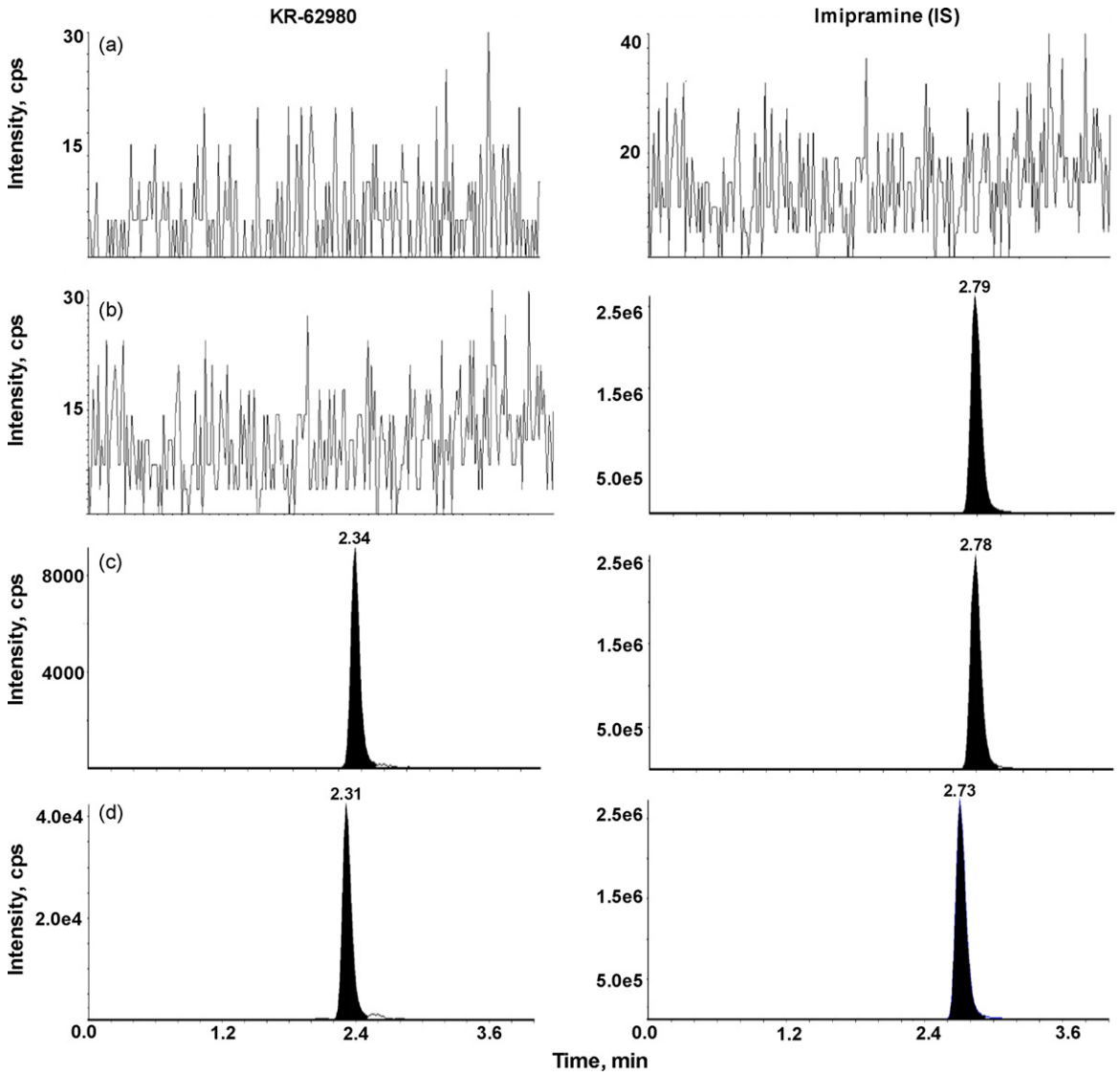

Fig. 2. Representative MRM chromatograms of (a) double blank rat plasma, (b) blank rat plasma spiked with IS, (c) blank rat plasma spiked 0.01 μg/ml (LLOQ) of KR-62980 and IS and (d) a plasma sample obtained at 1 h after intravenous administration of KR-62980 at 10 mg/kg to rats.

2.7. Stability

The stability of KR-62980 was analyzed 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-62980 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, 3, 6 h, and 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 of samples to −80 °C for 30 days, and (b) exposure to −20 °C for 30 days. The effect of esterase on KR-62980 stability was conducted by adding NaF (10 mg/ml) and PMSF (0.18 mg/ml) to the rat plasma, respectively. These samples were exposed to room temperature for 1, 3, 6, 24 h.

2.8. Pharmacokinetic study

Three male Sprague–Dawley rats, aged 8 weeks old and weighing 253 ± 28 g, were used for the pharmacokinetic disposition study. Animals were kept in plastic cages with free access to standard rat diet and water. 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.

The intravenous dose solutions (10 mg/kg) were formulated in PEG 400:distilled water:dimethyl sulfoxide (DMSO) at a ratio of 40:55:5. Blood (about 0.2 ml) was collected at predose, 0.033, 0.167, 0.5, 1, 2, 4, 8, 12, and 24 h after intravenous administration. Blood samples were centrifuged immediately and stored at −70 °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 ($AUC_{0\rightarrow\infty}$)

Table 1
Reproducibility and accuracy for KR-62980 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 (%)
0.01	0.01	3.4	−6.6	0.01	10.6	1.1
0.03	0.03	1.6	10.8	0.03	9.0	−5.3
0.5	0.57	1.2	13.2	0.50	9.2	−0.8
8	7.43	2.2	−7.1	7.57	8.5	−5.4

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

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-62980 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-62980 and the internal standard (imipramine) are presented in Fig. 1. KR-62980 and IS were investigated for the abundant precursor ions $[M+H]^+$ at m/z 437.2 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 114.2 (C–O bond cleavage) and at m/z 391.0 (ester bond cleavage) were the prominent products ions for KR-62980. Among them, the more sensitive compound at m/z 114.2 was chosen as the quantitative ion, and that at m/z 391.0 was used as the confirmative ion for KR-62980. Imipramine (IS) was fragmented to produce intense product ion signals at m/z $281.3 \rightarrow 86.1$.

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-62980 and IS eluted at 2.3 and 2.7 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.

3.2. Sample preparation

The sample was extracted with a suitable solvent to remove the sample matrix and extract the KR-62980. In this study, ethyl acetate, methylene chloride, and methyl-*t*-butyl ether (MTBE) were used as LLE solvent to extract KR-62980 from spiked plasma. No significant difference was found between the solvents used in extraction, probably due to their good dissolving capability for KR-62980 (recoveries > 80%). Although very effective, methylene chloride is of limited use due to its relatively high toxicity. Ethyl acetate and MTBE, a relatively polar solvent, extracted both target compounds and polar impurities, and no interference peaks and a stable baseline appeared in MRM mode. Thus, ethyl acetate was used as the extraction solvent for sample preparation, due to its lower toxicity than MTBE.

An 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) samples (Fig. 2) were analyzed. 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 0.01, 0.03, 1, 5, 8, and 10 μg/ml KR-62980. The calibration curves were generated by a linear least squares regression analysis of the KR-62980/IS peak area ratio versus the amount of spiked KR-62980. The correlation coefficient (R^2) for KR-62980 was greater than 0.99, indicating excellent linearity. The intra-day accuracy ranged from −7.1 to 13.2% (defined as RE), with RSD values ranging from 1.2 to 3.4%, and the inter-day accuracy ranged from −5.4 to 1.1%, with RSD values ranging from 8.5 to 10.6%, indicating excellent accuracy (Table 1).

Table 2
Recovery, matrix effect and process efficiency ($n = 3$).

	Concentration (μg/ml)	Matrix effect[a] (%)	Recovery[b] (%)	Process efficiency[c] (%)
KR-62980	0.03	90.0	96.4	86.8
	0.5	94.1	88.6	83.3
	8	96.5	85.4	82.4
	Mean	93.5	90.1	84.2
IS (Imipramine)	3	89.2	98.4	87.8

[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.

Recovery, matrix effect, and process efficiency results are presented in Table 2. The mean matrix effects at KR-62980 concentrations of 0.03, 0.5, and 8 μg/ml were 90.0, 94.1, and 96.5%, respectively, which are well within acceptable limits (<20%); the mean percentage recoveries at the three concentrations were 96.4, 88.6, and 85.4%, 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-62980 in plasma matrix (82.4–86.8%).

3.4. Stability

The stability of KR-62980 was investigated under a variety of conditions used for sample handling, and the stability of processed samples was evaluated. The stock solution of KR-62980 (1 mg/ml) was investigated for 3 weeks at −20 °C; KR-62980 was stable, ranging from 92.5 to 105.4%. The short-term and long-term stability of KR-62980 in plasma are shown in Table 3. There was no significant change when fresh plasma was kept −80 °C for short- and long-term after spiking with KR-62980 in QC samples (0.03, 0.5, 8 μg/ml). For the long-term stability of plasma samples stored at −20 °C, KR-62980 did decrease, to less than 77%. Three freeze–thaw cycles and post-preparative stability at low and high concentrations had little effect on the quantification. However, the concentration of KR-62980 (8 μg/ml) at room temperature and 4 °C for 1 day decreased significantly, to 8.6 and 57.3%, respectively.

The stability of a drug in plasma depends on the labile functional groups, such as esters, amide, carbamates, lactones, lactams, sulfates, sulfonamides, phosphates, peptides and peptide mimetics [17,18]. Such functional groups are vulnerable to hydrolysis by plasma enzymes. Fig. 3 shows the relative change of KR-62980 (0.5 μg/ml level) in rat plasma at room temperature without (control) or with NaF (10 mg/ml) or PMSF (0.18 mg/ml). The samples stored for 1 h at room temperature showed no apparent change in each condition. After 1 h, the concentration of KR-62980 appreciably decreased in control. However, the degradation rate of KR-62980 in rat plasma decreased with addition of NaF or PMSF, esterase inhibitors. These results indicated that one of the main reasons for the poor stability of KR-62980 in rat plasma is believed to be the ester groups of this compound. Therefore, careful sample storage conditions (below −80 °C) and sample preparation performed within 1 h are necessary.

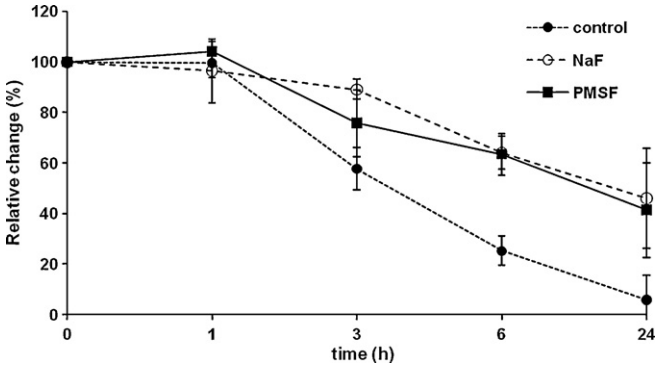

Fig. 3. Stability of KR-62980 in rat plasma samples (0.5 μg/ml level) at room temperature without (control), with NaF and PMSF ($n = 5$).

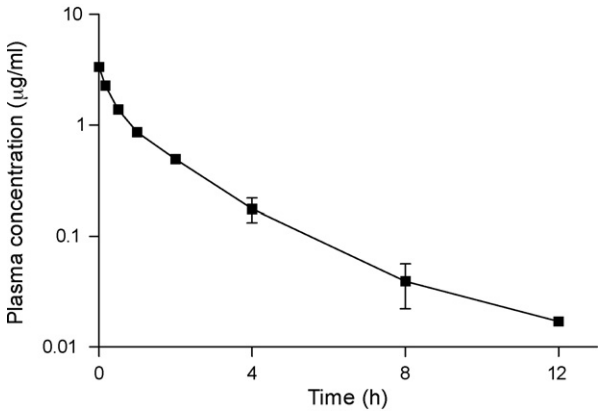

Fig. 4. Mean plasma concentration–time plot of KR-62980 after intravenous administration of KR-62980 at 10 mg/kg to rats (mean ± standard deviation, $n = 3$ rats).

3.5. Application to clinical testing

The proposed method was applied to the analysis of KR-62980 in plasma samples collected from male rats that had received intravenous KR-62980 at a dose of 10 mg/kg. The mean plasma concentration profiles of KR-62980 in rats are illustrated in Fig. 4. The concentration of KR-62980 was readily measurable in plasma samples collected up to 24 h post-dose. The terminal half-life and AUC_{∞} values of KR-62980 were 2.33 ± 0.43 h and 3.59 ± 0.43 μg h/ml. The $AUC_{0\rightarrow last}/AUC_{0\rightarrow\infty}$ ratio was higher than 99% for all subjects (mean values, 99.8 ± 0.1%). The systemic clearance (CL), volume of distribution (Vss) and mean residence time (MRT)

Table 3
Stability of KR-62980 in rat plasma ($n = 5$).

Condition tested	QCL (0.03 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)	–	7.9	–	–	5.2	–	–	4.3	–
Freeze–thaw (−80 °C, 3 cycle)	0.033	0.9	11.4	0.429	5.3	−14.2	6.992	5.7	−12.6
Bench (room temperature, 1 day)	0.003	12.2	−90.2	0.029	9.6	−94.2	0.688	11.7	−91.4
Refrigerator (4 °C, 1 day)	0.009	6.0	−70.2	0.160	7.9	−68.0	4.584	7.1	−42.7
Freezer (−20 °C, 1 day)	0.031	1.2	2.9	0.512	5.8	2.4	8.152	4.0	1.9
Freezer (−80 °C, 1 day)	0.031	4.3	3.1	0.477	14.2	−4.6	7.448	4.8	−6.9
Post-preparative stability (4 °C, 1 day)	0.031	0.8	4.3	0.491	7.8	−1.8	7.928	3.3	−0.9
Post-preparative stability (4 °C, 1 week)	0.032	8.4	5.7	0.482	1.2	−3.6	7.136	6.6	−10.8
Post-preparative stability (room temperature, 1 day)	0.027	2.3	−9.2	0.523	7.6	4.6	7.312	2.0	−8.6
Long-term stability									
Freezer (−80 °C, 30 days)	0.030	4.0	−0.2	0.484	4.9	−3.2	7.434	4.4	−7.1
Freezer (−20 °C, 30 days)	0.023	9.4	−22.7	0.375	4.0	−25.0	5.432	3.0	−32.1

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

was 2.82 ± 0.33 l/h/kg, 5.40 ± 0.90 l/kg and 1.91 ± 0.54 h, respectively.

4. Conclusions

The current analytical method provides a simple, sensitive, and selective method for the determination of KR-62980 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 90.1%. The intra and inter-day accuracy and precision for the analyte were less than 13.2 (defined as RE). The stability of KR-62980 was only maintained for 1 h at room temperature in rat plasma, so sample preparation should be performed carefully within 1 h. The method was successfully used in a pharmacokinetic study of KR-62980 in rats. This result provides beneficial information for the preclinical study of KR-62980.

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