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Short Communication

Determination of AJ-3941, a possible agent for the treatment of cerebrovascular disorders, in plasma and brain by means of high-performance liquid chromatography with fluorescence detection

Masuo Kurono, Koji Yoshida and Shunsuke Naruto

Exploratory Research Laboratories, Dainippon Pharmaceutical Co., Ltd., 33-94 Enoki-cho, Suita, Osaka 564 (Japan)

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic method with fluorescence detection is described for the determination of AJ-3941 (I), a possible agent for the treatment of cerebrovascular disorders, in plasma and brain tissue. A simple hexane extraction was used for plasma, and for brain homogenate the hexane extract was further purified by solid-phase extraction. The determination limit was *ca.* 3 ng/ml for both plasma (0.5 ml) and 10% (w/v) brain homogenate (1 ml). The method was applied to the determination of I in plasma and brain samples of experimental animals.

INTRODUCTION

(±)-(*E*)-1-(3-Fluoro-6,11-dihydrodibenz[*b*,*e*]-oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine dimaleate (I, AJ-3941, Fig. 1) [1] is a new agent with possible potential for cerebrovascular disorders [1,2]. The compound has several pharmacological activities, such as calcium antagonism, anti-hypoxia, anti-lipid peroxidation and anti-convulsion. In addition, it is expected that I is devoid of extrapyramidal symptoms which have been reported in some calcium antagonists [2,3].

Correspondence to: Dr. M. Kurono, Exploratory Research Laboratories, Dainippon Pharmaceutical Co. Ltd., 33-94 Enoki-cho, Suita, Osaka, 564, Japan.

This paper deals with a sensitive and selective determination method for I in plasma and brain samples by means of reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. The clean-up was based on a liquid–liquid extraction with hexane, and a solid-phase extraction was subsequently used with brain homogenates. The application of this method to a single-dose pharmacokinetic study in animals demonstrates its utility.

EXPERIMENTAL

Materials

I and (±)-(*E*)-1-(3-fluoro-6,11-dihydrodibenz-[*b*,*e*]oxepin-11-yl)-4-(3-(4-fluorophenyl)-2-propenyl)piperazine (II, internal standard, Fig. 1) were

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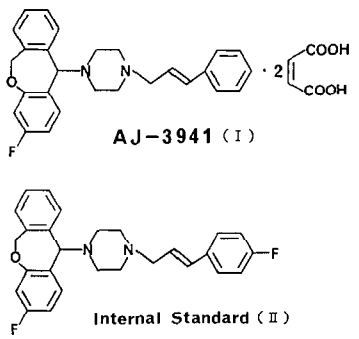

Fig. 1. Structures of I (AJ-3941) and II (internal standard).

synthesized in our laboratories [1]. Extrelut-1 columns and Bond Elut NH_2 cartridges (500 mg/2.8 ml) were obtained from Merck (Darmstadt, Germany) and Analytichem International (Harbor City, CA, USA), respectively. HPLC-grade acetonitrile and distilled water were purchased from Wako (Osaka, Japan). Other chemicals were of analytical grade.

Apparatus and chromatographic conditions

The chromatographic system consisted of a 1090L liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), an RF-535 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan) and an LC100W/F PC workstation with an LC100A A/D converter (Yokogawa, Tokyo, Japan). An STR ODS-H column (5 μm, 15 cm × 4 mm I.D., Shimadzu Techno-Research, Kyoto, Japan) was used and chromatography was carried out at 35°C. Acetonitrile–40 m*M* sodium phosphate buffer (pH 5.0) (52:48, v/v), which was degassed by bubbling helium, was used as the mobile phase at a flow-rate of 0.7 ml/min. The following wavelengths were used: excitation at 259 nm, emission at 316 nm.

Analytical procedure

Plasma samples. A 0.5-ml volume of plasma was pipetted into a test-tube. In the case of mouse plasma, blank plasma was added to the sample to make a total volume of 0.5 ml. Then 0.7 ml of 0.2 *M* carbonate buffer (pH 10) containing 70 ng of II was added and vortex-mixed. The mixture was applied to an Extrelut-1 column. After standing at room temperature for 15 min, I and II were eluted with 6 ml of hexane. The eluate was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 100 μl of methanol, and an aliquot of 15 μl was injected into the HPLC system.

Brain samples. Brain tissues were homogenized in nine volumes (v/w) of 0.2 *M* carbonate buffer (pH 10) at 0°C. A 1-ml volume of the homogenate was transferred to a glass-stoppered tube, then 3 ml of 0.2 *M* carbonate buffer (pH 10) containing 70 ng of II were added and extracted twice with 4 ml of hexane by shaking for 5 min followed by centrifugation at 1500 *g* for 5 min. The combined hexane layers were concentrated to *ca.* 2 ml in a gentle stream of nitrogen at room temperature, and the concentrated solution was passed through a Bond Elut NH_2 cartridge. After the cartridge was washed with 6 ml of 2-propanol–hexane (0.5:99.5, v/v), I and II were eluted with 3 ml of 2-propanol–hexane (4:96, v/v). The eluate was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 100 μl of methanol, and an aliquot of 15 μl was injected into the HPLC system.

RESULTS AND DISCUSSION

Extraction and purification

In the case of plasma samples, diluted plasma (pH 10) was first applied to a liquid–liquid extraction column, Extrelut-1. Several extraction solvents were evaluated for better recovery of I and II from an Extralut-1 column. Hexane or heptane extraction gave adequate recoveries and good chromatograms free from interfering plasma contaminants. Since hexane was more favourable for evaporation, hexane elution was selected as the best extraction process. The absolute recovery of I from plasma was 74 ± 7%, based on the comparison of the HPLC peak areas of the spiked plasma samples with those of methanolic standard solution.

Since the Extrelut-1 column extraction method was not applicable to tissue homogenates containing particulate substances, the clean-up of 10% brain homogenate samples was carried out

by a conventional liquid–liquid extraction. In order to raise the recovery of I and the reproducibility of the extraction procedure the homogenate was extracted twice with hexane. However, the hexane extract gave so many interfering peaks on the chromatogram that it needed to be further purified. Although re-extraction into an acidic aqueous layer followed by back-extraction into an organic layer, or an ion-exchange method using a strong cation-exchange resin under acidic conditions, has usually been applied to the purification of basic compounds such as flunarizine and cinnarizine [4–7], these methods were unfavourable for I because it is partially decomposed in strong acidic conditions. Thus, the hexane extract was further purified by use of a polar solid-phase cartridge. In order to find selective separation conditions, several polar solid-phase cartridges and elution solvents were examined. Consequently, an NH_2 cartridge was found to be excellent for reducing the amount of contaminants when 2-propanol–hexane was used as the elution solvent. After being washed with 2-propanol–hexane (0.5:99.5, v/v), both I and II were selectively eluted with 2-propanol–hexane (4:96, v/v); the recovery of I was *ca.* 93%. The overall absolute recovery in this procedure was *ca.* 87 ± 8%.

Calibration graph

Blank plasma (0.5 ml) was spiked with I at concentrations ranging from 2 to 200 ng/0.5 ml, and II at a fixed amount of 70 ng. In a similar way, 10% blank brain homogenate (1 ml) spiked with I in the range 4–300 ng/ml was assayed according to the procedure described above. The calibration graphs obtained by plotting the peak-height ratios *versus* concentrations were linear in this concentration range. The equations were $y = (0.0087 \pm 0.0001)x + (0.0336 \pm 0.0122)$ ($r = 0.999$) and $y = (0.0092 \pm 0.0001)x + (0.0321 \pm 0.0108)$ ($r = 0.999$) for plasma and 10% brain homogenate, respectively.

Precision and accuracy

A within-day variation was evaluated at concentrations of 4 and 400 ng/ml for plasma samples and 4 and 300 ng/ml for 10% brain homogenate samples. The precision and accuracy were ascertained by replicate analyses of each concentration. As shown in Table I, the coefficient of variation (C.V.) values were 1–2% for the higher concentrations and *ca.* 11% at the lower concentration.

Chromatography

Typical HPLC profiles of a dog plasma sample (dose 3 mg/kg), a mouse 10% brain homogenate sample (dose 30 mg/kg) and a rat blank plasma spiked only with II are shown in Fig. 2. The retention times of I and II were 14.5 and 15.8 min, respectively. The effective clean-up procedure and the selective fluorescence monitoring eliminate interfering peaks. The detection limit of I was 1.5 ng/ml (signal-to-noise ratio = 2) in both

TABLE I

PRECISION AND ACCURACY OF DETERMINATION OF I IN BIOLOGICAL FLUIDS

Biological matrix	Concentration added (ng/ml)	Concentration observed (mean ± S.D.) (ng/ml)	C.V. ($n=6$) (%)	Accuracy[a] (%)
Plasma samples	4.0	4.12 ± 0.46	11.2	3.0
(0.5 ml)	400.0	403.46 ± 4.82	1.2	0.9
10% Brain homogenate	4.0	3.91 ± 0.44	11.4	−2.3
samples (1 ml)	300.0	300.34 ± 6.80	2.3	0.1

[a] Accuracy = $\frac{\text{observed} - \text{added}}{\text{added}} \times 100$ (%).

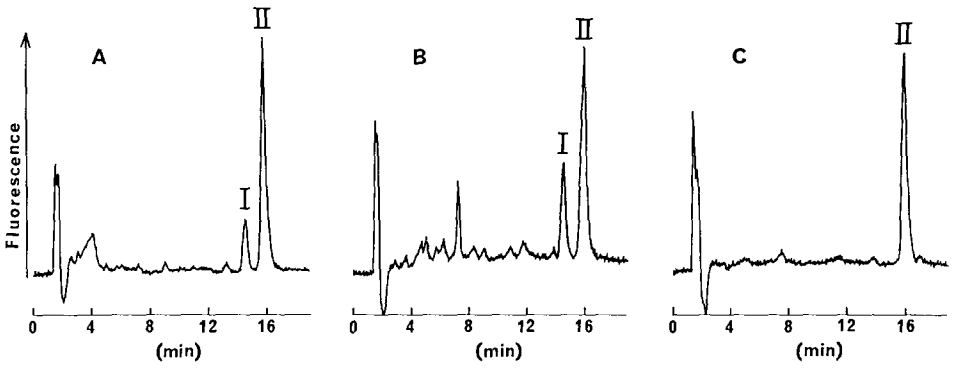

Fig. 2. HPLC of extracts from (A) a dog plasma sample at 2 h (I, 16 ng per 0.5 ml) after oral administration of 3 mg/kg I, (B) a 10% mouse brain homogenate at 24 h (I, 26 ng/ml) after oral administration of 30 mg/kg I and (C) a rat blank plasma spiked only with II.

plasma (0.5 ml) and 10% brain homogenate (1 ml). The determination limit of I was *ca.* 3 ng/ml when a C.V. of 15% is taken as the acceptance criterion, which is below the level expected in biological specimens from experimental animals.

Application to biological samples

The proposed method was applied to the determination of I in plasma and brain samples. Fig. 3 shows the mean plasma and brain levels of I in mice after a single oral dose of I at 30 mg/kg (n = 3). The maximum plasma level was attained 1 h after administration, and thereafter the plasma level declined slowly with an elimination half-life of 3.5 h. The brain level was maximal 2 h after dosing, and *ca.* twice as high as the corresponding plasma levels from 2 to 24 h.

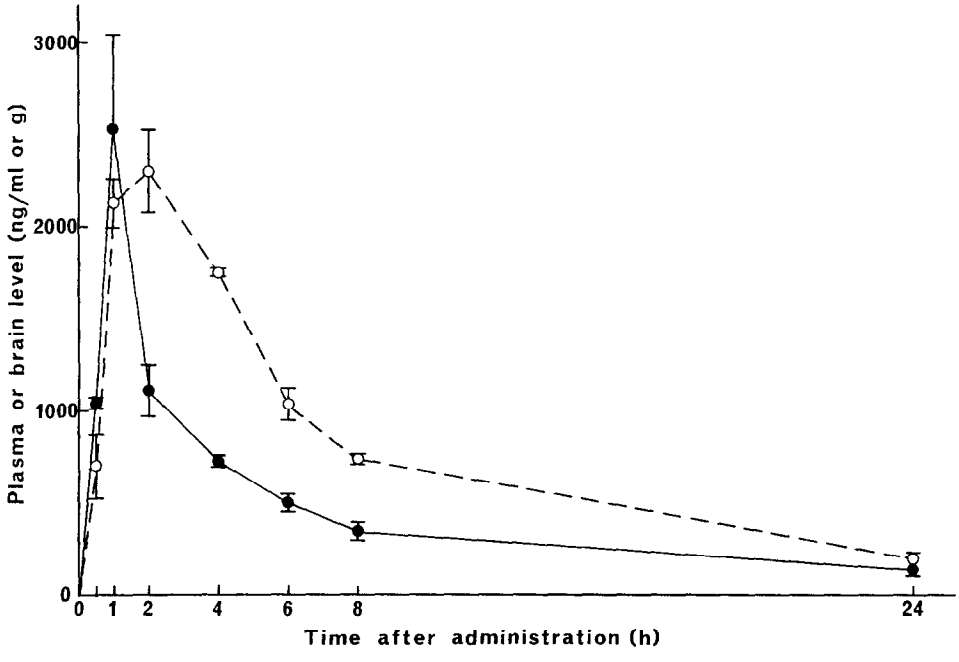

Fig. 3. Mean plasma (●) and brain (○) levels of I in three mice following a single oral administration of 30 mg/kg I. Each point represents the mean ± standard error for three mice.

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