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Pharmacokinetic studies of a novel 1,2,4-thiadiazole derivative, inhibitor of Factor XIIIa, in the rabbit by a validated HPLC method

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Abstract

Activated Factor XIII (FXIIIa) stabilizes fibrin clot by covalent cross-linking of fibrin strands in the fibrin, making it resistant to physiological and pharmacologically induced fibrinolysis. Inhibition of Factor XIIIa offers a novel approach to treatment of thrombosis. Selected derivatives of 1,2,4-thiadiazoles, presently in discovery and development, may offer new treatment strategies as inhibitors of Factor XIIIa. In order to evaluate its pharmacokinetic (PK) profile and to facilitate the selection of drug candidates for drug discovery and development process, we developed and validated a simple and selective reversed-phase high-performance liquid chromatographic method (RP-HPLC) with UV detection for the determination of *N*-[6-(imidazo[1,2-*d*][1,2,4]thiadiazol-3-ylamino)hexyl]-2-nitrobenzensulfonamide (5624) in rabbit plasma. The plasma protein precipitation and sample preparation was achieved by using acetonitrile, followed by organic phase evaporation to dryness and the residue reconstitution in the mobile phase. The 5624 recovery from the plasma was about 90%. Chromatography was performed on a C18 column using a gradient of acetonitrile in water as a mobile phase. A chemically related compound, *N*-[6-(imidazo[1,2-*d*][1,2,4]thiadiazol-3-ylamino)hexyl]naphthalene-1-sulfonamide (5422), was used as an internal standard. Limit of detection (LOD), based on signal to noise ratio > 3, was 0.2 μM (on-column amount of about 7 ng), while limit of quantification (LOQ), based on signal to noise ratio > 10, was 0.5 μM (on-column amount of about 20 ng). The plasma samples for the PK study were collected at defined time points during and after 5624 slow intravenous infusion (25 mg/kg) to male White New Zealand rabbits and analyzed by RP-HPLC method. The PK parameters, such as half-life, volume of distribution, total clearance, elimination rate constant etc., were determined. The PK profile of 5624 offered insights in the design and development of additional new compounds, derivatives of 1,2,4-thiadiazole, with desired PK properties.

Keywords: RP-HPLC; Factor XIIIa inhibitors; 1,2,4-Thiadiazole derivative 5624; PK study in rabbit

1. Introduction

Inhibition of activated plasma Factor XIII (FXIIIa) offers a novel approach to the treatment of thrombosis [1]. FXIIIa catalyses the formation of covalent linkages in fibrin and the covalent incorporation of α_2-antiplasmin into fibrin clot. Numerous in vitro studies demonstrated that plasma clots formed in the presence of FXIIIa inhibitors are characterized by less extensive cross-linking and increased susceptibility to fibrinolysis [2–5]. These observations have been corroborated in vivo in the animal models of arterial [6,7] and venous thrombosis [8]. Potent FXIIIa inhibitors have been isolated from natural sources, such as Tridegin [9] and alutacenoic acids [10], or obtained synthetically, such as halodihyroisooxazoles [11,12], 2-((2-oxopropyl)thio)imidazolium [3] and cyclopropenone derivatives [10].

Selected synthetic derivatives based on 1,2,4-thiadiazole pharmacophore, presently in discovery and development, exhibit in vitro Factor XIIIa inhibitory activity. The purpose of our work was to develop and validate an RP-HPLC method for the determination of a new 1,2,4-thidiazole

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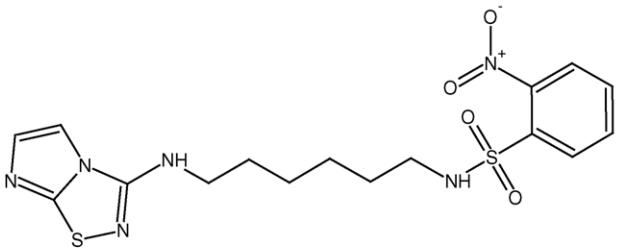
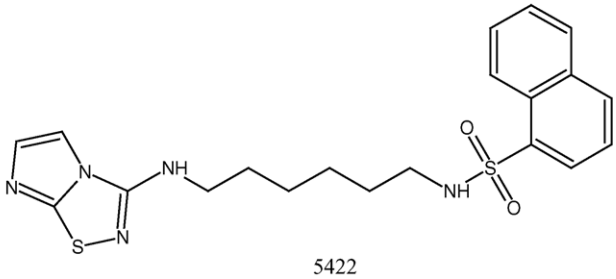


5624



5422

Fig. 1. 5624 and 5422 (IS) chemical structures.

derivative, 5624 (*N*-[6-(imidazo[1,2-*d*][1,2,4]thiadiazol-3-ylamino)hexyl]-2-nitrobenzensulfonamide, Fig. 1) in rabbit plasma and to establish its PK profile in rabbits in order to support and facilitate the design and selection of drug candidates with desirable PK properties.

2. Experimental

2.1. Chemicals

1,2,4-Thidiazole derivatives 5624 (M.W. 425.508) and *N*-[6-(imidazo[1,2-*d*][1,2,4]thiadiazol-3-ylamino)hexyl]naphthalene-1-sulfonamide (5422, the internal standard, M.W. 429.561, Fig. 1) were synthesized by Apotex IDD (Toronto, Canada). Sodium citrate (dihydrate), citric acid (anhydrous), and dimethylsulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile of HPLC grade purity was from Fisher Scientific (New Jersey, USA). Citrated blank rabbit plasma was obtained from White New Zealand rabbits (Charles River Laboratories, Montreal, Canada).

2.2. Standard solutions

Stock solutions: 10 mM solutions of 5624 and the internal standard 5422 were prepared separately in DMSO and stored at −20 °C before usage.

Solutions for an absolute reference calibration curve: stock solutions of 5624 and 5422 were diluted with acetonitrile–water mixture (40:60, v/v) to a final concentration of 400 and 200 μM, respectively. By mixing these solutions at 1:1 ratio, a calibration solution of 5624 (200 μM) and 5422 (100 μM) was obtained. This solution was diluted serially with 100 μM solution of 5422. Thus, the concentration of 5624 ranged from 0.2 to 200 μM and the concentration of 5422 was 100 μM. An aliquot of 80 μL was injected into the HPLC system.

Table 1
Gradient program

Time (min)	Acetonitrile (%)	Water (%)
0	40	60
5	40	60
15	60	40
16.5	40	60
22	40	60

Calibration curve in plasma: citrated rabbit plasma was spiked with stock solution of 5624 to obtain concentrations from 0.2 to 200 μM. A volume of 0.2 mL of spiked plasma samples was mixed thoroughly with 3 mL of acetonitrile, centrifuged for 20 min at 3000 rpm/min, and 2.7 mL of a clear supernate was transferred to a test tube and evaporated to dryness under air stream. The residue was dissolved in 0.18 mL of the internal standard solution (100 μM) in acetonitrile–water mixture (40:60, v/v) and an 80 μL aliquot of clear solution was injected into the HPLC system.

2.3. Chromatography

Column: Beckman Ultrasphere ODS (Beckman, USA); particle size 5 μm; column dimensions 4.6 mm × 150 mm; equipped with Zorbax RX-C18 guard column (Agilent Technologies, USA); mobile phase: acetonitrile (40%) and deionized water (60%) (Millipore) isocratically for 5 min followed by a gradient program as described in Table 1; flow rate 1 mL/min; analysis time 22 min.

HPLC system: Hewlett-Packard 1050 chromatograph, equipped with a quaternary pump, an autosampler, and a variable wavelength UV–vis detector. Detection wavelength was set at 260 nm (λ_{max} for 5624).

2.4. Rabbit experiment

Male New Zealand White rabbits weighing about 3 kg were housed one per cage at 22 °C and 50% humidity with a 12-h light–dark cycle. All animals were acclimatized for 1 week before the experiment. They were given a standard Purina rabbit chow and water ad libitum. Rabbits' marginal ear veins were catheterized and kept patent with heparin/saline. Animals were placed in metabolic cages 24 h before the experiment and fasted overnight before the experiment.

A 3-mL volume of an intravenous formulation in Cremophor EL (25 mg of 5624 per milliliter) was diluted with 12 mL of 0.9% saline solution (for infusion) to permit administration by slow intravenous infusion at a rate of 0.5 mL/min. Starting time and end of infusion were recorded. The administered dose was established by weighing the syringe before and after the infusion.

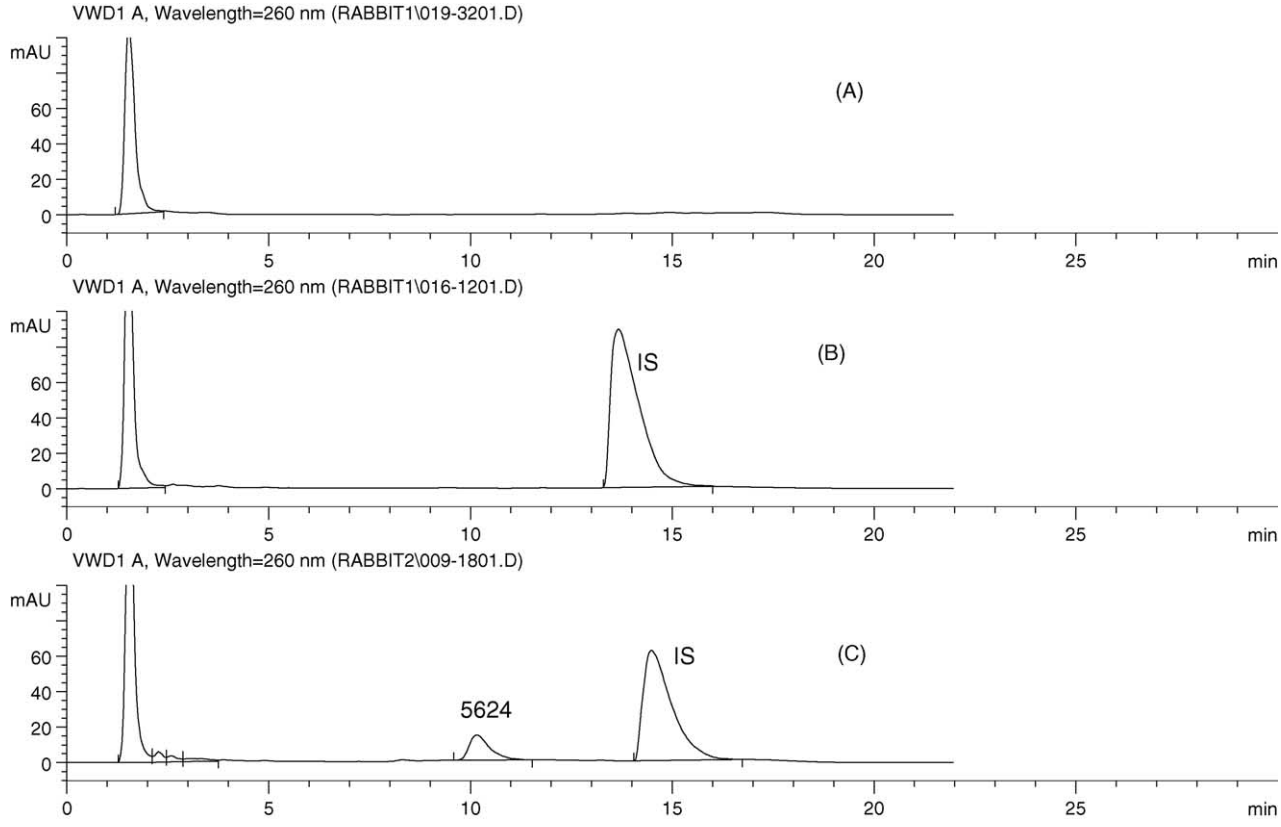


Fig. 2. Selectivity of HPLC method: chromatograms of blank rabbit plasma (A), rabbit plasma spiked with IS (B), and rabbit plasma spiked with 5624 (10 μM) and IS (C).

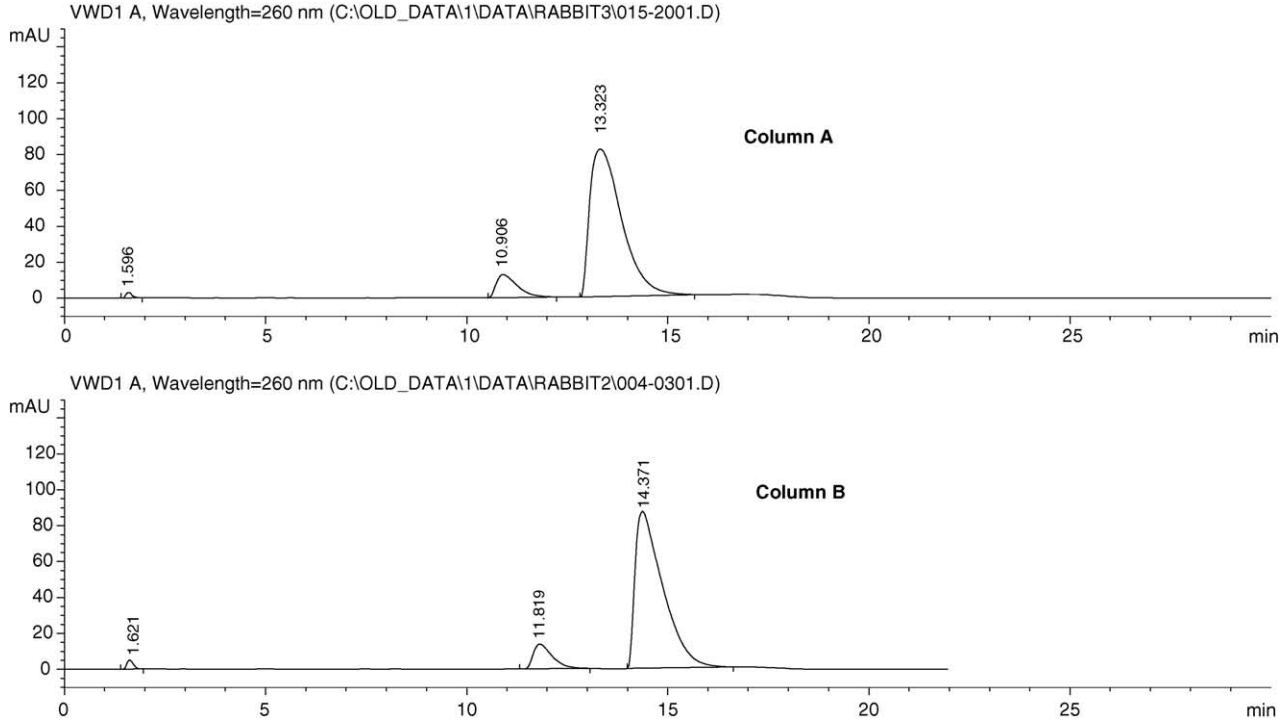


Fig. 3. Chromatography of 5624 (10 μM) and IS (100 μM) on Ultrasphere ODS (column A) and Zorbax RX C18 (column B) columns (4.6 mm × 150 mm, 5 μM).

Sequential blood samples (1 mL per sample, 1.5 mL for the blank) were collected (from the contralateral ear vein) before dosing (blank) and then at following time points: 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 4 h, 6 h, and 8 h after the start of infusion. Blood samples were replaced with an equal volume of 0.9% saline. The blood samples were centrifuged to obtain plasma (at 1500 rpm for 15 min). Citrate (320 mg of sodium citrate + 42 mg citric acid in 1 mL) was used as an anticoagulant (11 μL for the sample collection, 17 μL for the blank). Plasma protein precipitation was performed with 3 mL of acetonitrile as described for the calibration curve in the plasma. After evaporating the solvent, the residue was dissolved in 0.18 mL of the internal standard solution (5422, 100 μM in the mixture of acetonitrile/water, 40/60v/v). A clear solution (80 μL) was injected into the HPLC system.

2.5. Pharmacokinetic analysis

The PK parameters were calculated by noncompartmental analysis based on statistical moment theory [13]. The area under the curve (AUC), the area under the moment curve (AUMC), the rate constant for elimination (k), the half-life ($t_{1/2}$), and the volume of distribution (V_d) were determined using standard approaches.

3. Results and discussion

3.1. HPLC method

3.1.1. Mobile phase and column selection

The results of our prior studies on 1,2,4-thiadiazoles, including lipophilicity estimation by RP-HPLC [16] were used as a basis for mobile phase selection and optimization. Gradient chromatography with acetonitile as an organic modifier was necessary because of the highly lipophilic nature of the compounds. Furthermore, due to the large initial concentration of acetonitrile (40%), endogenous compounds from rabbit plasma were eluted first as one peak (Fig. 2). To test the method's robustness the chromatography was performed on Ultrasphere ODS (Beckman, USA) and Zorbax RX-C18 (Agilent, USA) of the same column dimensions (4.6 mm × 150 mm) and the particle size (5 μm). Representative chromatograms of 5624 (10 μM) and 5422 (100 μM) obtained using these two columns are presented in Fig. 3. The columns were comparable in terms of selectivity, the number of theoretical plates (>3600 for 5624, and >9500 for 5422), the peak symmetry (about 0.6), and the resolution (baseline resolution ≈1.4). The 5624 and 5422 resolution was calculated by the software (Chemstation, Agilent Technologies) taking into account the retention times and the peaks widths. The chromatography remained consistent and stable over the whole study and the above mentioned peak symmetry did not affect the method's applicability.

3.1.2. Method validation

The method validation was focused on the parameters essential for pre-clinical testing of a new compound under development. The HPLC method has been validated in terms of linearity, 5624 limit of detection (LOD), the lower limit of quantification (LLOQ), selectivity (blank plasma), robustness, accuracy, recovery, and sample stability (freeze and thaw cycles).

Linearity of the method was tested from 0.2 to 200 μM. The calibration dependence is described by the equation $Y = -0.0131\ (\pm 0.0015) + 0.0129\ (\pm 0.000058)X$, where Y is the peak area ratio (5624/5422), and X is 5624 concentration in μM. The coefficient of correlation (r^2) was 0.9991 (±0.0003). At concentrations below 1 μM, the negative intercept lead to overestimated 5624 concentrations. Thereby, to quantify 5624 below 1 μM, a single point calibration-method was used. Limit of detection (LOD), based on signal to noise ratio > 3, was 0.2 μM (on-column amount of about 7 ng), while the lower limit of quantification (LLOQ), based on signal to noise ratio > 10, was 0.5 μM (on-column amount of about 20 ng).

Chromatograms of blank rabbit plasma, rabbit plasma spiked with IS (100 μM), and rabbit plasma spiked with 5624 (0.5 μM) and IS (100 μM) is given in Fig. 2(A–C), illustrating the selectivity of the method. Shift in the retention times (RTs) for both 5624 (from about 9 to about 11 min) and 5422 (from about 12 to about 14.5 min) due to a non-linear gradient and day-to-day variations in the ambient temperature, did not affect the method selectivity and the resolution.

Accuracy and recovery of the method were tested by spiking blank rabbit plasma in quadruplets with 2.5, 25 and 75 μM of 5624. The samples were prepared as described above. The mean recoveries for these four set of samples were 93.73, 95.07, 89.87 and 87.03%, while the corresponding R.S.D.s were 2.44, 3.16, 3.91, and 4.67%.

To test the stability of 5624 in rabbit plasma samples spiked with 2.5, 25 and 75 μM of 5624 were subjected to freezing–thawing cycles. No degradation of the compound was observed after four freezing–thawing cycles over a 2-week period. The mean recovery was 91.61% and the R.S.D. was 5.48%.

3.2. Pharmacokinetic (PK) study in rabbits

Rabbit models of arterial thrombosis have been developed [6,14,15] and could potentially be used to test the efficacy of 1,2,4-thiadiazol derivatives. Therefore, male New Zealand White rabbits were chosen for the PK study. Single doses of 5624 were given as a slow intravenous infusion and the plasma samples were collected at specified time points as described above. After sample extraction and evaporation to dryness, the residue was dissolved in 5422 solution (100 μM in acetonitrile–water mixture (40:60, v/v)) and injected into the HPLC system. A representative plasma concentration versus time profile is illustrated in Fig. 4. Based on these

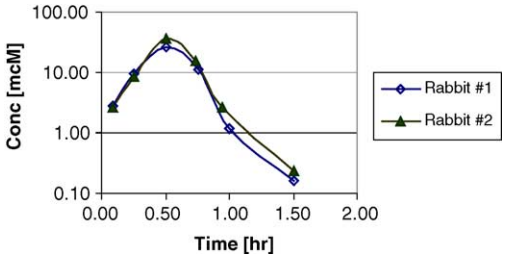


Fig. 4. 5624 plasma concentration vs. time profile in rabbits after slow i.v. infusion.

Table 2
5624 Pharmacokinetic parameters in rabbits

Parameter	Unit	Rabbit #1	Rabbit #2
Body weight	kg	3.2	3.0
Dose	μM	186.217	176.804
Ro	μM/h	429.732	408.010
AUC	μM h	12.056	15.194
AUMC	μM h^2	6.473	8.421
MRT	h	0.537	0.554
CL_{tot}	L/h/kg	4.827	3.879
k	1/h	5.410	5.170
$t_{1/2}$	h	0.128	0.134
V_d	L/kg	0.892	0.750

results the PK parameters were calculated. The calculated PK parameters are summarized in Table 2.

4. Conclusions

A simple and selective RP-HPLC method, using C18 column, acetonitrile–water as a mobile phase and UV detection at 260 nm, has been developed and validated for the determination of a novel 1,2,4-thiadiazole derivative 5624 in rabbit plasma in order to support and facilitate the design and selection of drug candidates with desirable PK properties. A high 5624 recovery (90%) from the rabbit plasma was achieved by plasma protein precipitation with acetonitrile, followed by the organic solvent evaporation to dryness and the residue reconstitution in the mobile phase. The method was applied to 5624 analysis in rabbit plasma. The PK study was conducted in male New Zealand White rabbits. The compound exhibits a moderately large volume of distribution ($V_d > 0.7$ L/kg), a short half-life ($t_{1/2} = 0.13$ h) and a high total clearance ($CL_{tot} > 3.8$ L/h/kg). Mean residence time is about 0.54 h. A target plasma concentration of about 80 μM as indicated by in vitro study, has not been achieved. Based on these findings, the design and development of new compounds, derivatives of 1,2,4-thiadiazole with improved PK parameters, is suggested to identify a more suitable preclinical candidate.

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