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Short Communication

Determination of the enantiomers of a novel 20,21-dinoreburnamenine derivative in rat plasma and brain by high-performance liquid chromatography using a chiral stationary phase

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ABSTRACT

A high-performance liquid chromatographic method with solid-phase extraction was developed for the assay of the enantiomers of a novel 20,21-dinoreburnamenine derivative (RU 49041) in rat plasma and brain using a chiral stationary phase (Nucleosil Chiral 2) and ultraviolet detection. The limit of detection was 10 ng/ml (or ng/g) in both tissues and the intra-assay precision was satisfactory (plasma, *ca.* 5%; brain, *ca.* 1%). The pharmacokinetic profiles of the two enantiomers were determined following oral administration of the racemate (10 mg/kg). The results show that their pharmacokinetics are very different: whereas both enantiomers appear in the brain, only the 3α,16β-enantiomer is detected in plasma.

INTRODUCTION

(±)-(16α)-11-Methyl-20,21-dinoreburnamenine (RU 49041, I, Fig. 1) is a racemic compound which, pharmacologically, has promnesic and anti-amnesic properties [1]. The two optical isomers, RU 52583 (II) (3α,16β) and RU 52582 (III) (3β,16α), have different pharmacological profiles which contribute to the overall activity of the racemate. In order to determine the pharmacokinetics of each enantiomer, it was necessary to develop a stereoselective assay procedure.

Attempts at separating the enantiomers using classical type III (cyclodextrin side-chain) or type V (serum protein side-chain) chiral stationary phases [2] were unsuccessful. A good separation was finally achieved, however, using high-performance liquid chromatography (HPLC) with a Nucleosil Chiral 2 column. Using this method, a preliminary pharmacokinetic study in rat plasma and brain was carried out.

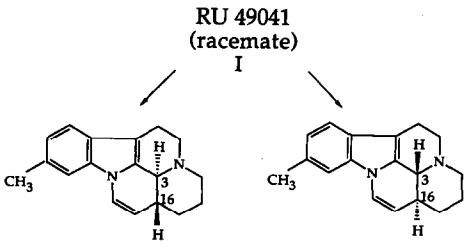

Fig. 1. Structures of the enantiomers of RU 49041 (I).

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EXPERIMENTAL

Chemicals

Compound I, its two enantiomers, II and III, and the internal standard, the 14,15-saturated derivative of II, were synthesized in the Chemistry Department at Roussel Uclaf.

All solvents and reagents were of HPLC or analytical-reagent grade. Water used for the preparation of buffers was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Stock solutions of I, II, III and the internal standard were prepared in methanol (10 μg/ml) and stored at 4°C.

Chromatography

The HPLC system consisted of a Spectra-Physics SP 8810 isocratic pump, an SP 8490 UV detector set at 260 nm (the absorbance maximum of RU 49041), coupled to an SP 4290 integrator and an SP 8880 refrigerated autoinjector equipped with a 20-μl loop.

A Nucleosil Chiral 2 (Macherey–Nagel, Düren, Germany) analytical column (250 mm × 4.6 mm I.D., 5 μm particle size) was used, in which two different chiral functions (tartaric acid and dinitrobenzylphenylethylamine) are bound by propyl spacer arms to the silica stationary phase. An organic, apolar mobile phase was used consisting of dichloromethane–*n*-heptane (75:25, v/v) to which methanol (0.3%) and trifluoroacetic acid (0.08%) were added. The flow-rate was 2.0 ml/min.

Extraction procedure

Plasma. To 1 ml of plasma were added 1 μg (100 μl of a methanolic solution) of internal standard, 500 μl of acetonitrile and 500 μl of 0.1 *M* potassium phosphate buffer (pH 7.0). The mixture was vortex-mixed for 10 s and centrifuged at 3000 *g* for 10 min. The supernatant was transferred on to octadecylsilane-bonded silica microcolumns (Bond Elut C_{18}, 1-ml capacity; Analytichem International, Harbor City, CA, USA) pretreated successively with 1 ml of methanol and 1 ml of purified water. The microcolumns were rinsed with 1 ml of 1 *M* NaCl and 1 ml of purified water and the bound compounds were eluted with 1 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 50°C and the residue was taken up in 300 μl of dichloromethane.

Brain. Each brain was homogenized in 3 ml of methanol using a PTFE–glass homogeniser. Internal standard (1 μg) was added to the homogenate, which was centrifuged at 3000 *g* for 20 min. The supernatant was removed and evaporated to dryness under a stream of nitrogen at 50°C. The residue was taken up in 400 μl of methanol and 800 μl of phosphate buffer. After vortex-mixing, the mixture was centrifuged at 3000 *g* for 10 min and the supernatant was purified on Bond Elut microcolumns as for plasma.

Calibration and calculation

The retention times of II and III were determined by separate injections of each enantiomer. The capacity factors were calculated as $k' = (t_R - t_0)/t_0$, the stereoselectivity factor, α_s, as $k'(\text{III})/k'(\text{II})$ and the stereochemical resolution, R_s, as $1/4\,[(\alpha_s - 1)/\alpha_s][k'_2/1 + k'_2]\,N_2^{1/2}$, where the subscript 2 refers to the compound with the longest retention time and N is the number of theoretical plates of the column [3].

Calibration graphs were constructed by addition of 25, 50, 100, 200, 300, 400 and 600 ng of each enantiomer, together with 1 μg of internal standard, to aliquots of control plasma (1 ml) or brain homogenate. Unweighted least-squares regression lines were generated using peak-height ratios (enantiomer/internal standard).

RESULTS AND DISCUSSION

Chiral chromatography

No separation of the two enantiomers was obtained using classical chiral stationary phases carrying either bound cyclodextrins (types α, β and γ) or bovine or human serum albumin. A good separation was achieved, however, using a less well known stationary phase, Nucleosil Chiral 2, to which two different chiral functions are bound. A typical chromatogram (Fig. 2a) shows the separation of the enantiomers after injection

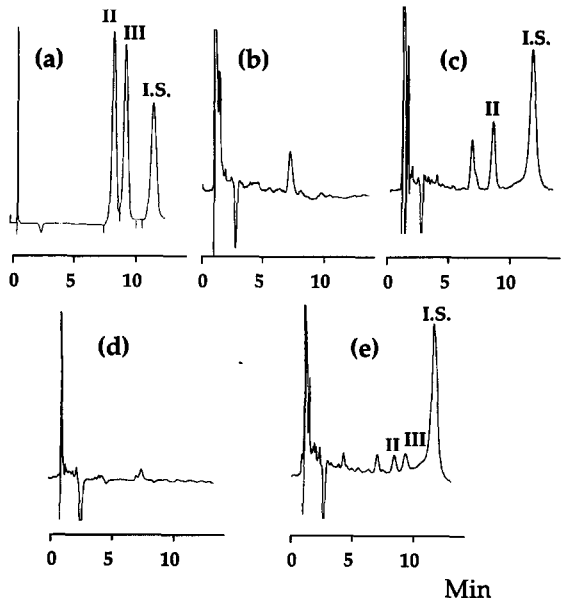

Fig. 2. Typical chromatograms (0.005 a.u.f.s.) after injection of I (a) and of rat plasma and brain extracts after oral administration of I (10 mg/kg): (b) plasma blank; (c) plasma sample, 1 h after treatment; (d) brain blank; (e) brain sample, 30 min after treatment. I.S. = internal standard.

of the racemate. Capacity factors, k', are 13.1 for II and 14.7 for III. The stereoselectivity factor, α_s, is 1.12 and the stereochemical resolution factor, R_s, is 1.39.

Linearity

The calibration graphs (four replicates) constructed with six different concentrations using spiked samples were found to be linear over the range used, with correlation coefficients ≥ 0.998 and y-intercepts close to zero (Table I).

Limit of detection and precision

The limit of detection of the assay for plasma or brain was evaluated as 10 ng/ml or ng/g, at which concentration the detector response was approximately equal to three times the detector noise. The limit of determination, in both tissues, was set at 25 ng/ml or ng/g and the precision ($n = 6$) at this level for II and III, was 9.0 and 9.2%, respectively, for the plasma and 2.8 and 4.9%, respectively, for the brain. Intra-assay precision was calculated using plasma or brain homogenate spiked with 300 ng/ml or ng/g tissue, respectively. The relative standard deviations (R.S.D.) for both enantiomers were 5.4% ($n = 7$) for plasma and 1.0% ($n = 6$) for brain. The between-assay R.S.D.s, calculated from five different assays over a period of several weeks, at 100 ng/ml or ng/g for II and III were 7.4 and 8.7%, respectively, for the plasma and 15.5 and 9.5%, respectively, for the brain.

Recovery

Plasma or brain samples spiked to a final concentration of 400 ng/ml or ng/g were taken through the entire preparation procedure described. The absolute recoveries of II and III were 72 and 75%, respectively, for the plasma and 64 and 62%, respectively, for the brain.

Pharmacokinetic study

The method was applied to the preliminary evaluation of the pharmacokinetic profiles of the enantiomers in rat plasma and brain after oral administration of the racemate (10 mg/kg). Typical chromatograms of plasma and brain extracts from treated rats (Fig. 2c and e) compared with

TABLE I

CALIBRATION GRAPHS FOR PLASMA AND BRAIN EXTRACTS

Extract	Compound	y-Intercept (mean)	Slope ($\times 10^3$) (mean ± S.D.)	Correlation coefficient
Plasma ($n = 4$)	II	0.00476	1.52 ± 0.09	0.999
	III	0.00254	1.50 ± 0.03	0.999
Brain ($n = 4$)	II	−0.00299	1.44 ± 0.09	0.999
	III	−0.00129	1.07 ± 0.05	0.998

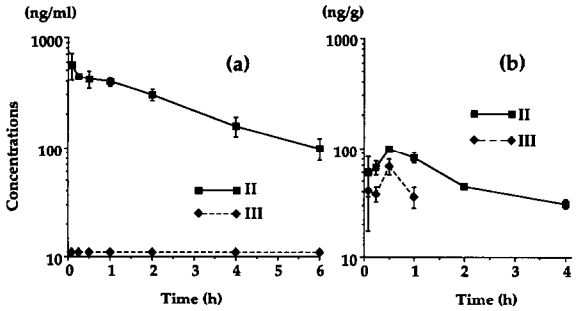

Fig. 3. Concentrations of II (solid line) and III (dashed line) as a function of time after oral administration of I (10 mg/kg) to rats: (a) plasma; (b) brain. Each point represents the mean ± standard error of the mean for four rats.

those of control tissues (Fig. 2b and d) are shown.

After administration of the racemate, only the enantiomer II was detected in plasma. The compound is rapidly absorbed, maximum concentrations being found at the first time point (5 min) (Fig. 3). The elimination half-life is of the order of 3 h and the area under the concentration curve (AUC) is 1480 (ng/ml) h. Both enantiomers are detected in the brain, which is the target tissue. As shown by the concentration curves (Fig. 3), they appear rapidly and maximum concentrations are seen at about 30 min. Whereas III is no longer detected at 2 h (< 10 ng/g of tissue), significant concentrations of II are still observed at 4 h. This result shows that although III is not seen in the plasma, it is nevertheless well absorbed and must therefore have a great affinity for tissues such as the brain, suggesting a large apparent volume of distribution. Such a striking difference in the pharmacokinetic profiles of two enantiomers appears to be an unusual occurrence [4].

CONCLUSION

A chiral HPLC method has been developed for the separation and assay of the enantiomers of the 20,21-dinoreburnamenine derivative I. A preliminary pharmacokinetic study in the rat showed that the pharmacokinetic profiles of the two enantiomers are very different.

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