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Enantiospecific determination of PNU-83894 and its major metabolite, PNU-83892, in plasma, and its application to the characterization of the enantioselective pharmacokinetics of PNU-83894 in the dog

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Abstract

A chiral method for the simultaneous analysis of the (+)- and (−)-enantiomers of PNU-83894 and its metabolite, PNU-83892, in plasma was developed to characterize the enantioselective pharmacokinetics of PNU-83894, a potential anticonvulsant candidate. The method involves solid-phase extraction (phenyl column) of the enantiomers from plasma followed by direct enantioselective separation on a β-cyclodextrin HPLC chiral column and UV detection at 230 nm. The linear range for this method was found to be 12.5 ng/ml to 5.00 μg/ml and the intra- and inter-assay precision and accuracy for each enantiomer were <11% in all cases. The validity of this assay was also demonstrated by its application to the pharmacokinetic evaluation of PNU-83894 in the dog. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; PNU-83894; PNU-83892; Dichloromethylaminocyclohexylbenzamide; Aminocyclohexyldichlorobenzamide

1. Introduction

PNU-83894 and PNU-83892 (Fig. 1) are active circulating metabolites of PNU-54494A, an anticonvulsant drug candidate [1]. PNU-54494A was found to be extensively first-pass metabolized in animal models and also in humans, which resulted in poor oral bioavailability and high inter-subject variability in plasma concentration [2]. PNU-83894 has been investigated as a potential back-up drug candidate and was found to have higher bioavailability (40%) in the dog compared with PNU-54494A (25%) [3]. It was also found that the major metabolite observed in dog plasma and urine of orally dosed PNU-83894 was PNU-83892, owing to biotransformation from the secondary amine to primary amine (unpublished data). PNU-83894 or PNU-83892 is a racemic mixture of the (+)- and (−)-enantiomers of the 1*R*,2*S*-diastereomer. A previous study of the two individual enantiomers of PNU-54494A in the dog showed significant differences in pharmacokinetics after both intravenous (i.v.) and oral (p.o.) administration, which appeared to be the contributing factor for the differences in pharmacological activities [4]. Because of the structural similarity between PNU-54494 and PNU-83894, enantioselective pharmacokinetics of PNU-83894 are also speculated. The difference in the amount of PNU-83892 enantiomers formed may also be important pharmacologically, following an oral dose of PNU-83894 to the dog. In order to investigate the pharmacokinetics of PNU-

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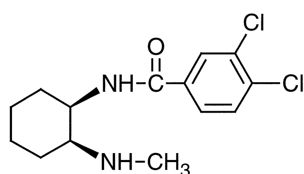
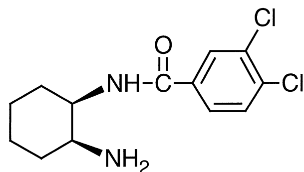


PNU-83894



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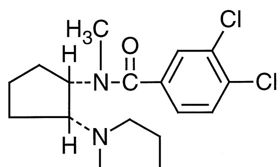

PNU-54490 (IS)

Fig. 1. Chemical structure of PNU-83894 {3,4-dichloro-*N*-[(1*R*,2*S*)-2-(methylamino)cyclohexyl]benzamide}, PNU-83892 {*N*-[(1*R*,2*S*)-2-aminocyclohexyl]-3,4-dichlorobenzamide}, and PNU-54590 {3,4-dichloro-*N*-methyl-*N*-[(1*S*,2*R*)-2-(1-pyrrolidinyl)cyclopentylbenzamide}.

83894 enantiomers in the dog, a chiral assay for the enantiomers of PNU-83894 and its metabolite PNU-83892 in plasma was developed. The plasma samples collected from an i.v./p.o. crossover study with PNU-83894 (racemate) in the dog were re-extracted and analyzed using this developed chiral assay.

2. Experimental

2.1. Chemicals

The racemate and individual enantiomers of PNU-83894 and PNU-83892 and the internal standard, PNU-54490 (Fig. 1), were provided by Pharmacia & Upjohn. HPLC-grade methanol, acetone and acetonitrile were obtained from Burdick and Jackson (Muskegon, MI, USA). All other chemicals were reagent grade. Acetic acid was purchased from Mallinckrodt (Paris, KY, USA). Triethylamine (TEA) was obtained from Aldrich (Milwaukee, WI, USA). Purified water was produced by a Milli-Q reagent water system (Millipore, Bedford, MA, USA).

2.2. Preparation of standard

PNU-83894 and PNU-83892 (racemates) were demonstrated to have an enantiomeric ratio of 1 by comparison with individual enantiomers. Stock standard solution of PNU-83894 or PNU-83892 was prepared by dissolving the drug in water to give a concentration of 200 μg/ml, which was equivalent to 100 μg/ml of the (+)- and (−)-enantiomers of PNU-83894 or PNU-83892. Working standard solutions (10.0, 1.00 and 0.100 μg/ml) were prepared by diluting stock solutions with water. The stock solution of the internal standard (I.S., PNU-54490) was prepared using a similar procedure. Stock and working solutions were stored at 4°C. Plasma standards were prepared freshly by aliquoting appropriate volumes of stock and working solutions to 1 ml of control dog plasma (drug-free) to produce a concentration series from 12.5 ng/ml to 5.00 μg/ml.

2.3. Chromatography

Direct HPLC separation of the (+)- and (−)-enantiomers of PNU-83894 and PNU-83892 was carried out on a β-cyclodextrin chiral column [Cyclobond I (β), 250×4.6 mm I.D., Astec, Whippany, NJ, USA] with a Spectra-Physics 8800 solvent delivery system (San Jose, CA, USA). The mobile phase was acetonitrile–water (20:80, v/v) containing 0.2% TEA with a final pH of 6.0±0.1 adjusted using acetic acid. The chromatographic system was operated at 21–23°C with a flow-rate of 0.7 ml/min. The effluent was monitored at an UV wavelength of 230 nm using a Spectra Focus detector. Quantification was accomplished by the peak-height ratio of analytes to an I.S. The concentrations of unknown samples were calibrated against fortified plasma standard curves for each enantiomer. Chromatographic peak heights were integrated by a Harris computer system.

2.4. Sample preparation

Unknown sample preparation was accomplished using the extraction method developed for PNU-54494A and its metabolites [3]. Briefly, 1 ml of each unknown plasma sample was mixed with 50 μl of I.S. working solution (1.00 μg/ml) and 1 ml of acetonitrile–water (3:7, v/v), and were loaded to each phenyl solid-phase extraction (SPE) column (100 mg/1.0 ml, Varian, Harbor City, CA, USA), which were prewashed with one column volume of methanol followed by one column volume of water. The columns were then washed with 300 μl of methanol–water (3:7, v/v) followed by 1 ml of water and were dried by vacuum aspiration for 10 min. The compounds of interest were then eluted from the column with 300 μl of acetone–methanol (2:3, v/v) followed by 350 μl of water into an autosampler vial, to which 350 μl of water was added. One hundred microliters of the mixture was injected for HPLC analysis.

3. Results and discussion

3.1. HPLC characteristics

Since the β-cyclodextrin chiral HPLC column has demonstrated selective separation of a variety of optical isomers [5,6], it was our first choice for testing the chiral separation of PNU-83894 and PNU-83892. Adequate capacity and resolution of the two enantiomers of PNU-83894 and PNU-83892 were achieved when using an isocratic run on a reversed-phase HPLC system with a Cyclobond I (β) column mobile phase of acetonitrile–water (20:80, v/v) containing 0.2% TEA delivered at a flow-rate of 0.7 ml/min. Apparently, both PNU-83894 and PNU-83892 have a suitable size to tightly bind to the β-cyclodextrin cavity, thereby interacting with its hydrophobic cavity for chiral recognition. Under the conditions described above, the enantiomeric resolution factor, R, was 0.98 and 0.94 for PNU-83894 and PNU-83892, respectively. R was determined using $R = 2\Delta t/(W_1 + W_2)$, where Δt is the difference between the retention times of the two enantiomers and W is the width of peaks 1 and 2 at their bases. The capacity factors, k', for the (+)- and (−)-enantiomers of PNU-83894 and PNU-83892 were 3.45 and 3.76, and 2.48 and 2.86, respectively. k' was calculated using $k' = (V_r - V_0)/V_0$, where V_r and V_0 are the experimentally measured elution volumes of analytes and solvent, respectively. These data suggest that the enantiomeric resolution is sufficient to analyze the enantiomers for investigation of their pharmacokinetic behavior. Inclusion of 0.2% TEA in the mobile phase greatly improved the peak shape, resulting in better separation of the enantiomers. PNU-54490 (Fig. 1) was selected as an internal standard for this assay since it has similar characteristics in extraction efficiency as PNU-83894 and PNU-83892. Although PNU-54490 is a racemic mixture of the (+)- and (−)-enantiomers of the 1*S*,2*R*-diastereomer, the two enantiomers of this compound could not be separated under the assay conditions and, thus, was used as an internal standard for the four enantiomers.

Fig. 2 depicts chromatograms of extracts of pre-dose dog plasma with I.S., dog plasma spiked with 0.5 μg/ml of PNU-83894 and 0.5 μg/ml of PNU-83892, and plasma samples collected 1 h after i.v. administration of PNU-83894 to the dog. No significant interfering peaks were observed in the chromatograms of pre-dose and post-dose dog plasma samples.

3.2. Extraction recovery

The mean absolute extraction recovery for PNU-83894 and PNU-83892 at three concentrations ranged from 89 to 92%, as described previously [3]. The extraction recovery for the individual enantiomers was also evaluated using this chiral method. Slightly but consistently lower extraction recovery for the (+)-enantiomer compared with the (−)-enantiomer was seen for both PNU-83894 and PNU-83892 at three different concentrations. The absolute extraction recovery ranged from 79 to 87%, 92 to 95%, 80 to 88%, and 90 to 96%, for the (+)- and (−)-enantiomers of PNU-83894 and PNU-83892, respectively. The differences in the extraction recovery between enantiomers might be related to the different protein-binding characteristics of individual enantiomers.

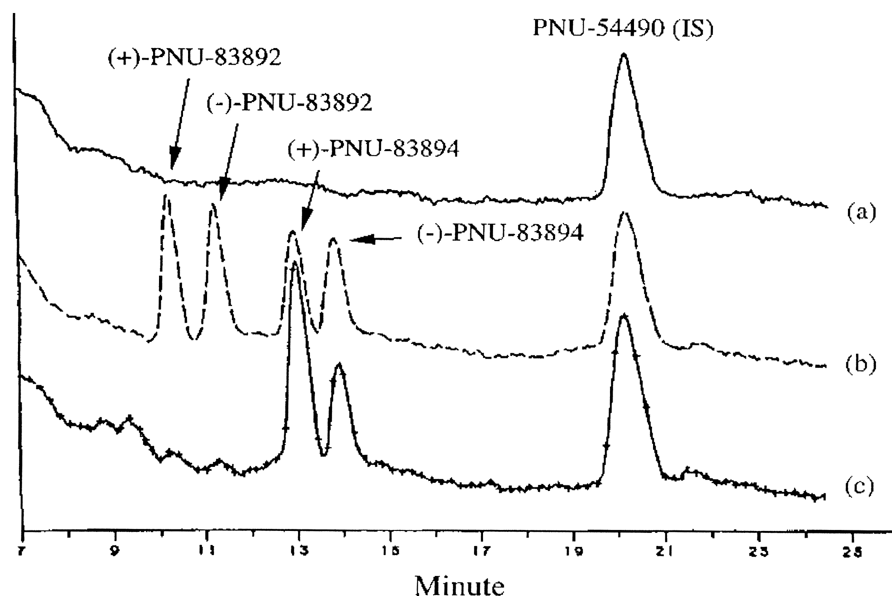

Fig. 2. Representative chromatograms of PNU-83894 and PNU-83892 enantiomers in dog plasma: (a) a pre-dose dog plasma, (b) a plasma standard fortified with 0.25 μg/ml each of (+)- and (−)-enantiomers of PNU-83894 and PNU-83892, and (c) a 1-h post-dose plasma sample collected from a dog administered 5 mg/kg PNU-83894 intravenously.

3.3. Method validation

The linearity, sensitivity, precision and accuracy were evaluated from four analytical runs. Nine concentrations ranging from 25.0 ng/ml to 10.0 μg/ml of the racemate, corresponding to the equivalent of 12.5 ng/ml to 5.00 μg/ml of each enantiomer, were used to construct calibration curves. Calibration curves were fitted by least-squares linear regression without a weighting factor. The detailed calibration curve data for each enantiomer are listed in Table 1. A linear range from 12.5 to 5.00 μg/ml with a correlation coefficient of >0.999 was obtained for each enantiomer. A level of 25.0 ng/ml was determined to be the lowest concentration of the four enantiomers which could be quantified with an acceptable assay precision and accuracy (<15%). The minimum detectable amount was determined to be 12.5 ng/ml. When the concentration is ≥25.0 ng/ml, the intra- and inter-assay precision and accuracy were less than 11% in all cases (Table 2). These data demonstrate the validity of this assay.

3.4. Application to enantioselective pharmacokinetic evaluation

The pharmacokinetic parameters obtained from plasma concentration–time data of (+)- and (−)-

Table 1
Calibration data for determination of the (+)- and (−)-enantiomers of PNU-83894 and its metabolite, PNU-83892, in dog plasma (model: $y = ax + b$, no weighting)

Compound	Slope (a)	Intercept (b)	Correlation coefficient (r)
(+)-PNU-83894	2.63±0.07	0.01±0.04	0.9998±0.0002
(−)-PNU-83894	3.52±0.3	−0.015±0.026	0.9998±0.0001
(+)-PNU-83892	3.02±0.10	0.015±0.017	0.9995±0.0002
(−)-PNU-83892	3.34±0.20	0.00±0.04	0.9996±0.0002

Table 2
Intra- and inter-assay precision and accuracy for determination of PNU-83894 and PNU-83892 enantiomers

Compound	Concentration (μg/ml)	Intra-assay ($n=3$)		Inter-assay ($n=4$)	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
(+)- PNU-83894	0.025	6	−3.5	4	−2.8
	0.25	2.5	4	1.4	2.8
	5.0	0.3	−0.65	0.4	0.7
(−)-PNU-83894	0.025	5.4	−4.2	10	−8.0
	0.25	8	5.5	10	4.4
	5.0	0.8	0.3	0.5	0.8
(+)-PNU-83892	0.025	5	−7.2	6	6
	0.25	6.3	−6.8	7	−9.2
	5.0	0.6	−0.9	0.4	−1.6
(−)-PNU-83892	0.025	9	3.3	5	1.2
	0.25	8.8	4.6	8	−7.2
	5.0	0.5	−1.8	0.6	−1.0

enantiomers of PNU-83894 and its metabolite PNU-83892 following i.v. and p.o. administration of PNU-82894 are given in Table 3. With a considerably higher plasma concentration for (+)-PNU-83894 than its antipode, the results are indicative for a significant difference in the pharmacokinetics of the two PNU-83894 enantiomers. The average bioavailability of the two enantiomers (38.2%) following racemic administration was consistent with that obtained from a previous achiral assay (39.1%) (unpublished data). The enantiomers of PNU-83892 were also detectable in plasma using this chiral assay. In contrast to the parent drug, the (−)-enantiomer of PNU-83892 had a higher plasma concentration than its antipode, suggesting that the enantioselective metabolism of PNU-83894 in the dog

Table 3
Pharmacokinetic parameters[a] of the (+)- and (−)-enantiomers of PNU-83894 and PNU-83892 in the dog following a 5 mg/kg intravenous and oral administration of PNU-83894

Compound	Route	C_{max} (μg/ml)	t_{max} (h)	$AUC_{0-\infty}$ (μg h/ml)	$t_{1/2}$ (h)	Cl_p ($l\ h^{-1}\ kg^{-1}$)	F (%)
(+)-PNU-83894	i.v.	0.93	0.033	4.28	4.12	1.15	
	p.o.	0.33	1	1.79	3.55		40.4
(−)-PNU-83894	i.v.	0.54	0.033	1.59	2.81	3.09	
	p.o.	0.095	2.0	0.596	2.49		36.2
(+)-PNU-83892	i.v.	0.064	6	0.548	3.76		
	p.o.	0.056	3	0.433	3.29		
(−)-PNU-83892	i.v.	0.089	6	1.26	6.11		
	p.o.	0.088	4	0.482	3.88		

[a] Pharmacokinetic parameters were evaluated using a model-independent method [7]. The maximum plasma concentration (C_{max}) and the time at which C_{max} is reached (t_{max}) were obtained from the plasma concentration–time data. $AUC_{0-\infty}$, the area under the plasma concentration–time curve from time zero to infinity, was calculated from $AUC_{0-t} + C_t/\lambda_z$, where AUC_{0-t} is the area under the plasma concentration–time curve from zero to the time of the last plasma sample with measurable drug concentration (C_t) obtained using the linear trapezoidal rule, and λ_z is the terminal elimination rate constant determined from the log-linear regression slope of the terminal portion of the plasma concentration–time curve. The terminal elimination half-life ($t_{1/2}$) was calculated from $0.693/\lambda_z$. The total plasma clearance (Cl_p) was determined from $Dose/AUC_{0-\infty}$ following i.v. administration, and the oral bioavailability (F) was obtained from $AUC_{p.o.} \cdot Dose_{i.v.} / AUC_{i.v.} \cdot Dose_{p.o.} \cdot 100\%$.

appears to be one of the contributing factors for the difference in systemic clearance between enantiomers.

4. Conclusions

The versatility of the reported method for direct chromatographic separation of enantiomers of PNU-83894 and its metabolite, PNU-83892, was demonstrated by the results from analyzing plasma samples collected in a pharmacokinetic study with PNU-83894 in the dog. These results enable one to readily obtain the enantioselective pharmacokinetics of PNU-83894 and reveal the contributing factors responsible for the enantioselectivity. The most significant benefit of this method is that it is simple, reliable, and uses a common reversed-phase HPLC system, in addition to the good enantiomeric resolution and adequate sensitivity.

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