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Short communication

High-performance liquid chromatographic determination of a new oral thrombin inhibitor in the blood of rats and dogs

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Abstract

A reliable reversed-phase high-performance liquid chromatographic method has been developed for the determination of a new oral thrombin inhibitor (compound I) in the blood of rats and dogs. The analyte was deproteinized with a 1.5 volume of methanol and a 0.5 volume of 10% zinc sulfate, and the supernatant was injected into a 5-μm Capcell Pak C_{18} column (150×4.6 mm I.D.). The mobile phase was a mixture of acetonitrile and 0.2% triethylamine of pH 2.3 (31:69, v/v) with a flow-rate of 1.0 ml/min at UV 231 nm. The retention time of compound I was approximately 9.3 min. The calibration curve was linear over the concentration range of 0.05–100 mg/l for rat blood (r^2>0.9995, n=6) and dog blood (r^2>0.9993, n=6). The limit of quantitation was 0.05 mg/l for both bloods using a 100-μl sample. For the 5 concentrations (0.05, 0.1, 1, 10, and 100 mg/l), the within-day recovery (n=4) and precision (n=4) were 98.1–104.1% and 1.5–6.8% for rat blood and 95.4–105.7% and 1.4–5.3% for dog blood, respectively. The between-day recovery (n=6) and precision (n=6) were 99.8–105.3% and 3.7–12.6% for rat blood and 87.5–107.1% and 2.9–15.3% for dog blood, respectively. The absolute recoveries were 82.4–93.3%. No interferences from endogenous substances were observed. In conclusion, the presented simple, sensitive, and reproducible HPLC method proved and was used successfully for the determination of compound I in the preclinical pharmacokinetics.

Keywords: Thrombin inhibitors; (*S*)-*N*-Cyclopentyl-N-methyl-3-(4-amidrazonophenyl)-2-(2-naphtylsulfonylamino)-propionamide

1. Introduction

Thrombin is a trypsin-like serine protease which plays a central role in both hemostasis and thrombosis [1–4]. Since many cardiovascular diseases such as myocardial infarction, unstable angina, and deep vein thrombosis are caused by thrombosis, a wide variety of thrombin inhibitors have been synthesized and tested for the prevention of thrombosis and acceleration of thrombolysis in conjunction with a thrombolytic agent [5]. Among them, D-Phe-Pro-Arg-H analogs [6–9] and argatroban [10] are the typical examples of synthetic small molecular thrombin inhibitors. However, these first generation compounds have limitations in specificity, half-life, and oral bioavailability [11].

Compound I [(*S*)-*N*-cyclopentyl-*N*-methyl-3-(4-amidrazonophenyl) -2- (2-naphthylsulfonylamino)-propionamide] is a new oral thrombin inhibitor (Fig.

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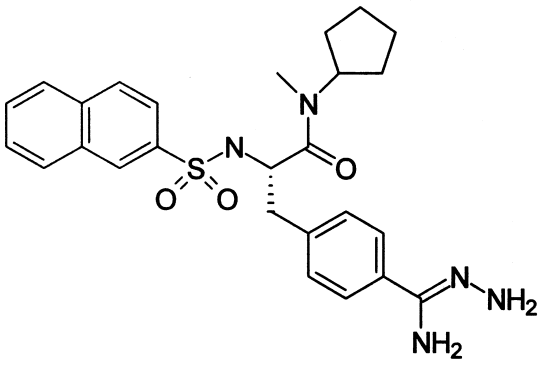

Fig. 1. The structure of compound I.

1), which is potent (human thrombin K_i=0.38 n*M*) and selective (trypsin=3290 n*M*, plasmin K_i=47 900 n*M*, t-PA K_i=27 200 n*M*) where inhibition is constant [12,13]. It inhibits clot-bound thrombin and in vitro fibrin generation with 50% inhibition concentration (IC_{50}) of 16 n*M* and 1.1 n*M*, respectively [14]. The dose-dependent antithrombotic effect of compound I was observed in several experimental animal models such as venous thrombosis, arterial thrombosis, AV-shunt, and disseminated intravascular coagulation in rats [14]. Therefore, compound I is a promising anticoagulant, which is under evaluation in clinical situations. Pharmacokinetic studies of this compound in animals require sensitive and reproducible analytical methods for the quantitation of the drug in biological fluids, such as blood, urine, bile, and tissue homogenates. For the quantitation of compound I in the blood of rats and dogs, a simple isocratic reversed-phase high-performance liquid chromatographic (HPLC) assay method with ultraviolet detection has been developed. This method was successfully applied for the preclinical pharmacokinetics of compound I.

2. Experimental

2.1. Chemicals and reagents

Compound I (Lot No.: TP-07, purity: 98.8%) was synthesized at LG Chem (Taejon, South Korea). Triethylamine hydrochloride and zinc sulfate were obtained from Sigma (St. Louis, MO. USA). Acetonitrile and methanol (Burdick and Jackson, Muskegon, MI, USA) were of HPLC grade, and all other reagents were of analytical grade. Deionized water was purified using a Milli-Q filter system (Millipore, Milford, MA, USA).

2.2. Preparation of standard solutions

Stock solution (1 g/l) and diluted working solutions (0.05, 0.1, 0.2, 1, 2, 10, 20, and 100 mg/l) of compound I were made in methanol in polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ. USA). They were stored at −70°C and were stable for at least for 1 month. A 100-μl volume of the corresponding working solutions was mixed with 100 μl of blank blood, 50 μl of blank methanol, and 50 μl of 10% $ZnSO_4$, over a six-day period to construct 6 calibration standards (n=6).

2.3. Method validation

Calibration curves were constructed using compound I peak area against nominal concentration, and linear regression analysis with $1/x$ weight used to determine the slope (i.e. the response factor), correlation coefficient, and intercept which would best fit the data. The within-day variation for replicated assays (n=4) and the between-day variation over a six-day period (n=6) were determined from validation samples to assess the recovery (%) and precision (%) of the analytical method over 5 concentration levels (0.05, 0.1, 1, 10, and 100 mg/l). The accuracy and the precision of the assay were determined by measuring the concentrations of compound I using validation samples and comparing them with the nominal concentrations. The limit of quantitation was determined as the lowest concentration at validation samples with acceptable accuracy (80–120%) and precision (within 20%) [15]. The absolute recovery of compound I was determined by comparing peak area of treated validation samples with that of untreated standard of corresponding concentration in the mixture of methanol–water $ZnSO_4$ (1.5:1:0.5, v/v/v).

2.4. Sample preparation

For the analysis of compound I in the blood of rats and dogs, a deproteinization method was applied

using methanol and 10% zinc sulfate. A 100-μl aliquot of sample was mixed with 150 μl of methanol and 50 μl of 10% $ZnSO_4$ for validation samples, study samples, and stability samples (blood and plasma) in a 1.5-ml polypropylene tube. The tubes were then tightly capped, vortex mixed for approximately 10 s, centrifuged for 20 min at 15 800 *g* (Eppendorf centrifuge 5402, Hamburg, Germany) to achieve a clear supernatant, and 100 μl of the supernatants was injected directly into HPLC. All procedures for sample handling and processing were carried out at 4°C using a Labtop cooler (Nalgene, Rochester, NY, USA).

2.5. Chromatography

Compound I was quantified using a reversed-phase (RP) HPLC on a Capcell Pak C_{18} column (150×4.6 mm, 5-μm particle size, Shiseido, Chuo-ku, Tokyo, Japan) fitted with a Waters Nova Pak C_8 Guard-Pak precolumn (Millipore, Milford, MA, USA). The HPLC system (Shimadzu, Tokyo, Japan) consisted of a Class-LC10A system control software, a CBM-10A communication bus module, two LC-10AD pumps, a SIL-10A autoinjector with sample cooler set at 4°C, a SPD-10AV UV–VIS detector, and a GLP-2050+ laser printer (LG Elect., Seoul, South Korea). The mobile phase was a mixture of acetonitrile and 0.2% triethylamine hydrochloride of pH 2.3 adjusted with HCl (31:69, v/v), and the flow-rate was 1.0 ml/min. Detection was by UV absorption at 231 nm wavelength.

2.6. Blood concentration

The pharmacokinetics of compound I were studied in the male Sprague-Dawley rat (200–300 g, $n=2$) and male Beagle dog (8–12 kg, $n=4$). Compound I was orally administered into 18-h fasted animals at a dose of 30 mg/kg for rat and 10 mg/kg for dog via gavage. Afterwards, approximately 150 μl of blood was collected from the right femoral artery of the rat or the cephalic vein of the dog for up to 720 min, and 100 μl of sample was mixed immediately with 150 μl of methanol and 50 μl of 10% $ZnSO_4$ at 4°C. The blood extracts were stored at 4°C and injected onto HPLC within 12 h after sample collection.

3. Results and discussion

The UV radiation absorption of compound I was maximum at 231 nm, and this wavelength was therefore used for the HPLC analysis. Fig. 2 shows typical chromatograms of drug-free rat and dog blood, drug standard containing 0.1 mg/l in blood, and real samples at 60 min after oral administration of the drug. The deproteinization of samples with methanol and 10% zinc sulfate gave no interference in the chromatogram from endogenous substances. The peak of compound I was symmetrical and eluted at approximately 9.3 min by the isocratic mobile phase.

The limit of detection was 0.03 mg/l using 0.1 ml of blood based on the signal-to-noise ratio of 3. The plot of compound I peak area versus nominal concentration was linear over the concentration range of 0.05–100 mg/l in the blood of rats [$r^2>0.9995$; slope, 217341=14937 (mean=S.D.); $n=6$] and dogs [$r^2>0.9993$; slope, 208527±22405 (mean=S.D.); $n=6$].

Assay variability was calculated at 5 concentrations (0.05, 0.1, 1, 10, and 100 mg/l). The within-day and between-day recoveries were listed in Table 1. In this assay method, the within-day recovery ($n=4$) and precision ($n=4$) were 98.1–104.1% and 1.5–6.8% for rat blood and 95.4–105.7% and 1.4–5.3% for dog blood respectively, while the between-day recovery ($n=6$) and precision ($n=6$) were 99.8–105.3% and 3.7–12.6% for rat blood and 87.5–107.1% and 2.9–15.3% for dog blood, respectively. The limit of quantitation was 0.05 mg/l based on the lowest concentration with acceptable accuracy and precision. The absolute recoveries were 84.2–93.3% for rat blood and 82.4–91.7% for dog blood. These results meet the criteria for a valid analytical assay defined by Shah et al. [15] with acceptable accuracy: 85–115% and precision: within 15%.

The time courses of compound I in the blood following oral administration into rats and dogs are shown in Fig. 3. The peak concentration was 1.0 mg/l at 20 min for rats and 1.2 mg/l at 60 min for dogs. The dog (10 mg/kg dose) showed higher blood concentration than the rat (30 mg/kg dose) in the post-absorption phase.

The stability of compound I was studied in methanol, water, rat blood, rat plasma, and rat blood

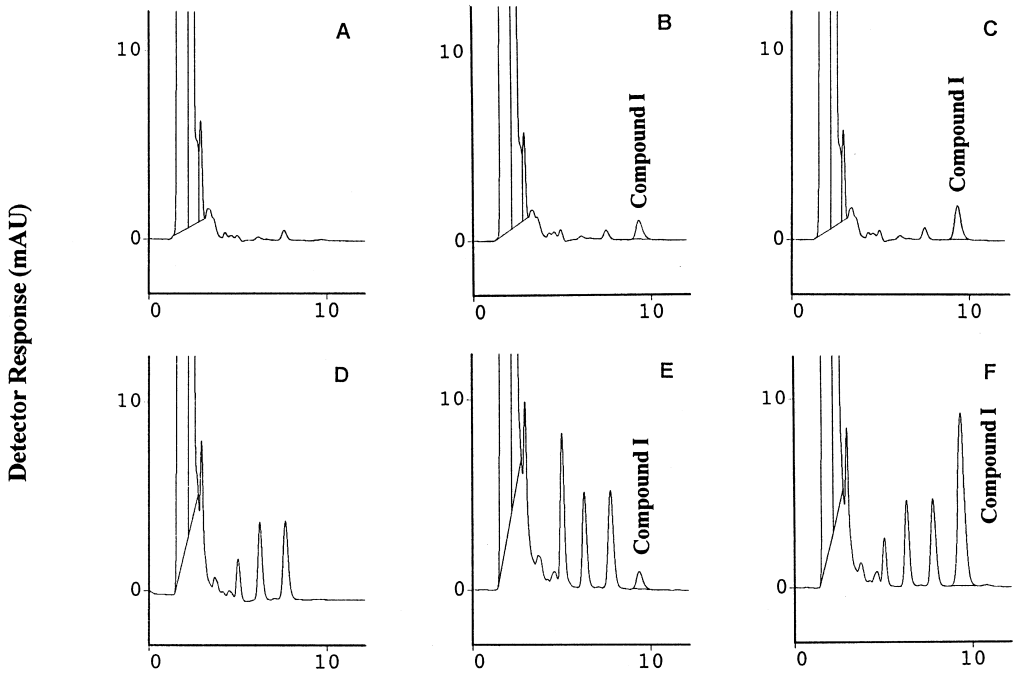

Fig. 2. HPLC chromatograms of (A) drug-free rat blood, (B) rat blood spiked with 0.1 mg/l of compound I, (C) rat blood obtained at 60 min after oral administration of compound I at 30 mg/kg dose, (D) drug-free dug blood, (E) dog blood spiked with 0.1 mg/l of compound I, and (F) dog blood obtained at 60 min after oral administration of compound I at 10 mg/kg dose.

extract at the concentration of 10 mg/l. A 100-μl aliquots of various stability samples in eppendorf tubes were stored at −70, −20, and 4°C for 1 month. Duplicate samples were analyzed to assess the stability at min 0, 1, 5, 10, 15, 30, 60, 90, 120, 180, 360, and 720 for blood and plasma, and at day 0, 1, 5, 10, 20, and 30 for methanol, water, and blood extract. Compound I was completely stable at −70°C for 1 month in methanol, water, and blood extract (recovery >95%). However, compound I was unstable even at −70°C in rat blood and plasma, showing approximately 80% recovery after 12 h

Table 1
Recovery (mean±S.D.) of compound I in the blood of rats and dogs

Blood	Added conc. (mg/l)	Within-day (%, n=4)	Between-day (%, n=6)	Absolute between-day (%, n=6)
Rat	0.05	104.1±6.8	99.8±12.6	85.0±11.3
	0.1	99.5±2.5	103.6±4.4	93.3±15.5
	1	100.2±3.0	103.5±6.9	86.8±6.5
	10	98.1±2.7	105.3±6.2	88.0±6.2
	100	100.1±1.5	100.8±3.7	84.2±5.8
Dog	0.05	95.4±3.6	87.5±15.3	82.4±4.8
	0.1	100.2±2.7	101.9±4.4	91.7±9.4
	1	105.7±2.0	107.1±4.2	86.4±4.9
	10	103.7±5.3	106.3±7.3	85.1±7.2
	100	105.3±1.4	99.5±2.9	79.5±5.8

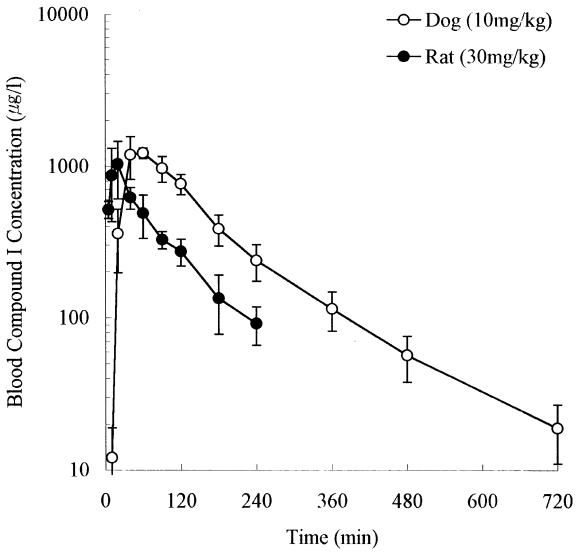

Fig. 3. The concentration–time profiles of compound I in the blood of rats ($n=2$) and dogs ($n=4$) following oral administration of compound I at the dose of 30 mg/kg for rats and 10 mg/kg for dogs (mean±S.D.).

storage. Overall the stability of compound I changed with the increase of temperature. The stability samples stored at −20°C showed approximately 85% recovery at day 10 in methanol and at day 5 in water and blood extract. The stability samples stored at 4°C showed approximately 85% recovery at day 10 in methanol and water, and at day 1 in blood extract. By contrast, the stability samples of rat blood and plasma stored at 4°C showed approximately 80% recovery at 180 min. That is why the blood source was chosen and deproteinized immediately after blood sampling, and injected onto the HPLC for the measurement of compound I blood concentration.

In summary, a simple and reproducible reversed-phase high-performance liquid chromatographic assay method using deproteinization and UV detection was developed for the determination of compound I in the blood of rats and dogs. The absolute recoveries were 84.2–93.3% for rat blood and 82.4–91.7% for dog blood, and the limit of quantitation was 0.05 mg/l using 0.1 ml of blood. Applicability of this method to a pharmacokinetic study was demonstrated. Thus, the above assay method appears to be suitable for the determination of compound I in biological samples for preclinical pharmacokinetics.

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