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Short communication

Simultaneous determination of a novel calcium entry blocker, monatepil maleate, and its metabolites in rat plasma by means of solid-phase extraction and reversed-phase liquid chromatography with electrochemical detection

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Abstract

A reversed-phase LC method with electrochemical detection is described for the simultaneous determination of monatepil maleate (AJ-2615, AJ), a novel calcium entry blocker, and its three S-oxidized metabolites in plasma. These compounds were extracted from plasma by solid-phase extraction and injected onto an ODS column. The determination limit in plasma (0.5 ml) was 10 ng/ml for AJ and 5 ng/ml for the three metabolites. The method was applied to the determination of AJ and the metabolites in rat plasma samples.

Keywords: Monatepil maleate

1. Introduction

Monatepil maleate, (±)-*N*-(6,11-dihydrodibenzo[*b*,*e*]thiepin-11-yl)-4-(4-fluorophenyl)-1-piperazinebutanamide monomaleate (AJ-2615, AJ, Fig. 1) [1], is a novel calcium entry blocker with α_1-adrenoceptor antagonistic properties. AJ exhibits gradual and long-lasting antihypertensive activity and myocardial protective effects in experimental models [2–4]. A determination method for AJ in plasma by reversed-phase liquid chromatography with electrochemical detection (LC–ED) has been developed in our laboratories [5]. Its S-oxidized metabolites [a sulfoxide A (I), a sulfoxide B (II) and a sulfone (III), Fig. 1], which may contribute to the pharmacological effects in vivo [6], have been identified in rat plasma by tandem mass spectrometry [7].

This paper describes a sensitive and simple method for the simultaneous determination of AJ and the three S-oxidized metabolites in rat plasma by means of a solid-phase extraction and LC–ED method. The application of this method to a single-dose pharmacokinetic study in rats demonstrates its utility.

2. Experimental

2.1. Materials and reagents

Monatepil maleate (AJ), the sulfoxide A (I) [(5*R**,11 *R**)-*N*-(6,11-dihydrodibenzo [*b*,*e*]thiepin-

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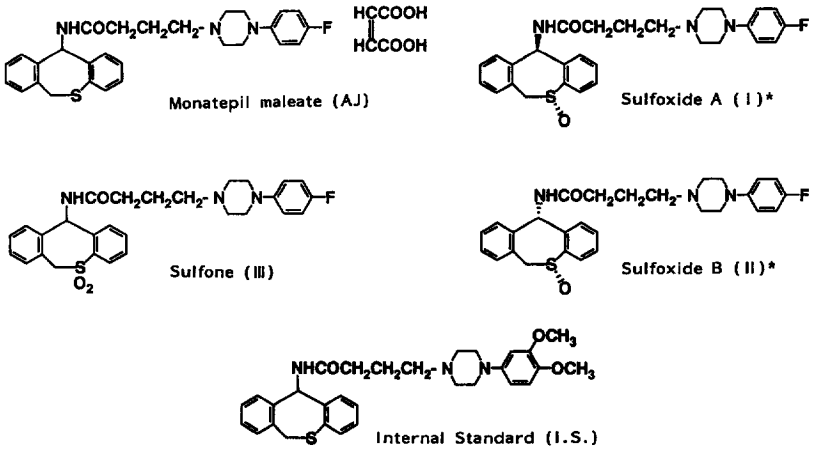

Fig. 1. Structures of monatepil maleate (AJ), its S-oxidized compounds (I, II and III) and the internal standard (I.S.). * Sulfoxides are shown in relative configuration.

11-yl)-4-(4-fluorophenyl)-1-piperazinebutanamide 5-oxide], the sulfoxide B (II) [(5*R**,11*S**)-*N*-(6,11-dihydrodibenzo[*b*,*e*]thiepin-11-yl)-4-(4-fluorophenyl)-1-piperazinebutanamide 5-oxide], the sulfone (III) [(±)-*N*-(6,11-dihydrodibenzo[*b*,*e*]thiepin-11-yl)-4- (4-fluorophenyl) -1-piperazinebutanamide 5,5-dioxide] and the internal standard (I.S.) [(±)-*N*-(6,11-dihydrodibenzo[*b*,*e*]thiepin-11-yl)-4-(3,4-dimethoxyphenyl)-1-piperazinebutanamide) (Fig. 1) were synthesized in our laboratories [8]. Sep-Pak Vac C_{18} cartridges (500 mg, Part No. WAT020805) were obtained from Waters (Milford, MA, USA). Bovine serum albumin (BSA, fraction V, essentially fatty acid free) was purchased from Sigma (St. Louis, MO, USA). LC-grade acetonitrile and distilled water were from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were of analytical grade. Acetate buffer (0.2 *M*) containing 0.2% (w/v) BSA was prepared by dissolving 16.4 g of sodium acetate in 1 l of distilled water, then adjusting to pH 6.0 with acetic acid, and adding 2 g of BSA to this solution. Phosphate buffer (20 m*M* and 50 m*M*) pH 6.0 was prepared from equimolar solutions of sodium dihydrogenphosphate and disodium hydrogenphosphate. Citrate buffer (0.1 *M*) was obtained by dissolving 21.0 g of citric acid monohydrate in 1 l of distilled water, and adjusting to pH 3.0 with 1 *M* sodium hydroxide. The mobile phase was prepared by mixing 330 ml of acetonitrile and 670 ml of 50 m*M* phosphate buffer containing 0.1 m*M* disodium EDTA that was pre-filtered through a 0.45-μm cellulose acetate filter (Advantec Toyo, Tokyo, Japan).

2.2. Animals and sample collection

Ten male Sprague–Dawley rats (seven weeks old, body weight 269–303 g, Clea Japan, Tokyo, Japan) were used. They were orally administrated with 30 mg/kg AJ as a suspension in 0.5% (w/v) aqueous tragacanth (a gummy exudation from *Astragalus* species; used as a suspending agent for drugs) solution, and supplied with water and food ad libitum. The rats were divided into two groups of five animals, and plasma samples at 0.5, 3 and 9 h after administration were collected from one group and at 1, 6 and 24 h from the other group. All samples were stored at −20°C until taken for assay.

2.3. Preparation of stock and working solutions

Fresh stock solutions of AJ, I, II, III and of I.S., 0.2 mg/ml, were prepared weekly in acetonitrile and stored at −20°C. The working solution of the analytes (AJ, I, II and III), 5 μg/ml of each compound, was prepared from the stock solutions by

diluting with 0.2 *M* acetate buffer (pH 6.0) containing 0.2% (w/v) BSA and the working solution of I.S., 200 ng/ml, by diluting with the acetate buffer.

2.4. Analytical procedure

To a 10-ml test-tube, 0.5 ml of plasma, 0.5 ml of the working solution of I.S. and 1 ml of 0.1 *M* sodium hydroxide were added. The mixture was vortexed for 15 s and centrifuged for 3 min at 1500 *g*. The supernatant was passed through a Sep-Pak Vac C_{18} cartridge, which was preconditioned by successive washings with 3 ml of methanol and 10 ml of distilled water. The cartridge was then washed with 10 ml of distilled water and subsequently with 3 ml of 20 m*M* phosphate buffer (pH 6.0)–methanol (50:50, v/v). After being dried under reduced pressure, the cartridge was placed on a glass-stoppered 10-ml tube. Analytes in the cartridge were eluted with 3 ml of 0.1 *M* citrate buffer (pH 3.0)–methanol (30:70, v/v). The eluate was made basic with 1 ml of 1 *M* sodium hydroxide and extracted with 3 ml of chloroform by shaking for 10 min, followed by centrifugation at 1500 *g* for 5 min. The organic layer (3 ml) was transferred to another tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 80 μl of 20 m*M* phosphate buffer (pH 6.0)–acetonitrile (40:60, v/v) and an aliquot of 20 μl was injected onto the column.

2.5. Chromatographic conditions

The LC–ED system consisted of a 1090L liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) and an L-ECD-6A electrochemical detector with a glassy carbon electrode set at +0.9 V vs. Ag/AgCl (Shimadzu, Kyoto, Japan). Chromatograms were recorded on an LC100W/F PC workstation with an LC100A A/D converter (Yokogawa, Tokyo, Japan). A YMC-Pack ODS-AM column (5 μm, 15 cm×6 mm I.D., Yamamura Chemical, Kyoto, Japan) was used at 37°C. Acetonitrile–50 m*M* phosphate buffer (pH 6.0) containing 0.1 m*M* disodium EDTA (33:67, v/v) was used as the mobile phase at a flow-rate of 1 ml/min. The detection was performed at ambient temperature.

2.6. Recoveries and calibration curves

The recoveries of the analytes from plasma were determined by comparing their peak heights obtained from spiked samples with those obtained from equivalent standard solutions without extraction. Plasma calibration standards were prepared by appropriately diluting the working solution of the analytes with blank plasma to give concentrations ranging from 2 to 400 ng/ml, and extracted and analyzed as mentioned above. The peak-height ratios (y, each analyte/I.S.) and the concentrations (x) were used for calculation of the calibration curves. A weighting factor of $1/x$ was applied in the least squares regression analysis.

3. Results and discussion

3.1. Optimization of solid phase extraction procedure

The retention of the S-oxidized compounds on C_{18} cartridge was not satisfactory under acidic conditions (pH 4.0) described in the earlier papers [5,7]. In order to retain these compounds as well as AJ, the quantity of cartridge sorbent was increased to 500 mg and the plasma sample was made basic before being applied onto the cartridge. Despite successive washing with water and phosphate buffer (pH 6.0)–methanol (50:50, v/v), no analytes could be lilberated from the cartridge. These compounds were well eluted with 0.1 *M* citrate buffer (pH 3.0)–methanol (30:70, v/v) and further purified by chloroform extraction under basic conditions.

3.2. Electrochemical detection and LC separation

Under the present conditions AJ and the S-oxidized compounds are all electrochemically detectable. From the electrochemical behavior of related ("fragment") compounds of AJ, it has become apparent that the 4-fluorophenylpiperazine moiety would be electrochemically active.

The pH of the mobile phase was set to 6.0 in order

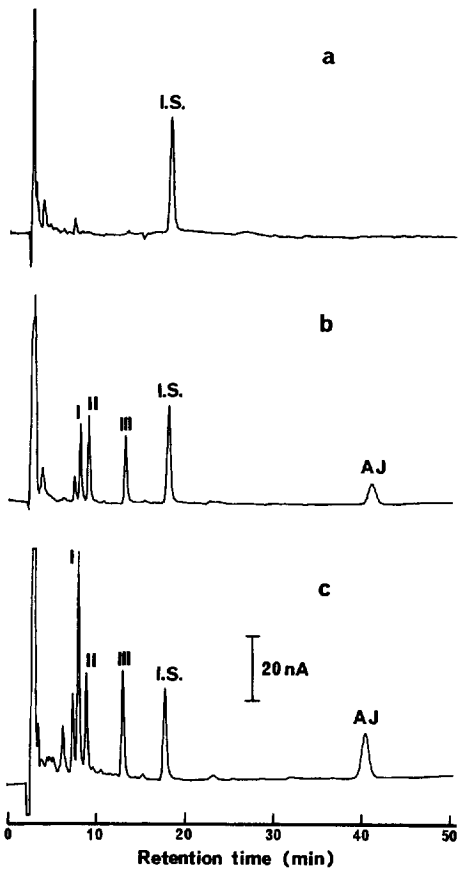

Fig. 2. Representative liquid chromatograms for extracts from (a) blank plasma spiked only with I.S. (I.S., 200 ng/ml), (b) plasma calibration standard (AJ, I, II and III, 100 ng/ml; I.S., 200 ng/ml) and (c) plasma sample at 6 h after a single oral administration of 30 mg/kg AJ (AJ, 233 ng/ml; I, 296 ng/ml; II, 114 ng/ml ; III, 160 ng/ml; I.S., 200 ng/ml).

to obtain sharp, well-resolved chromatographic peaks of the diastereomeric sulfoxides (I and II). Typical chromatograms of extracts from a blank plasma, a plasma calibration standard and a plasma sample are shown in Fig. 2. The separation of the sulfoxides was satisfactory and blank plasma extracts did not show any interfering peaks. The retention times for I, II, III, I.S., and AJ were 8.0, 9.0, 13.1, 18.0 and 40.6 min, respectively.

3.3. Stability of the analytes during the analytical procedure

For the survey of the interconversion among I, II, III and AJ during the assay, extracts from plasma samples (0.5 ml) spiked with each analyte were injected into the LC–ED system. Values for the relative ratios of their peak intensities are given in Table 1. These data indicate that only a slight S-oxidation occurred in AJ and I, and a little interconversion of I and II was accompanied during the procedure. However, such a variation in quantity should not lead to serious errors in determinations.

3.4. Recoveries and calibration curves

The overall absolute recoveries ($n=5$) of I, II, III and AJ were 93±3, 89±3, 86±2 and 84±4% at 400 ng/ml plasma, and 95±3, 89±1, 87±2 and 79±4% at 50 ng/ml plasma. Calibration curves were linear in the range 2–400 ng/ml for all analytes. The linear regression equations and the correlation coefficients (for $n=19$–23) were as follows: I, $x=(99.54\pm2.09)y-(1.172\pm0.598)$ ($r=0.992$); II, $x=(95.01\pm1.99)y-(1.082\pm0.598)$ ($r=0.993$); III, $x=(129.6\pm2.7)y-(1.512\pm0.600)$ ($r=0.993$); AJ, $x=(367.1\pm7.8)y-(2.446\pm0.799)$ ($r=0.993$).

Table 1
Interconversion of each analyte during the procedure ($n=5$)

Spiked compound (at 400 ng/ml)	Mean±S.D. of detected compound (% in relative intensity)			
	AJ	I	II	III
AJ	99.5±0.4	0.1±0.1	0.5±0.2	N.d.
I	N.d.	93.5±3.0	6.5±2.5	0.9±1.1
II	N.d.	7.8±2.1	92.2±2.0	≤0.1
III	N.d.	N.d.	N.d.	100

N.d.=not detected.

Table 2
Intra-assay precision and accuracy of the present method ($n=5$)

Analyte	Spiked concentration (ng/ml)	Measured concentration (ng/ml)		
		Mean±S.D.	C.V.[a](%)	Accuracy[b](%)
I	4.48	4.52±0.62	13.8	+0.9
	28	30.9±2.8	9.1	+10.2
	400	389±28	7.2	−2.6
II	4.48	4.83±0.71	14.7	+7.8
	28	30.1±3.8	12.6	+7.6
	400	389±30	7.6	−2.7
III	4.48	4.85±0.57	11.7	+8.3
	28	29.3±4.3	14.7	+4.8
	400	387±21	5.5	−3.3
AJ	11.2	10.1±1.3[c]	13.0	−10.1
	28	28.7±4.2	14.6	+2.3
	400	373±28	7.5	−6.8

[a] Coefficient of variation: S.D.×100/mean.
[b] Accuracy: (measured−spiked)/spiked×100 (%).
[c] $n=4$.

3.5. Precision and accuracy

By analyzing blank plasma samples spiked with various concentrations of I, II, III and AJ, the intra- and inter-assay variations were evaluated (Tables 2 and 3). Adequate precision and accuracy for pharmacokinetic studies were demonstrated. The determination limits, defined as the lowest concentration within C.V. 15% based on a 0.5-ml plasma sample, were ca. 5 ng/ml for I, II and III, and ca. 10 ng/ml for AJ.

3.6. Application to biological samples

Fig. 3 shows the time courses of the mean plasma levels of I, II, III and AJ in male normal rats ($n=5$) after a single oral administration of 30 mg/kg of AJ. The plasma levels of I, II, III and AJ were maximal around 3 h after the administration. From the differences in plasma levels between the diastereomeric sulfoxides I and II, an oxidative metabolism at the sulfur atom of AJ appears to be stereoselective in rats.

Table 3
Inter-assay precision and accuracy of the present method ($n=3$)

Analyte	Spiked concentration (ng/ml)	Measured concentration (ng/ml)		
		Mean±S.D.	C.V.[a](%)	Accuracy[b] (%)
I	25	25.0±1.9	7.8	−0.2
	400	382±19	5.0	−4.4
II	25	21.8±1.9	8.9	−12.9
	400	373±16	4.2	−6.7
III	25	21.9±2.2	10.1	−12.3
	400	354±16	4.5	−11.6
AJ	25	27.9±1.3	4.5	+11.8
	400	416±13	3.2	+4.1

[a] Coefficient of variation: S.D.×100/mean.
[b] Accuracy: (measured−spiked)/spiked×100 (%).

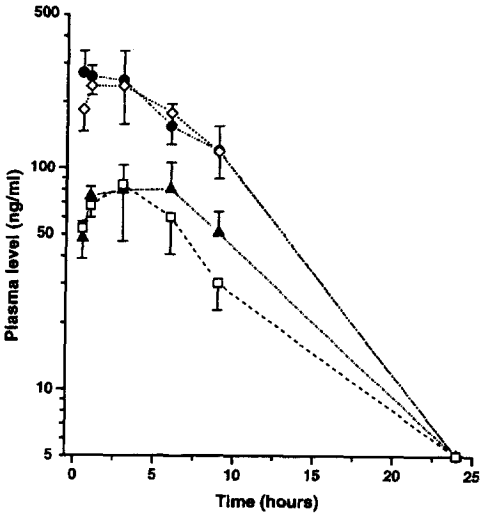

Fig. 3. Mean plasma levels of AJ (●) and its three S-oxidized metabolites I (◇), II (□) and III (▲) in male normal rats after a single oral administration of 30 mg/kg AJ. Each point represents the mean±S.E.M. for five rats.

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