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Short communication

Determination of a novel cognitive enhancer, X9121, and its mono N-oxide metabolite, XG696, in dog plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A selective and sensitive high-performance liquid chromatographic assay for a novel cognitive enhancer, X9121 (I), and its mono N-oxide metabolite, XG696 (II), in dog plasma has been developed. Compounds I, II and internal standard (I.S.) were first extracted from dog plasma using a solid-phase Bond Elut Certify I 10-ml LRC reservoir extraction cartridge. Chromatographic separation of I, II and I.S. was conducted on a reversed-phase Zorbax Stable Bond cyano column. Ammonium acetate buffer (0.05 *M*, pH 6)–acetonitrile–triethylamine (75:25:0.1, v/v) was used as the mobile phase. Detection of all three compounds was by UV light absorbance at 313 nm. Using 0.5 ml of dog plasma for extraction, the minimum quantifiable limit was 10 ng/ml and the assay was linear from 10 to 5400 ng/ml. The coefficients of variation for intra-day precision ranged from 2.2 to 8.5% for I and from 2.5 to 9.8% for II. The coefficients of variation for the inter-day precision for these two compounds ranged from 2.6 to 9.0% and from 3.6 to 16.2%, respectively. The absolute percent differences for the accuracy results were within 11.0% of the spiked concentrations. Compounds I and II were stable in frozen plasma at −20°C for at least 67 days.

1. Introduction

X9121, 5,5 - bis(4 - pyridinylmethyl) - 5H - cy - clopental [2,1-b:3,4-b'] dipyridine (I), is a novel compound (Fig. 1) intended for use as a cognitive enhancer for the treatment of Alzheimer's disease. The postulated mechanism of action is through enhanced release of selected neurotransmitters [1]. In rat brain slices, I has been shown to enhance the depolarization-induced release of acetylcholine, dopamine and serotonin [1]. Compound I is sparingly soluble in water (0.94 mg/ml at pH 7.6), and has ionization constants (pK_as) for the two pyridine nitrogens of 5.2 and 5.5 [2]. To facilitate pharmacokinetic investigations of I and its active metabolite II, a specific reversed-phase HPLC assay with UV detection has been developed using 4,4'-(9H-fluorene-9,9-diyl-

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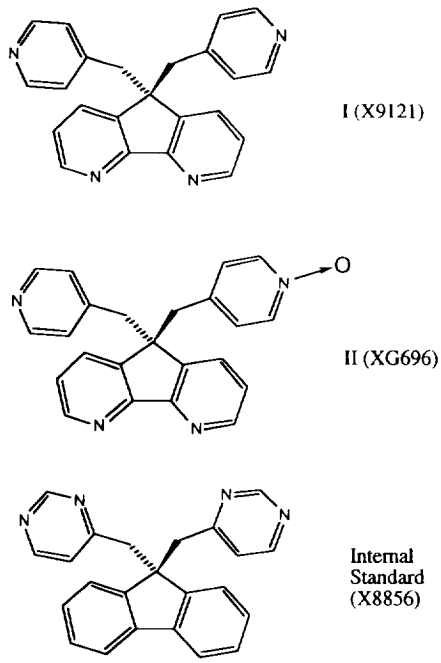

Fig. 1. Structures of I (X9121), II (XG696) and internal standard (X8856).

methylene)bispyrimidine (X8856) as internal standard (I.S.). This report describes the validation of the HPLC method in dog plasma. The assay is suitable to characterize the disposition of I and II in dog following oral dosing.

2. Experimental

2.1. Chemicals and solutions

Compounds I, II, and I.S. were synthesized and characterized (>99% purity) by The DuPont Merck Pharmaceutical Company and stored as dry powders at room temperature. Ammonium acetate, methanol, acetonitrile and ammonium hydroxide were HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Isopropyl alcohol and methylene chloride were HPLC grade (Baxter/Burdick and Jackson, Muskegon, MI, USA). Triethylamine was HPLC grade (Pierce Chemical Co., Rockford, IL, USA). All other chemicals were reagent grade (J.T. Baker). Bond Elut Certify I 10-ml LRC reservoir extraction cartridges (Catalog No. LR 29904) were purchased from Analytichem International (a division of Varian, Harbor City, CA, USA). Blank dog plasma, containing the disodium salt of ethylenediamine tetraacetic acid (EDTA) as anticoagulant, was obtained from male beagle dogs (Marshall Farms, North Rose, NY, USA).

2.2. Instrumentation

The HPLC system consisted of an isocratic solvent delivery pump (Spectroflow 400, Applied BioSystems, Ramsey, NJ, USA), an automated liquid sampler (Waters WISP 710B, Milford, MA, USA), a reversed-phase cyano column (5 μm, 250 × 4.6 mm I.D.; Zorbax Stable Bond-CN, Mac-Mod Analytical, Chadds Ford, PA, USA), a precolumn (300 Å pore size, 7 μm diameter, 15 × 3.2 mm I.D.; MPLC NewGuard cartridge, Applied Biosystems, San Jose, CA, USA), and a UV absorbance detector (Spectroflow 783 variable wavelength, Applied BioSystems). The data system consisted of an A/D converter (Nelson Analytical Model 760, Cupertino, CA, USA), a 16-bit desktop computer (Hewlett-Packard, HP 9200, Wilmington, DE, USA) a hard disk drive (HP 9133), a Hewlett-Packard thermal printer (HP 2671G) and Xtrachrome 4400 software (version 7.2, Nelson Analytical).

2.3. Chromatographic conditions

The mobile phase was acetonitrile–0.05 *M* ammonium acetate–triethylamine (25:75:0.1, v/v). The column temperature was kept at ambient and the flow-rate was 1.5 ml/min. The detector wavelength was set at 313 nm.

2.4. Stock solutions

Primary stock solutions (1.0 mg/ml of I and II; 100 μg/ml of I.S.) were prepared in methanol and stored refrigerated at 4°C. These solutions were periodically analyzed and compared to the

peak-area results of freshly prepared methanolic solutions. Working stock solutions (10 μg/ml, 500 ng/ml and 50 ng/ml of I and II; 10 μg/ml of I.S.) were prepared fresh each week from the primary stocks. These solutions were also stored refrigerated at 4°C and were found to be stable for at least 3 months.

2.5. Preparation of plasma standards and samples, and extraction procedure

Compound I and II plasma standards were prepared by adding appropriate aliquots of working stock solutions into separate 16 × 100 mm disposable glass culture tubes. One hundred μl of I.S. working stock solution (10 μg/ml) was added to all tubes and the methanol was evaporated under a stream of nitrogen gas. Subsequently, 0.5 ml of blank dog plasma was added to each tube and subjected to vortex-mixing for 20 s. The resultant plasma concentrations of I and II were 10, 25, 50, 100, 200, 600, 1800, and 5400 ng/ml. For plasma samples in pharmacokinetic studies, only the internal standard was added to the 0.5 ml sample of dog plasma.

The spiked plasma standards or pharmacokinetic samples (0.5 ml each) were pipetted into a Bond Elut Certify I 10-ml LRC reservoir disposable extraction cartridge which had been previously conditioned with 3 ml each of methanol, distilled water and isotonic sodium phosphate (0.033 *M*) buffer at pH 7.4. The column was rinsed with 3 ml of dilute hydrochloric acid (0.1 *M*). The cartridge was then dried, under vacuum, on a VacElut SPS 24 System (Analytichem International) for 15 min. After drying, the column was rinsed with 3 ml of tetrahydrofuran. The compounds of interest were subsequently eluted with two 1-ml rinses of elution solvent containing methylene chloride, N-isopropyl alcohol and 1% ammonium hydroxide (80:20:2, v/v). The elution solvent was evaporated under a gentle stream of nitrogen gas and the remaining residue was reconstituted in 200 μl of mobile phase. The sample was placed in WISP vials and 150 μl of the sample were injected onto the HPLC.

2.6. Sample preparation for accuracy and stability evaluation

Precision

Quadruplicate plasma samples of I and II were prepared in the concentration range 10–5400 ng/ml for intra- and inter-day reproducibility. The samples were extracted and analyzed on the same day on different conditions.

Accuracy

Plasma samples (0.5 ml) of varying concentrations of I and II were prepared and coded by a co-worker of the analyst. The assay of unknown samples containing I and II was then carried out by the analyst in the aforementioned manner.

Stability

Plasma samples containing 50, 200 and 1800 ng/ml of both I and II were prepared in 15-ml glass tubes and stored frozen at −20°C. The tubes were thawed and analyzed at various times to assess stability.

2.7. Calibration and calculation of plasma concentrations

The peak-height ratios (PHR) of I and II relative to the I.S. of the extracted plasma samples were evaluated against the plasma standards. A single calibration curve, constructed by weighted least square linear regression with a weighting factor of $1/\text{conc.}^2$, was used to determine slope, intercept and the coefficient of determination. The concentration range for the calibration curve was from 10 to 5400 ng/ml for both I and II. The equation for the calculation of unknown sample concentration was: concentration = (PHR − *y*-intercept)/slope.

2.8. Dog pharmacokinetics

Female beagle dogs, approximately 8–10 months old weighing 9–13 kg at the time of dosing (Hazelton Research Products, Kalamazoo, MI, USA) were used in the studies. The animals were housed in stainless steel meta-

bolic cages equipped with an automated watering valve. The animal room environment was controlled (temperature 20 ± 3°C; humidity 50 ± 20%; 12 h light/12 h dark cycle; 10–15 air changes per hour). The animals received standard certified commercial dog food (400 g Wayne certified Dog Chow No. 8727) 4 h postdose over a 2-h feeding period.

Four female dogs received I as a single oral dose by gavage via an intubation tube at 1.0 mg/kg in aqueous solution (0.1 ml/kg) adjusted to pH 3 with 0.1 *M* hydrochloric acid. Blood samples (1 ml) were collected from the jugular vein by venipuncture at predose and at 15, 30, 45 min, 1, 2, 3, 5, 7 and 9 h postdose into tubes containing EDTA. The plasma was harvested following centrifugation (for 15 min at 2100 *g*). Plasma samples were stored frozen at −20°C prior to analysis for I and II.

3. Results and discussion

Solid-phase extraction as a sample cleanup procedure was preferred over the alternative liquid–liquid extraction to avoid the use of large volumes of nonpolar organic solvents (e.g. toluene, ethyl acetate) required for the assay of I and II. A simple solid-phase extraction utilizing 3-ml C-8 Bakerbond (J.T. Baker, Phillipsburg, NJ, USA) columns, preconditioned with methanol followed by 0.1 *M* sodium hydroxide, and methanol elution yielded excellent recoveries (> 98% for both I and II). The limit of quantitation for the preliminary assay was 50 ng/ml for each compound. However, the potency of the compounds necessitated better sensitivity for their complete pharmacokinetic characterization. A modified extraction and cleanup procedure utilizing 10-ml LRC reservoir Bond Elut Certify I columns, preconditioned with methanol and isotonic sodium phosphate (0.033 *M*) buffer at pH 7.4, a tetrahydrofuran rinse step and an elution mixture containing methylene chloride, N-isopropyl alcohol and 1% ammonium hydroxide (80:20:2, v/v) was optimized and adapted to yield the desired sensitivity of 10 ng/ml.

3.1. Selectivity

Fig. 2a,b show representative chromatograms obtained from dog plasma blank and dog plasma blank containing I, II and I.S. No interference from endogenous substances in plasma was detected at the retention times (t_R) of I (t_R = 6.6 min), II (t_R = 3.2 min) or I.S. (t_R = 9.3 min). Fig. 2c depicts a chromatogram from a 2-h sample after an oral 1.0 mg/kg dose of I in a dog. The chromatographic peaks of the three compounds were well resolved. Standard curves for I and II assay in dog plasma were linear between 10 and 5400 ng/ml using 0.5 ml of dog plasma. The slope and *y*-intercept values for a representative calibration curve of I were 0.008 and −0.027, respectively. Whereas, the slope and *y*-intercept

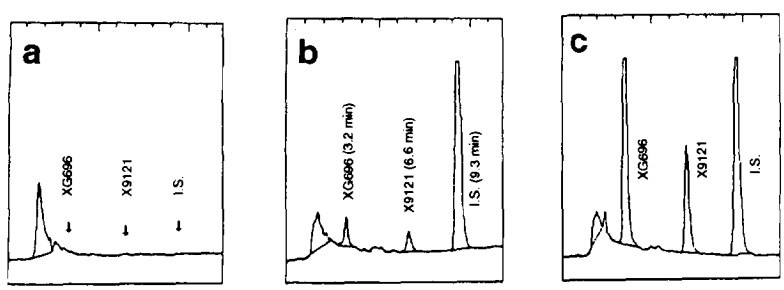

Fig. 2. Representative chromatograms of extracted samples; (a) 0.5 ml of control dog plasma; (b) 0.5 ml of control dog plasma spiked with 5 ng of I and II, and 1 μg of I.S.; and (c) 0.5 ml of dog plasma at 2 h after receiving a single 1.0 mg/kg oral dose of I. The found concentrations of I and II were 40 and 309 ng/ml, respectively. The *y*-axis represents absorbance at 313 nm (one full scale deflection = 10 mV); the *x*-axis represents time after injection (each tick on the *x*-axis = 1 min).

values for a representative calibration curve of II were 0.010 and −0.026, respectively. The coefficient of determination of calibration curves for the dog plasma assay of I and II usually exceeded 0.99.

3.2. Intra-day reproducibility

Dog plasma blanks spiked with I and II were prepared in replicates of 6 in concentrations ranging from 10 to 5400 ng/ml for I and II. The samples were extracted and analyzed on the same day. The results are summarized in Table 1. The coefficients of variation (C.V.) for the intra-day results ranged from 2.2 to 8.5% and from 2.5 to 9.8% for I and II, respectively.

3.3. Inter-day reproducibility

Dog plasma blanks spiked with I and II in the concentration range of 10 to 5400 ng/ml for both compounds were prepared fresh and analyzed on three different occasions. The measured concentrations of each analyte from these three studies are shown in Table 2. The C.V.s ranged from 2.6 to 9.0% and from 3.6 to 16.2% for I and II, respectively.

3.4. Extraction recovery

The extraction recovery was determined by comparing the peak heights of extracted and unextracted standards of I and II. The recoveries of I from 0.5-ml dog plasma at 50 and 600 ng/ml were 74.6 and 83.5%, respectively. The recoveries of II at similar concentrations were 75.6 and 80.3%.

3.5. Accuracy

The results from the accuracy studies in which unknown concentrations of I and II in dog plasma were measured are also shown in Tables 1 and 2. The percent difference between the spiked and found concentrations during intra-day ranged from −5.0 to 11.0% and from −12.5 to 7.5% for I and II, respectively, over the entire validated concentration range of the assay. The percent difference between the spiked and found concentrations during inter-day ranged from −5.6 to 6.3% and from −5.4 to 4.5% for I and

Table 1
Intra-day precision for the quantitation of I and II in dog plasma

Added concentration (ng/ml)	Found concentration[a] of I (ng/ml)	C.V.[b] (%)	Difference[c] (%)	Found concentration[a] of II (ng/ml)	C.V.[b] (%)	Difference[c] (%)
10	11.10 ± 0.24	2.2	11.0	10.63 ± 0.71	6.6	6.3
25	26.5 ± 2.1	8.0	6.0	25.3 ± 2.0	7.9	1.2
50[d]	49.4 ± 3.4	6.8	1.2	51.1 ± 5.0	9.8	2.2
100	103.2 ± 3.4	3.2	3.2	103.5 ± 3.4	3.3	3.5
200	210 ± 16	7.9	5.0	215 ± 17	7.9	7.5
600[e]	570 ± 48	8.5	−5.0	551 ± 14	2.5	−8.2
1800	1730 ± 96	5.5	−3.9	1700 ± 140	8.0	−12.50
5400	5400 ± 150	2.7	0.0	5410 ± 220	4.1	0.2

[a] Values are mean ± S.D., $n = 6$.
[b] Coefficient of variation = (S.D./mean) · 100.
[c] % Mean difference = 100 · (mean of found − added)/added.
[d] Extraction recoveries of I and II were 74.6 and 75.6%, respectively.
[e] Extraction recoveries of I and II were 83.5 and 80.3%, respectively.

Table 2
Inter-day precision for the quantitation of I and II in dog plasma

Added concentration (ng/ml)	Found concentration[a] of I (ng/ml)	C.V.[b] (%)	Difference[c] (%)	Found concentration[a] of II (ng/ml)	C.V.[b] (%)	Difference[c] (%)
10	10.45 ± 0.89	8.5	4.5	10.44 ± 0.73	7.0	4.4
25	23.9 ± 1.8	7.5	−4.4	23.65 ± 0.88	3.7	−5.4
50	47.2 ± 3.0	6.4	−5.6	47.3 ± 3.3	7.0	−5.4
100	102.6 ± 2.7	2.6	2.6	103.9 ± 9.0	8.7	3.9
200	194 ± 17	9.0	−3.0	209 ± 33	16.2	4.5
600	617 ± 29	4.7	2.8	599 ± 41	6.9	0.2
1800	1790 ± 84	4.7	−0.6	1790 ± 97	5.4	−0.6
5400	5740 ± 310	5.2	6.3	5550 ± 210	3.6	2.8

[a] Values are mean ± S.D., $n = 6$.
[b] Coefficient of variation = (S.D./mean) · 100.
[c] % Mean difference = 100 · (mean of found − added)/added.

II, respectively, over the entire validated concentration range of the assay.

3.6. Stability

Triplicate stability samples of I and II were analyzed at concentrations of 50, 200 and 1800 ng/ml on days 1, 15 and 67. Both I and II appeared to be stable in dog plasma, stored frozen at −20°C, for at least 67 days.

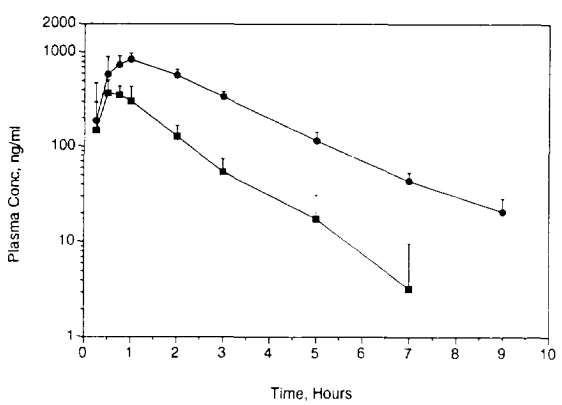

Fig. 3. Plasma concentration–time profiles of I (■) and II (●) in female beagle dogs following a single 1.0 mg/kg oral dose of I (mean ± S.D., $n = 4$).

3.7. Dog pharmacokinetic study

The mean plasma concentration–time curves of I and II in female dogs following a single oral dose of I at 1.0 mg/kg is illustrated in Fig. 3. The mean terminal elimination half-lives of I and II were 65 and 87 min, respectively. Plasma concentrations of I and II could be quantified up to 7 and 9 h, respectively, after oral dosing of I at 1.0 mg/kg. These results indicate that the assay is sufficiently sensitive and specific to be used in preclinical investigations of I and II.

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