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Development and validation of a UPLC–MS/MS method for quantification of SKLB010, an investigational anti-inflammatory compound, and its application to pharmacokinetic studies in beagle dogs

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ABSTRACT

SKLB010 is currently under development as a potential therapeutic agent for the treatment of acute hepatitis and rheumatoid arthritis. The purpose of this paper was to investigate the pre-clinical pharmacokinetics of SKLB010 in beagle dogs. An ultra performance liquid chromatographic tandem mass spectroscopy (UPLC–MS/MS) method was developed and validated for the quantitative determination of SKLB010 in dog plasma, using rosiglitazone as the internal standard (I.S.). Plasma samples were prepared by a simple solid phase extraction (SPE) method. The analyte and internal standard were separated by an Acquity UPLC BEH C18 (2.1 mm × 50 mm) column with a mobile phase of methanol–water (80/20, v/v) over 2 min. Detection was based on the multiple reaction monitoring with the precursor-to-product ion transitions m/z 234.10 → 147.92 (SKLB010) and m/z 356.15 → 150.00 (I.S.). The method was validated according to FDA guidelines on bio-analytical method validation. The selectivity, sensitivity, linearity, accuracy, precision, extraction recovery, ion suppression and stability were within the acceptable ranges. The method described above was successfully applied to reveal the single- and multi-pharmacokinetic profiles of SKLB010 in beagle dogs and should be extendable to pharmacokinetic studies in other species as well.

1. Introduction

SKLB010, (Z)-5-(4-methoxybenzylidene)thiazolidine-2,4-dione, a novel derivative of thiazolidinediones, showed potent anti-inflammatory effects *in vitro* and *in vivo* in our previous pharmacological studies [1,2]. *In vitro* studies, SKLB010 showed potency of inhibition of chemotaxis of RAW264.7 cells stimulated by monocyte chemotactic protein-1 (MCP-1) [1], and depressed cells migration and the production of NO in Raw264.7 cells [2]. *In vivo* studies, SKLB010 showed potential hepatoprotective effects against concanavalin A (Con A)-induced acute liver injury in BALB/c mice, as demonstrated by decreasing the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [1]. Besides, our study also proved SKLB010 can ameliorate the adjuvant-induced arthritis in Lewis rats through inhibiting the migration of macrophages and the expression of pro-inflammatory cytokines [2]. Based on these evidences, SKLB010 could be developed as a candidate of anti-inflammatory therapeutic agent for the treatment of acute hepatitis and rheumatoid arthritis.

The pharmacokinetic profile of any new drug candidate is one of the key determinants for its success in drug development. For further research, it is important to investigate the pharmacokinetic profile of SKLB010 to understand its absorption and clearance characteristics *in vivo*. Hence, SKLB010 has been tested in a pre-clinical pharmacokinetic study in beagle dogs. During this study phase, a reliable bioanalytical method was needed for the quantification of SKLB010 in dog plasma.

Several methods have been reported for the detection of thiazolidinedione drugs in biological fluids, such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [3], fluorescence detection [4,5] and capillary zone electrophoresis [6]. In our early studies, we utilized HPLC with ultra-violet (UV) detection to quantify SKLB010 in biological matrices. The lower limit of quantification of the method was 100 ng/mL in plasma, which was not adequate for the study. Thus a more sensitive and selective analytical method is required.

The combination of HPLC with the tandem mass spectrometer (MS/MS) has become more and more attractive for the quantification of drugs at trace levels [7,8]. This technique has been used to quantify thiazolidinedione drugs including rosiglitazone,

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Table 1
Compounds dependent parameters for SKLB010 and I.S. in MRM mode for UPLC–MS/MS analysis.

Analyte	$[M-H]^-$	Product ion	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
SKLB010	234.10	147.92	0.2	20	25
Rosiglitazone	356.15	150.00	0.2	25	30

pioglitazone and other derivatives in biological matrices [9–11]. Compared to conventional HPLC analyses, ultra performance liquid chromatography (UPLC) performed with Acquity BEH 1.7 μm columns can provide greater peak capacity, higher speed and efficiency [12]. Moreover, in comparison with HPLC–MS/MS, UPLC–MS/MS technique produces significant improvements in method sensitivity, speed, and resolution [13,14]. Therefore, in this study, we developed a UPLC–MS/MS method to quantify SKLB010 in small volumes of plasma. Solid phase extraction was chosen to extract SKLB010 from plasma samples which resulted in good recovery, lack of endogenous interference, and moderate matrix effect.

The fast, selective and highly sensitive UPLC–MS/MS method with simple pretreatment procedures was successfully applied to assess the pharmacokinetic profiles of SKLB010 in beagle dogs. To our knowledge, this is the first report on the pharmacokinetics of SKLB010. The method can also be used into pharmacokinetic studies of other animals.

2. Experiments

2.1. Chemicals and reagents

SKLB010 was synthesized at the Medicinal Chemistry Department of the State Key laboratory of Biotherapy according to the published procedure [1]. The purity of SKLB010 was 99.5% as determined by HPLC with UV detection at 367 nm. Rosiglitazone, which served as internal standard, was purchased from Chengdu Changzheng Chemical Reagent Company (Sichuan, China). The chemical structures of SKLB010 and rosiglitazone are shown in Fig. 1. Methanol (HPLC grade) was purchased from Fisher Scientific (American). Water used in the experiment was purified using a Milli-Q water purification system (Millipore Corp., American). Other reagents and chemicals were of analytical grade. Blank plasma was obtained from untreated animals and frozen at −20 °C until analysis.

2.2. Chromatographic conditions

The ultra performance liquid chromatography conditions for analyzing SKLB010 in plasma were: system, Acquity™ UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary pump, a thermostatically controlled column compartment and a photodiode-array detector; column, Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 μm, Waters); mobile phase, methanol–water (80/20, v/v). The column temperature was maintained at 30 °C and sample temperature at 10 °C. The flow rate was 0.2 mL/min and the injection volume was 5 μL.

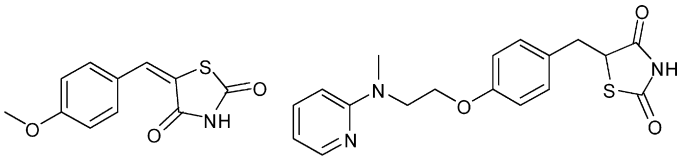

Fig. 1. The chemical structures of (I) SKLB010 and (II) rosiglitazone.

2.3. Mass spectrometry conditions

A Quattro Premier XE™ triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA), operated in electrospray negative mode was used for detection. The quantification was performed in multiple reactions monitoring (MRM) mode with the precursor-to-product ion transitions of m/z 234.10 → 147.92 for SKLB010, m/z 356.15 → 150.00 for rosiglitazone. The mass spectrometric parameters were optimized to improve the MRM sensitivity by infusing an approximately 100 ng/mL solution of SKLB010 and the I.S. in methanol/water (80/20, v/v). The optimized ESI source parameters were as follows: capillary voltage, 3.0 kV (negative mode); extractor voltage, 5 V; source temperature, 110 °C; desolvatation temperature, 400 °C; desolvation gas (nitrogen) flow, 800 L/h; cone gas (nitrogen) flow, 40 L/h; and collision gas (argon) flow, 0.20 mL/min. The cone voltage and collision energy were optimized to maximize the intensity of the transition ions for SKLB010 and I.S. Analyte-specific cone voltages, collision energies and MRM transitions are provided in Table 1. Detection conditions were: inter channel delay, 0.02 s; span, 0.2 Da; dwell time, 0.2 s; start time, 0.0 min; end time, 2.0 min. All the operations, acquisition and data analyses were controlled by the MasslynxV4.1 software.

2.4. Preparation of calibration standards and quality control samples

The stock solutions of SKLB010 (100 μg/mL) and the internal standard (50 μg/mL) were separately prepared in methanol. These stock solutions remained stable for three months if stored in a refrigerator at 4 °C. Standard solutions of SKLB010 at desired concentrations were prepared by serial dilution of the stock solution with methanol. The internal standard solution was diluted with methanol to 1000 ng/mL. All the solutions were stored at 4 °C and warmed to room temperature before use. Calibration standards were prepared by spiking 10 μL of the appropriate standard solutions to 100 μL of blank dog plasma. The final drug concentrations after sample extraction corresponded to 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL. Each sample also contained 10 ng (10 μL × 1000 ng/mL) of the internal standard. In each run, a blank sample (no I.S.) was also analyzed. One calibration curve was constructed on each analysis day using freshly prepared calibration standards. The quality control (QC) samples were prepared at low (10 ng/mL), mid (200 ng/mL), and high (1600 ng/mL) concentrations in the same way as the plasma samples for calibration, and QC samples were stored at −20 °C until analysis.

2.5. Sample processing

Plasma samples were thawed at room temperature before processing. Solid phase extraction was carried out using an Oasis HLB 30 mg extraction cartridge (Waters, Milford, MA, USA). The HLB cartridge was conditioned and equilibrated with 1 mL methanol followed by 1 mL deionized water before use. The plasma sample (100 μL) spiked with 10 μL of I.S. (rosiglitazone, 1 μg/mL) was vortex mixed and then loaded on the cartridges. Cartridges were then washed with 1 mL 5% methanol in water, and eluted with 1.0 mL 100% methanol. The methanol fraction was collected and

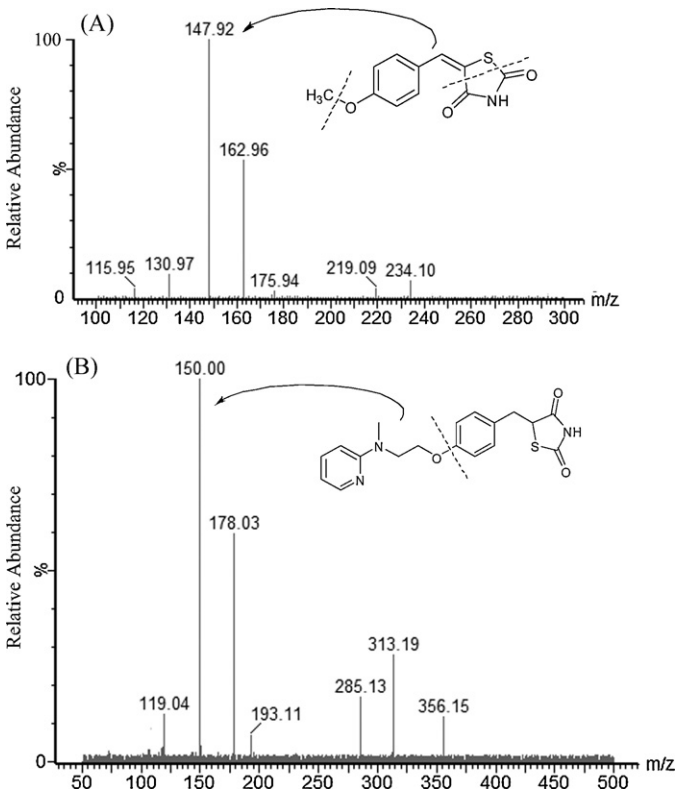

Fig. 2. Product ion mass spectra of SKLB010 (A) and rosiglitazone (B) in negative electrospray ionization mode.

concentrated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 100 μL of mobile phase and centrifuged at 13,000 rpm for 20 min. 5 μL of supernatant was injected into the UPLC–MS/MS system for quantitative analysis.

2.6. Validation procedures

The validation of SKLB010 determination in dog plasma was carried out according to FDA guidelines for bio-analytical method validation [15], including selectivity, sensitivity, linearity, accuracy, precision, recovery, ion suppression and stability.

2.6.1. Specificity

The selectivity of the method was evaluated by comparing the chromatograms of six different batches of blank dog plasma with the corresponding spiked plasma (LLOQ samples), so as to ensure no interfering peak of SKLB010 and I.S. from endogenous plasma components.

2.6.2. Linearity and sensitivity

The calibration standards of the following concentrations 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL were used to assess linearity, which were prepared according to Section 2.4. Calibration curves were plotted as the peak area ratio (drug/internal standard) versus the SKLB010 nominal concentration. Least-squares linear regression method was used to determine the slope, intercept and correlation coefficient. The limit of quantification is defined as the concentration that produced a signal-to-noise (S/N) ratio of at least 10. To investigate the ability to dilute and analyze samples containing SKLB010 at concentrations above the upper limit of quantification, plasma samples ($n = 6$) with SKLB010 concentrations of 5000 and 10,000 ng/mL were diluted 5- and 10-fold with blank plasma and assayed. The accuracy and precision should be within acceptable limits. The carryover was evaluated by analyzing blank samples after the highest concentration samples (2000 ng/mL). The carryover should be less than 20% of LLOQ.

2.6.3. Accuracy and precision

The precision and accuracy of this analytical method were evaluated by assay QC samples at the LLOQ, QCL, QCM and QCH concentrations ($n = 6$). The QC samples were analyzed three times a day to evaluate intra-day precision and accuracy. The same procedure was performed once a day for three consecutive days to determine inter-day precision and accuracy. SKLB010 concentrations in samples were calculated using a calibration curve prepared on the same day. The precision was calculated using the relative standard deviation (%R.S.D.) and the accuracy was expressed as a percentage of measured concentrations versus nominal concentrations.

2.6.4. Extraction recovery and matrix effect

The extraction recovery of SKLB010 through the SPE method was evaluated at three QC concentrations: 10, 200 and 1600 ng/mL ($n = 6$). Extraction recovery was determined by comparing the peak areas obtained from blank plasma spiked with analytes before the extraction with those from samples to which analytes were added after the extraction. Matrix effect was assayed to compare the peak

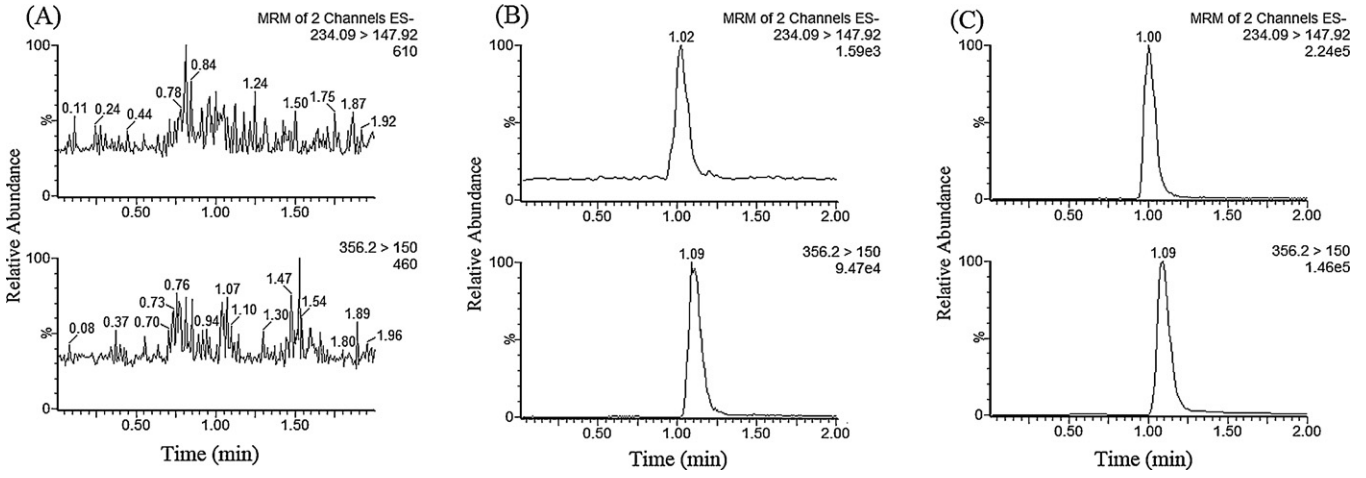

Fig. 3. Representative MRM chromatograms of: (A) blank dog plasma; (B) blank plasma spiked with SKLB010 at a LLOQ level (5 ng/mL) and I.S.; and (C) a plasma sample at 3 h after the dose of 30 mg/kg and spiked with I.S. Peaks I and II refer to SKLB010 and rosiglitazone, respectively. The m/z 234.05 > 149.92 transition was monitored for SKLB010 and the m/z 356.2 > 150.00 transition for rosiglitazone.

Table 2
Precision and accuracy (intra-day and inter-day) of the UPLC–MS/MS method for determining SKLB010 in dog plasma ($n = 6$).

Nominal conc. (ng/mL)	Measured conc. (mean ± S.D., ng/mL)	Precision (%R.S.D.)	Accuracy (mean ± S.D., %)
Intra-day ($n = 6$)			
5 (LLOQ)	4.52 ± 0.14	3.08	90.42 ± 2.79
10 (QCL)	9.10 ± 0.15	1.62	90.98 ± 1.47
200 (QCM)	204.32 ± 7.04	3.45	102.16 ± 3.52
1600 (QCH)	1560.14 ± 34.64	2.22	97.51 ± 2.17
Inter-day (3 days, six replicates per day)			
5 (LLOQ)	4.53 ± 0.16	3.58	90.56 ± 3.25
10 (QCL)	9.74 ± 0.83	3.76	91.09 ± 3.43
200 (QCM)	198.95 ± 8.41	4.23	99.48 ± 4.20
1600 (QCH)	1575.06 ± 47.82	3.04	98.44 ± 2.99

Table 3
Extraction recovery, matrix effects for the assay of SKLB010 in dog plasma ($n = 6$, data are mean ± S.D.).

Nominal conc. (ng/mL)	Recovery (mean ± S.D., %)	Matrix effects (%)
10 (QCL)	92.83 ± 7.99	91.63 ± 7.18
200 (QCM)	90.78 ± 2.17	89.97 ± 5.54
1600 (QCH)	90.12 ± 3.58	89.98 ± 7.34

areas of blank plasma extracts spiked with analytes with those of the standard solutions dried and reconstituted with mobile phase.

2.6.5. Stability

The stability experiments were performed to evaluate the stability of the analyte in dog plasma under the following conditions: short-term stability at room temperature for 24 h; long-term stability at −20 °C for 30 days; three freeze (−20 °C)–thaw (room temperature) cycles on consecutive days; post preparative stability of the processed samples kept under the auto-sampler conditions (10 °C) for 24 h. All stability testing in plasma was performed at the QCL, QCM and QCH concentrations ($n = 6$). All the samples were analyzed and the experimental concentrations were compared with the nominal values.

2.7. Pharmacokinetic study

2.7.1. Animals

Mature beagle dogs were provided by Gaoyao Kangda Laboratory Animal S & T Co., Ltd. (SCXK, Kwangtung Province, 2009-0009, China). Prior to experimental initiation the attending veterinarian certified that the animals were healthy and free from disease and parasites. All the dogs were housed under controlled environmental conditions (temperature, 21 ± 5 °C; humidity, 55 ± 15%; 12 h light/dark cycle and 8–10 air changes per hour). Filtered tap water and a standard animal diet was available ad libitum. Animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Sichuan University.

2.7.2. Single-dosing pharmacokinetic study in beagle dogs

A total of 12 beagle dogs weighing 8–12 kg (half male and half female) were randomly divided into three dosage groups of 4 animals each. The dosages were 10, 30 and 90 mg/kg with the volume of 5 mL/kg for intragastric administration. Blood-sampling time was arranged before dosing and at 0.083, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h post-dosing. All blood samples were centrifuged at 4000 rpm (4 °C) for 10 min, and the plasma samples were collected and stored at −20 °C until analysis.

2.7.3. Multi-dosing pharmacokinetic study in beagel dogs

In multi-dosing experiments, 4 beagle dogs of both sexes weighing 9–14 kg received 30 mg/kg of SKLB010 by intragastric administration at 24 h intervals from the morning of the first day and lasting for 4 consecutive days. Dogs were fasted overnight and were further fasted for 4 h after i.g. administration with free access to water. On Day 1, serial blood samples (2 mL) were collected from the forelimb vein at pre-dose (0), 0.083, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 h post-dose. On the mornings of Day 2 and Day 3, blood was sampled pre-dose. After administration on the Day 4 morning, serial blood samples were collected according to the same time points as on Day 1. The plasma was separated from heparinized blood by centrifugation and was stored at −20 °C. All of the plasma samples were analyzed within one week after collection.

3. Results and discussion

3.1. Method development

3.1.1. Selection of internal standard

For a LC–MS/MS analysis, the internal standard should have similar chromatographic and mass spectrometric behavior to the analyte, and mimic the analyte in any sample preparation steps. Rosiglitazone, troglitazone and piglitazone, which are structurally and chemically similar to SKLB010, were considered. Rosiglitazone was adopted in the end because of its similarity of retention action and ionization as well as its less endogenous interference with the analyte.

3.1.2. Optimization of the extraction method

Due to the complex nature of plasma, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS/MS analysis. However, the sample preparation procedure usually negatively affects detection and quantification, even when

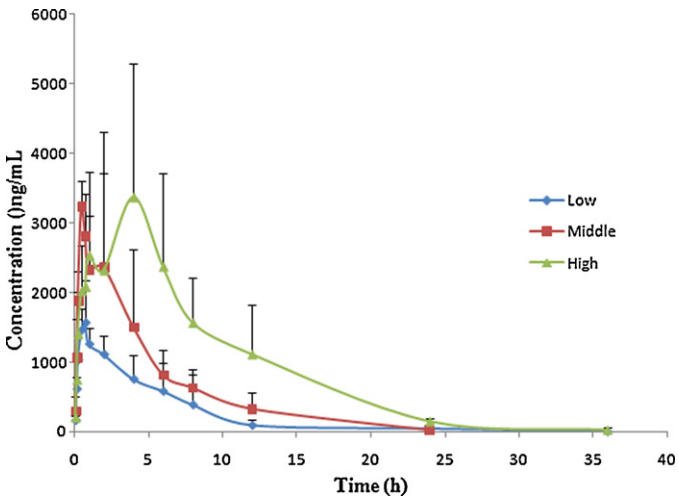

Fig. 4. Mean plasma concentration–time profiles of SKLB010 after single oral doses of 10, 30 and 90 mg/kg ($n = 4$) to beagle dogs, respectively. Each point represents mean ± S.D.

Table 4
Stability of SKLB010 in dog plasma under various storage conditions ($n = 6$, data are mean ± S.D.).

Storage conditions	Remaining percentage[a] (mean ± S.D.)		
	10 ng/mL	200 ng/mL	1600 ng/mL
Short-term stability (25 °C, 24 h)	102.58 ± 12.26	103.97 ± 7.87	94.48 ± 4.95
Long-term stability (−20 °C, 30 days)	86.69 ± 3.56	86.07 ± 1.37	91.10 ± 6.05
Freeze–thaw stability (three cycles, −20 °C/25 °C)	89.71 ± 5.27	100.07 ± 2.24	98.55 ± 2.13
Post-preparative stability (10 °C, 24 h)	96.83 ± 10.07	93.88 ± 9.53	100.05 ± 3.09

[a] Remaining percentage = (concentration found)/(concentration added) × 100.

Table 5
Main pharmacokinetic parameters of SKLB010 following single dose of 10, 30 and 90 mg/kg to beagle dogs, respectively ($n = 4$, mean ± S.D.).

Parameters	10 mg/kg	30 mg/kg	90 mg/kg
C_{max} (μg/L)	1768.85 ± 530.55	3649.98 ± 1098.04	3781.16 ± 1796.22
T_{max} (h)	0.94 ± 0.72	1.75 ± 1.66	3.25 ± 1.50
$t_{1/2}$ (h)	3.02 ± 1.49	3.87 ± 1.58	5.49 ± 1.86
$AUC_{0-24\,h}$ (μg/L h)	8262.92 ± 3662.13	16,378.19 ± 6460.25	33,684.92 ± 9514.07
$AUC_{0-\infty}$ (μg/L h)	8368.87 ± 3828.20	16,427.95 ± 6494.63	33,965.88 ± 9718.54
C_{lz}/F (L/h/kg)	1.43 ± 0.72	2.02 ± 0.69	2.84 ± 0.92
V_z/F (L/kg)	5.19 ± 1.42	10.41 ± 2.28	23.84 ± 13.73
MRT (h)	4.49 ± 1.65	5.14 ± 0.52	8.16 ± 1.83

Table 6
Main pharmacokinetic parameters of SKLB010 following multiple dose of 30 mg/kg to beagle dogs ($n = 4$, mean ± S.D.).

Parameters	Day 1	Day 4
C_{max} (μg/L)	3747.42 ± 897.01	3921.21 ± 237.16
T_{max} (h)	1.44 ± 0.66	1.19 ± 0.55
$t_{1/2}$ (h)	2.59 ± 0.88	3.76 ± 0.90
$AUC_{0-24\,h}$ (μg/L h)	19,025.16 ± 3778.90	15,323.99 ± 2166.58
$AUC_{0-\infty}$ (μg/L h)	19,112.34 ± 3778.08	15,640.27 ± 1932.72
C_{lz}/F (L/h/kg)	1.62 ± 0.36	1.94 ± 0.24
V_z/F (L/kg)	5.86 ± 1.71	10.45 ± 2.44
MRT (h)	4.22 ± 0.75	3.83 ± 0.22

many modern and sophisticated techniques are applied. For the present study, different extraction techniques, including protein precipitation (PPT), solid phase extraction (SPE) and liquid–liquid extraction (LLE), were evaluated to improve extraction efficiency, reduce endogenous interference, and minimize the matrix effect of SKLB010. Our initial approach of developing an assay for SKLB010 and rosiglitazone in dog plasma was based on PPT with methanol and acetonitrile. However, this technique resulted in strong interferences from the sample matrix and low recovery (<50%) of SKLB010. Then liquid–liquid extraction (LLE) using methyl tert-butyl ether (MTBE), ethyl acetate (EA) and dichloromethane (DCM) were tried. However, there was ion suppression and a low recovery was obtained. Solid phase extraction was adopted in the end because this technique cannot only purify but also concentrate the sample.

3.1.3. Optimization of UPLC/MS/MS method

Initial attempt in our laboratory to use HPLC–UV for the analysis of SKLB010 in plasma samples was proved to be unsuccessful due to the poor sensitivity of the analyte. Then we developed a simple, rapid UPLC–MS/MS method for the determination of SKLB010 in dog plasma samples collected in pharmacokinetic studies.

The chromatographic conditions, especially the composition of mobile phase, could affect the separation and ionization of SKLB010 and the I.S. Therefore, the optimization of mobile phase components was critical. Several mobile phases have been reported for the separation of thiazolidinediones on C18 column, such as methanol–water [16], acetonitrile–formic acid–water [17], acetonitrile–ammonium acetate–TFA–water [18], and acetonitrile–ammonium acetate–water [19]. In this work we have trialed different mobile phase combinations to achieve good resolution and symmetric peak shapes for the analyte and I.S., as well as a short run time. Modifiers, such as ammonium acetate and formic acid alone or in combination in different concentrations were added. It was found that methanol–water (80/20, v/v) could achieve this purpose and was finally adopted as the mobile phase.

Fragments of high intensities were selected for ion monitoring. The structural formulae of analyte and I.S. with prominent sites of fragmentation are shown in Fig. 2(A) and (B). For selectivity and specificity of the method, detection of eluted peaks was done in MRM mode, wherein the most abundant fragmentation ion of SKLB010, m/z 147.92 was selected for sensitive quantification of the drug, while I.S. response was monitored using the most abundant fragment of m/z 150.00. The retention times were 1.02 min for SKLB010 and 1.09 min for I.S., and the total chromatographic run time was 2.0 min.

3.2. Validation of the analytical method

3.2.1. Specificity

The selectivity was evaluated by comparing the chromatograms of blank dog plasma, the lower limit of quantification (LLOQ, 5 ng/mL) samples and real plasma samples in pharmacokinetic studies (shown in Fig. 3) Compared to spiked plasma samples, blank plasma showed no significant peaks interfering with the quantification of the analyte and I.S. and the representative chromatogram of real plasma samples showed similar chromatographic behavior to QCs.

3.2.2. Linearity and sensitivity

The calibration curves were all linear with regression correlation coefficients (r^2) > 0.9970 over the concentration range of 5–2000 ng/mL on 3 continuous days. Typical equation of the calibration curves was: $y = 3.60 \times 10^{-3}x + 5.7 \times 10^{-3}$, $r = 0.9993$. Where x represents the plasma concentration of SKLB010 and y is the peak area ratio of SKLB010 to I.S. The lower limit of quantification (LLOQ) was found to be 5 ng/mL in dog plasma (S/N ≥ 10), which was sufficient to support pharmacokinetic studies of SKLB010. The accuracy of the diluted samples of SKLB010 was 97.50 ± 2.57% at 5000 ng/mL and 98.10 ± 3.95% at 10,000 ng/mL. The precision determined at each concentration level was within 5% of the relative standard deviation (%R.S.D.). Dilution study found that the assay was reasonable to quantify SKLB010 in samples that exceeded the upper limit by an appropriate dilution. After analyzing the samples of

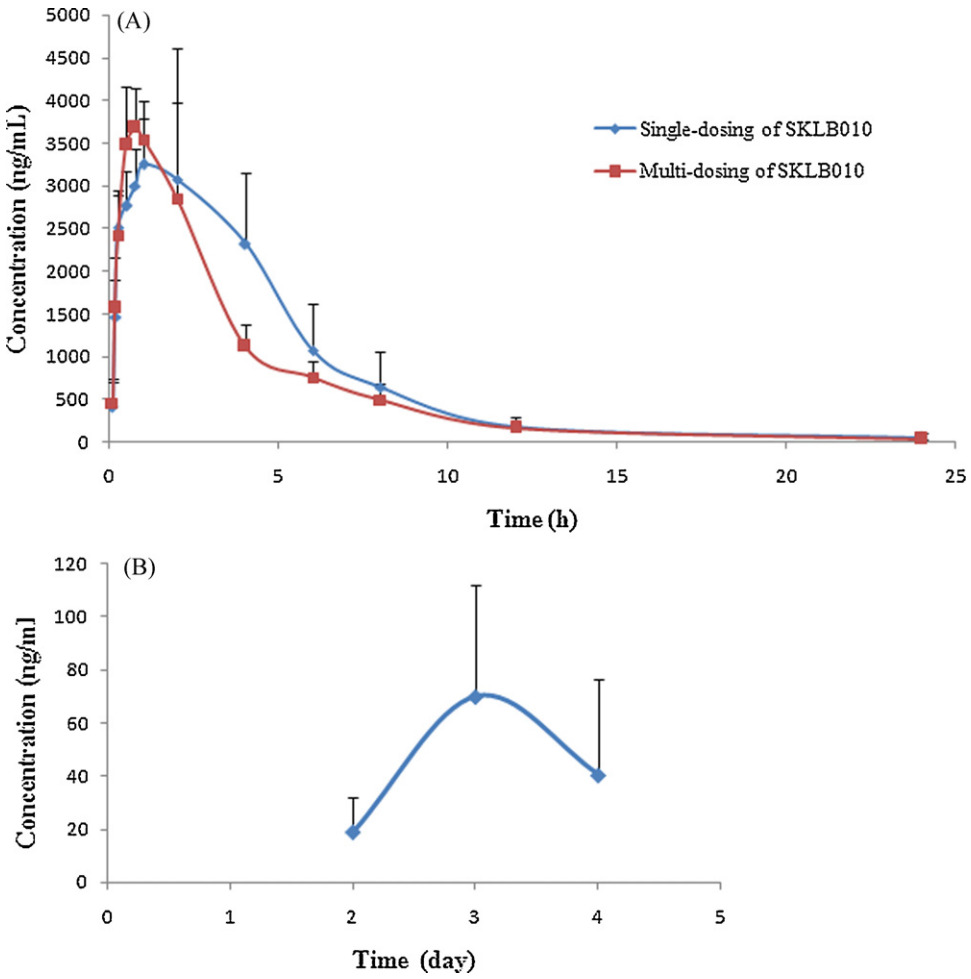

Fig. 5. Plasma concentration versus time profiles of SKLB010 following oral multi-dosing administration of SKLB010 at 30 mg/kg to beagle dogs ($n = 4$). (A) Plasma concentration–time curve after dosing on the Day 4 morning, comparing to the curve after oral single-dosing administration of SKLB010 at 30 mg/kg to beagle dogs. (B) The valley concentrations of plasma SKLB010 in multi-dosing study.

2000 ng/mL, the concentration of the blank samples detected was 0.6 ± 0.1 ng/mL. The carryover was 12% of LLOQ, which was within the acceptable limits.

3.2.3. Accuracy and precision

The intra-day and inter-day precision and accuracy for the tested concentrations (LLOQ, QCL, QCM, and QCH) are all within acceptable limits, as presented in Table 2. Intra-day precision (expressed as percent relative standard deviation, %R.S.D.) ranged from 1.62 to 3.45% and the inter-day precision ranged from 3.04 to 4.23%, which indicated that the method was reproducible. Intra- and inter-day accuracy ranged from 90.42 to 102.16% and 90.56 to 99.48%, respectively, which indicated that the method was accurate and reliable.

3.2.4. Extraction recovery and matrix effect

Table 3 shows a summary of extraction recovery and matrix effect of SKLB010 in dog plasma. Average recovery of SKLB010 was 92.83 ± 7.99%, 90.78 ± 2.17%, and 90.12 ± 3.58% for the low (10 ng/mL), mid (200 ng/mL), and high (1600 ng/mL) level QC samples, respectively, which was consistent over the range of concentrations investigated. The recovery of I.S. was 65.99% at the concentration used in the assay (100 ng/mL). Recovery of I.S. was low, but it was consistent and reproducible. As for ionization, the peak areas of SKLB010 after spiking evaporated plasma samples at three concentration levels were comparable to neat standard solutions (ranged from 89.97% to 91.63%), suggesting that there was no measurable matrix effect interfered with SKLB010 determination in dog plasma.

3.2.5. Stability

On storage at ambient temperature (25 °C) for 24 h, the concentrations of analytes in plasma deviated less than ±15% from their nominal concentrations, showing that the samples were stable during preparation processes. On storage in the autosampler at 10 °C for 24 h, the analytes showed good stability, wherein, the concentration varied no more than 6.12% of their nominal concentrations. In freeze–thaw stability experiment, no significant degradation of SKLB010 was observed. After 4 weeks of storage at −20 °C, QC samples had a certain degree of degradation, but the concentrations obtained deviated less than ±15% from their nominal concentrations. The results of stability experiments are presented in Table 4.

Taken together, the above results showed that a sensitive, reproducible, and robust method for the analysis of SKLB010 in dog plasma had been developed and validated.

3.3. Pharmacokinetic study

The validated analytical method was successfully used to quantify plasma concentrations of SKLB010 in the pharmacokinetic study in beagle dogs. The high sensitivity of our assay allowed to measure plasma concentrations up to 24 h post dose. Samples

whose concentrations exceeded the upper limit of quantification were diluted to within the assay range with blank plasma and re-analyzed.

The plasma concentration–time curves of SKLB010 involving three dose administrations (10, 30, and 90 mg/kg) are shown in Fig. 4. The pharmacokinetic parameters including AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, T_{max}, $t_{1/2}$, V_d, C_l and MRT were estimated by a non-compartmental analysis based on statistical moments using Drug and Statistics 2.0 (DAS 2.0) (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The major pharmacokinetic parameters are listed in Table 5.

After intragastric administration of SKLB010 at the doses of 10, 30, and 90 mg/kg, AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, V_d and MRT_{0-t} showed significant differences ($P > 0.05$). The C_{max} values for SKLB010 were estimated to be of 1768.8 ± 530.5, 3649.9 ± 1098.0, and $3{,}783{,}781.1 \pm 1796.2$ ng/mL, respectively. The mean $AUC_{0-\infty}$ values were 8368.8 ± 3828.2, $16{,}427.5 \pm 6494.6$, and $33{,}965.8 \pm 9718.5$ μg/L h, respectively. The $AUC_{0-\infty}$ values versus doses were linear over the administered dose range ($r = 0.995$) while the C_{max} values versus doses were not. SKLB010 may have linear pharmacokinetic characteristics. After reaching maximal plasma concentration (C_{max}), plasma SKLB010 concentration declined gradually with a half-life of 3.02 ± 1.49, 3.87 ± 1.58 and 5.49 ± 1.86 h, respectively, which showed no significant difference. In addition, we identified multi-peak phenomena in the concentration–time profiles after beagle dogs received single oral doses of SKLB010. These phenomena suggested a possible enterohepatic cycle. However, this hypothesis needed further investigation.

In multi-dosing experiments, non-compartmental analysis was applied to calculate the pharmacokinetic parameters. The mean plasma concentration–time profiles for SKLB010 on Day 1 and Day 4 are shown in Fig. 5(A), while the pharmacokinetic parameters are summarized in Table 6.

In the multi-dosing study, pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, $t_{1/2}$, T_{max} and C_l obtained on Day 1 and Day 4 showed no significant differences ($P > 0.05$). In this way, multi-dosing for 4 days of SKLB010 at 30 mg/kg did not affect the pharmacokinetic profiles. Moreover, the exposures on Day 1 and Day 4 showed no significant accumulation of the SKLB010 in beagle dogs.

4. Conclusions

In conclusion, this paper describes the development, validation and application of a UPLC–MS/MS method for the quantitative analysis of SKLB010 in beagle dog plasma. SKLB010 is extracted by a simple and fast SPE method. The relatively simple sample preparation together with the short LC run time (2 min) makes the present method more practical for high throughput sample analysis. This method is satisfactory in terms of accuracy, precision, sensitivity, matrix effects and reproducibility. It has been successfully applied in pre-clinical pharmacokinetic research and will be used in future clinical pharmacokinetic studies.

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