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Enantioselective determination of pazinaclone, a new isoindoline anxiolytic, and its active metabolite in rat plasma by high-performance liquid chromatography

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Abstract

A sensitive and specific high-performance liquid chromatographic method has been developed for the simultaneous determination of the enantiomers of pazinaclone (DN-2327), a new anxiolytic agent, and those of its active metabolite, M-II, in rat plasma. Organic solvent extraction of pazinaclone, M-II, and internal standard (I.S.) in plasma was followed by separation of the analytes from other metabolites using an achiral reversed-phase column. Fluorescence detection was employed with excitation and emission wavelengths of 328 and 367 nm, respectively. Separation of all the enantiomers and I.S. was then accomplished with normal- and chiral-phase columns connected in series. For each analyte, the lower quantitation limit was 0.5 ng/ml. The assay has been applied to a chiral inversion study in rats. Chiral conversion from one enantiomer of pazinaclone to the other hardly occurred. This method is suitable for enantioselective pharmacokinetic and toxicokinetic studies in animals.

1. Introduction

Racemic pazinaclone [DN-2327; (±)-2-(7-chloro-1, 8-naphthyridin-2-yl)-3-[(1, 4-dioxa-8-azaspiro[4.5]dec-8-yl) carbonylmethyl] isoindolin-1-one, Fig. 1)] is a new benzodiazepine receptor ligand, having potent anxioselective activities in several animal species after oral dosage [1–5]. Pazinaclone acts as an agonist with respect to the anticonflict and anti-convulsant effects but as an antagonist to the muscle-relaxant and sedative effects of diazepam. In rats, dogs and monkeys, pazinaclone is metabolized to M-I, M-II, M-III, M-IV and M-V, of which M-II is the predominant pharmacologically active circulating metabolite (Fig. 1) [6,7]. Unpublished results showed that pharmacological activity, measured both *in vitro* and *in vivo* in rats, resides primarily in the *S*-(+)-enantiomers of pazinaclone and M-II [8]. Thus, the enantioselective determination of pazinaclone and M-II in biological fluids is necessary for the characterization of the pharmacodynamic and toxicological effects after administration of racemic pazinaclone. Recently, enantioselective high-performance liquid chromatographic (HPLC) methods for pazinaclone

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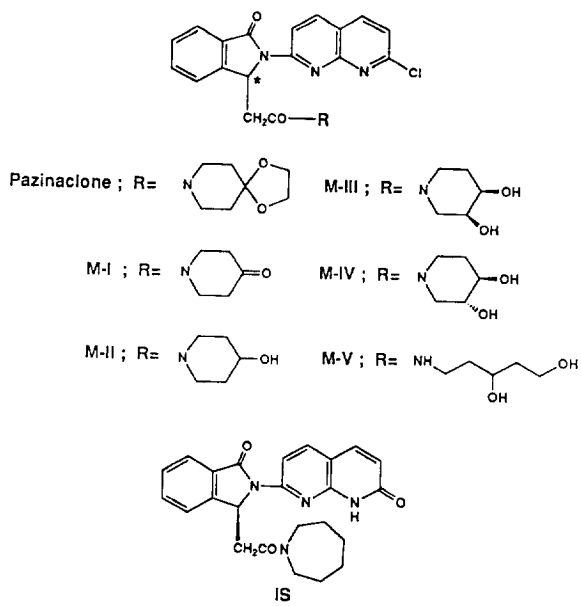

Fig. 1. Chemical structures of pazinaclone metabolites and the internal standard (I.S.). * = Chiral center.

and M-II in dog and human plasma have been reported [9,10]. These methods were, however, not applicable to rat plasma, because M-III, M-IV, M-V and other unidentified metabolites formed predominantly in rats [6] were found to interfere. Described here is a sensitive, specific, and reproducible method for the simultaneous determination of the *S*- and *R*-enantiomers of both pazinaclone and M-II in rat plasma. We also indicate an application, i.e. a chiral inversion study as part of the enantioselective pharmacokinetic studies of pazinaclone and M-II in rats, of this method (the other studies were reported in a separate paper [11]).

2. Experimental

2.1. Reagents

Pazinaclone, *S*-(+)-pazinaclone [100% enantio-excess (ee)], *R*-(−)-pazinaclone (100% ee), M-II [(±)-2-(7-chloro-1, 8-naphthyridin-2-yl)-3-[(4-hydroxypiperidin-1-yl) carbonylmethyl] isoindolin-1-one], *S*-(+)-M-II (100% ee), *R*-(−)-M-II (100% ee) and *S*-(+)-2-[2(1*H*)-oxo-1, 8-naphthylidin-7-yl]-3-[(hexamethyleneimin-1-yl) carbonylmethyl] isoindolin-1-one [internal standard (I.S.); 100% ee, Fig. 1], were synthesized in house by Production Research Laboratories and Pharmaceutical Research Laboratories. HPLC-grade acetonitrile, ethanol, *n*-hexane and dichloromethane, analytical grade diethyl ether and $Na_2HPO_4 \cdot 12H_2O$ were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Instrumentation

The HPLC system (Waters Assoc., Milford, MA, USA) consisted of a Model 600E pump, a WISP 712 automatic sample injector with a cooling system, a Model 470 fluorescence detector and a Model 825 or 840 data processor. A model 2211 Superrac fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden), a column heater U-620 TYPE 30 (Sugai Chemical Industry Co., Wakayama, Japan) and an automated switching valve (Waters Assoc.) were also used.

2.3. Chromatographic conditions

The HPLC columns used were LiChrospher 100 RP-18 (5 μm, 250 × 4 mm I.D.; Merck, Darmstadt, Germany), YMC-Pack SIL-06 (250 × 4.6 mm I.D.; YMC Co., Kyoto, Japan) and Opti-Pak TA (cellulose derivative-type column, 300 × 3.9 mm I.D.; Waters Assoc.). The LiChrospher 100 RP-18 column was maintained at 45°C and the mobile phase was acetonitrile–water (1:1, v/v) at a flow-rate of 1 ml/min. The YMC-Pack SIL-06 and Opti-Pak TA columns were connected in series and maintained at 50°C. The mobile phase was *n*-hexane–ethanol (4:1, v/v) at a flow-rate of 1 ml/min.

In both methods, the eluate was monitored with a fluorescence detector at λ_{EX} 328 nm and λ_{EM} 367 nm.

2.4. Preparation of standard solution

For pazinaclone and M-II, a stock solution (25 μg/ml as enantiomer) was prepared. About 5

mg of each compound were weighed accurately into a 100-ml volumetric flask and dissolved to volume with acetonitrile. This was further diluted 1:100 with acetonitrile to obtain a working solution. A working I.S. solution (100 ng/ml) was obtained by diluting a stock solution (50 μg/ml in acetonitrile) 1:500 into acetonitrile–water (1:1, v/v). The stock solutions were refrigerated at 5°C and used within one month. The working solutions were prepared daily.

2.5. Preparation of standard curves and quality control (QC) samples in rat plasma

The working solution (250 ng/ml each) was evaporated to dryness under a stream of nitrogen at a temperature below 40°C and the residue was dissolved in a drug-free rat plasma, followed by serial dilution with plasma to prepare standard samples with concentrations between 0.5 and 250 ng/ml. Similarly, a QC sample containing 50 ng/ml (high) of each analyte was prepared by evaporating the working solution to dryness under a stream of nitrogen and dissolving the residue in drug-free rat plasma; this was serially diluted to provide QC samples at 10 ng/ml (medium) and 2 ng/ml (low). Samples were stored at −20°C until analysis.

2.6. Extraction

A 0.2-ml aliquot of plasma was mixed with 0.2 ml of 0.05 *M* Na_2HPO_4 solution and 0.05 ml of working I.S. solution, and vortex-mixed. The enantiomers of pazinaclone and M-II, and I.S. were extracted with diethyl ether–dichloromethane (4:1, v/v, 2 ml) using a vortex-mixer. The organic phase was evaporated to dryness, and the residue dissolved in acetonitrile–water (1:1, v/v, 200 μl). This solution (150 μl) was injected onto an HPLC system with the reversed-phase column. The eluate containing pazinaclone, M-II and I.S. (t_R; ca. 4–10 min) was collected together as one fraction by the fraction collector. The retention times of pazinaclone and M-II were checked each day before analysis.

2.7. Chiral separation

The eluate containing pazinaclone, M-II and I.S. was concentrated to a volume of ca. 0.5 ml under a stream of nitrogen at a temperatures below 40°C. The residue was dissolved in 0.05 *M* Na_2HPO_4 solution (0.2 ml) and extracted with diethyl ether–dichloromethane (4:1, v/v, 4 ml). The organic phase was evaporated to dryness and the residue dissolved in *n*-hexane–ethanol (4:1, v/v, 200 μl). The solution (100 μl) was injected onto an HPLC system equipped with YMC-Pack SIL-06 and Opti-Pak TA columns connected in series.

2.8. Calculation

The concentrations of *S*-(+)- pazinaclone, *R*-(−)-pazinaclone, *S*-(+)-M-II, and *R*-(−)-M-II in plasma were calculated with a computer from the standard curve constructed with a weighting factor of 1/conc., using peak-height ratio of analyte to I.S.

Absolute recovery from the plasma spiked with pazinaclone and M-II enantiomers was calculated by the following equation:

$$\text{Recovery (\%)} = [(\text{peak height for treated plasma sample})/(\text{peak height for untreated standard})] \cdot 100$$

The untreated standard samples were prepared by serial dilution of working solution with *n*-hexane–ethanol (4:1, v/v).

The relative error (R.E.) of the observed concentration of analyte to the calculated concentration was also calculated by the following equation:

$$\text{Relative error (\%)} = [(\text{observed conc.} - \text{calculated conc.})/(\text{calculated conc.})] \cdot 100$$

2.9. Application

The assay has been applied to a chiral inversion study. Concentrations of the enantiomers were measured in plasma samples obtained from

male Wistar rats (229–242 g) given a single oral 25 mg/kg dose of either *S*-(+)- or *R*-(−)-pazinaclone. Blood samples were obtained from the abdominal aorta at 0.5, 1 and 4 h after administration.

3. Results and discussion

3.1. Separation of enantiomers

Our attempts to determine simultaneously the enantiomers of pazinaclone and M-II on a chiral column failed. *R*-(−)-Pazinaclone and *R*-(−)-M-II could not be separated under any conditions. To resolve these enantiomers, we connected an achiral normal-phase column and a chiral-phase column in series. Pazinaclone was separated from M-II in the achiral column and the enantiomers of these compounds were resolved on the chiral stationary phase. Fig. 2 shows the complete separation of all the enantiomers of interest.

As reported previously [6], pazinaclone, M-II, M-III, M-IV, M-V and other unidentified metabolites were found in the plasma of rats given an oral dose of pazinaclone. The enantiomers of pazinaclone and M-II, and I.S. were not separated from other metabolites by the normal-phase column connected with the chiral column in a series. However, a reversed-phase column was found to be suitable for separation of these compounds from other metabolites. The eluate containing pazinaclone, M-II and I.S. was collected together and the enantiomers of pazinaclone and M-II, and I.S. were further separated by the normal- and chiral-phase columns.

Fig. 3 shows chromatograms of the analytes extracted from the plasma of rats after oral administration of *S*-(+)-pazinaclone or *R*-(−)-pazinaclone (samples from chiral inversion study described below): Fig. 3A,C, separation of pazinaclone, M-II and I.S. (fraction I) from other metabolites; Fig. 3B,D, separation of the enantiomers.

3.2. Linearity

The standard curves for *S*-(+)-pazinaclone, *R*-(−)-pazinaclone, *S*-(+)-M-II and *R*-(−)-M-II were obtained by the analysis of a standard solution over the sample concentration ranges. The least squares regression fit showed good linearity with correlation coefficients higher than 0.999 for each enantiomer. The limit of quantitation was 0.5 ng/ml for each enantiomer.

The linearity of the standard curves for the four analytes added to rat plasma was reproducible as indicated by coefficients of variation (C.V.) in the slopes of the different standard curves.

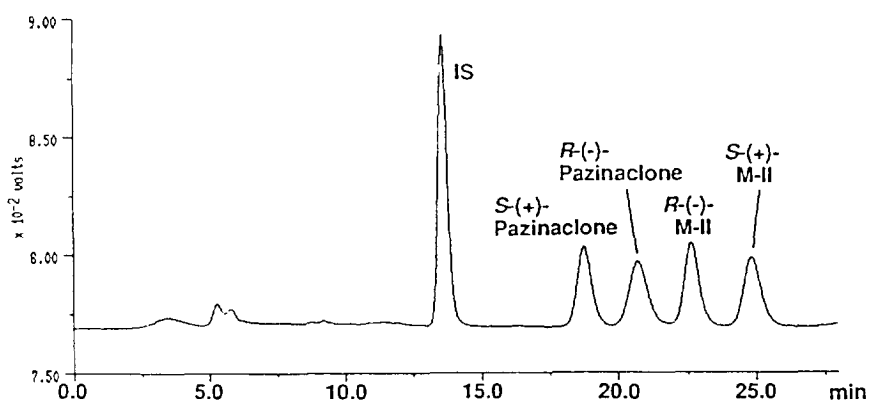

Fig. 2. Typical chromatogram from rat plasma spiked with the enantiomers of pazinaclone and M-II. Analytical conditions: column, YMC-Pack SIL-06 (250 × 4.6 mm I.D.) connected in a series with Opti-Pak TA (300 × 3.9 mm I.D.); flow-rate, 1 ml/min; mobile phase, *n*-hexane–ethanol (4:1, v/v); column temperature, 50°C; detection, fluorescence (λ_{EX} 328 nm, λ_{EM} 367 nm).

Table 1
Intra-day precision and accuracy of pazinaclone and M-II enantiomers in spiked rat plasma

Enantiomer		Concentration (ng/ml)				
		0.5	2	10	50	250
S-(+)-Pazinaclone	Mean	0.48	2.03	10.05	51.7	249
	C.V. (%)	6.3	3.4	0.9	4.1	0.4
	R.E. (%)	4.0	1.5	0.5	3.4	0.4
R-(−)-Pazinaclone	Mean	0.55	1.84	9.72	50.1	250
	C.V. (%)	3.6	4.9	3.4	2.8	0.8
	R.E. (%)	10.0	8.0	2.8	0.2	0.0
S-(+)-M-II	Mean	0.49	2.01	9.98	51.2	249
	C.V. (%)	8.2	7.5	4.6	5.5	1.2
	R.E. (%)	2.0	0.5	0.2	2.4	0.4
R-(−)-M-II	Mean	0.53	1.95	9.67	50.5	250
	C.V. (%)	11.3	11.3	5.4	5.7	1.2
	R.E. (%)	6.0	2.5	3.3	1.0	0.0

Data were calculated from 5 samples. R.E. = relative error.

3.3. Recovery

The recoveries of *S*-(+)-pazinaclone, *R*-(−)-pazinaclone, *S*-(+)-M-II and *R*-(−)-M-II from rat plasma in the concentration range 2–250 ng/ml spiked with standard samples were 87.6–95.8, 89.9–97.4, 80.2–90.6 and 81.7–86.0%, respectively, with reproducibilities of 2.0–3.7, 2.6–4.7, 4.4–7.4 and 4.3–7.1% ($n = 5$), respectively.

3.4. Precision and accuracy

The precision and accuracy of the procedure were checked by analyses of rat plasma spiked with pazinaclone and M-II enantiomers in the range 0.5–250 ng/ml: the precision and accuracy were evaluated by C.V. and R.E., respectively. The intra-day C.V.s of *S*-(+)-pazinaclone, *R*-(−)-pazinaclone, *S*-(+)-M-II and *R*-(−)-M-II were below 6.3, 4.9, 8.2 and 11.3%, respectively (Table 1). The R.E. was less than 10.0% for the four analytes.

The inter-day variabilities associated with the concentrations of 2, 10 and 50 ng/ml indicated that the C.V.s for *S*-(+)-pazinaclone, *R*-(−)-pazinaclone, *S*-(+)-M-II, and *R*-(−)-M-II were within 12.4, 13.5, 10.7 and 6.2%, respectively (Table 2).

3.5. Applications

The described analytical method was used for a chiral inversion study in rats. Plasma concentrations of the enantiomers of pazinaclone

Table 2
Inter-day precision of pazinaclone and M-II enantiomers in spiked rat plasma

Enantiomer		Concentration (ng/ml)		
		2	10	50
S-(+)-Pazinaclone	Mean	1.94	9.33	47.6
	C.V. (%)	12.4	2.8	1.7
R-(−)-Pazinaclone	Mean	1.85	9.22	46.5
	C.V. (%)	13.5	2.7	1.1
S-(+)-M-II	Mean	2.06	9.15	47.3
	C.V. (%)	10.7	5.5	2.3
R-(−)-M-II	Mean	1.94	9.00	46.8
	C.V. (%)	6.2	5.9	2.1

Data were calculated from samples on 3 days.

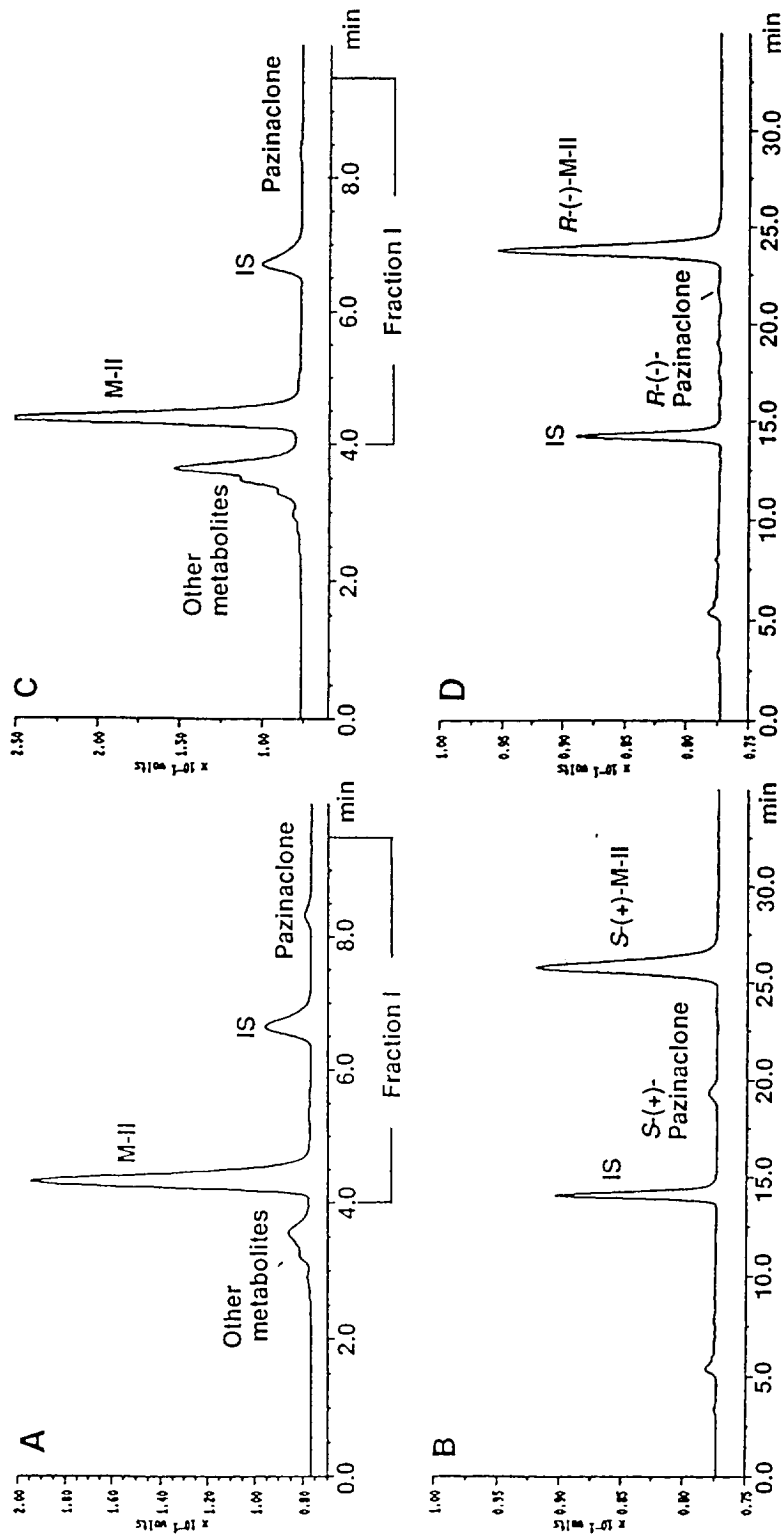

Fig. 3. Typical chromatograms from pazinaclone, M-II and I.S. extracted from rat plasma after an oral dose of *S*-(+)-pazinaclone (A, B) or *R*-(−)-pazinaclone (C, D). Analytical conditions of A and C: column, LiChrosphere 100 RP-18 (250 × 4 mm I.D.); flow-rate, 1 ml/min; mobile phase, acetonitrile–water (1:1, v/v); column temperature, 45°C; detection, fluorescence (λ_{EX} 328 nm, λ_{EM} 367 nm). Analytical conditions of B and D: same conditions as in Fig. 2.

and its metabolite were determined over a 4-h period. After oral administration of *S*-(+)-pazinaclone to rats, concentrations of *R*-(−)-enantiomers of both pazinaclone and M-II were lower than the limit of quantitation (0.5 ng/ml) in all plasma samples; in the plasma of rats given *R*-(−)-pazinaclone, the *S*-(+)-enantiomers of the two compounds could also not be detected (Fig. 4A,B). Thus, it seems unlikely that chiral inversion from one enantiomer of pazinaclone to its antipode occurs in rats.

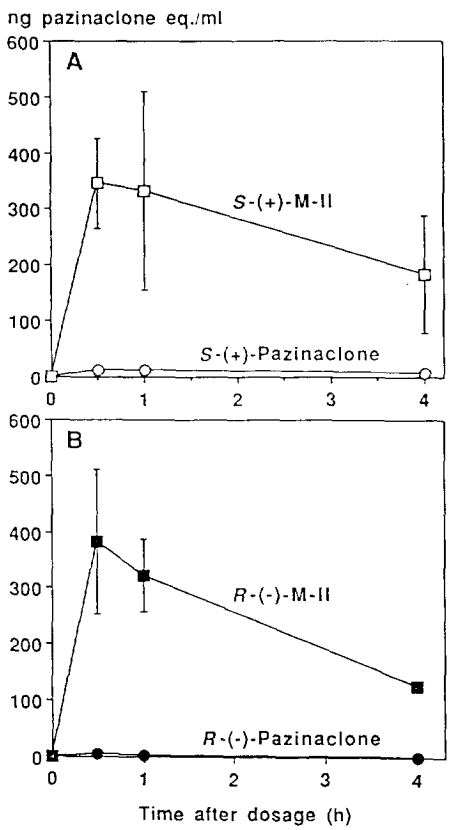

Fig. 4. Concentration of the enantiomers of pazinaclone and M-II in plasma of rats given an oral 25 mg/kg dose of *S*-(+)-pazinaclone (A) or *R*-(−)-pazinaclone (B).

4. Conclusions

This paper describes a reproducible, sensitive and specific HPLC method for determining the enantiomers of pazinaclone and M-II in rat plasma. The method using normal- and chiral-phase columns can also be applied to mouse, dog, and monkey plasma without reversed-phase column separation [11] and is thus very useful for both pharmacokinetic and toxicokinetic studies in animals.

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