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Stereoselective high-performance liquid chromatographic assay for pirmenol enantiomers in dog plasma

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ABSTRACT

Pirmenol enantiomers in dog plasma were quantified using a stereospecific high-performance liquid chromatographic method with ultraviolet detection at 262 nm. Racemic pirmenol and internal standard, (+)-propranolol, were isolated from dog plasma by a three-step extraction procedure using toluene, 0.1 *M* hydrochloric acid and hexane, respectively. A chiral analytical column (Chiralcel OJ) was used with a mobile phase consisting of hexane–isopropanol–diethylamine (98.9:1.0:0.1). Linear calibration curves were obtained in the concentration range 0.0200–5.00 μg/ml for each enantiomer. Precision of the method, expressed as coefficient of variation for nine quality control samples, was 7.1% for (+)-pirmenol and 6.4% for (−)-pirmenol. Bias was ±2.2% for (+)-pirmenol and ±1.5% for (−)-pirmenol in quality control samples.

INTRODUCTION

Pirmenol, (±)-*cis*-α-[3-(2,6-dimethyl-1-piperidinyl)propyl]-α-phenyl-2-pyridinemethanol monohydrochloride monohydrate, is a chiral compound (Fig. 1) being investigated as an antiarrhythmic agent. Pirmenol is administered as a racemic mixture and its pharmacokinetics have been studied in healthy subjects and patients with dysrhythmia [1–4]. Analytical methods used for these studies quantified pirmenol as a racemic mixture without distinguishing between enantiomers [5,6]. Investigation of possible stereoselective differences in pharmacokinetics, metabolism and pharmacodynamics of pirmenol requires a method for quantitation of its enantiomers in biological samples.

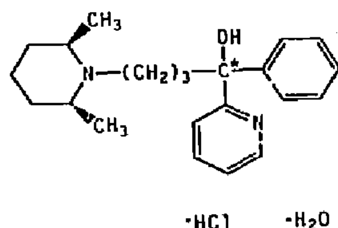

Fig. 1. Structure of pirmenol (CI-845). The asterisk denotes the location of the chiral center.

Since derivatization of pirmenol was not successful, several chiral columns were investigated for the separation of pirmenol enantiomers. A chiral Hypro-Cu-Daltosil column (Serva, New York, NY, USA) was first tried since it was reported to separate pirmenol enantiomers in dog and human urine [7]. However, this column did not provide adequate separation at typical racemic pirmenol concentrations in plasma (< 1.0 μg/ml) following therapeutic doses. An α_1-acid glycoprotein column (LKB EnantioPac, Bromma, Sweden) also separated pirmenol enantiomers, but racemic concentrations less than 0.5 μg/ml could not be quantified accurately. For pharmacokinetic studies, a detection limit of at least 0.1 μg/ml racemic pirmenol would be required.

The method described uses cellulose tris(4-methylbenzoate) adsorbed on silica gel [8] as a chiral stationary phase (Chiralcel OJ, Daicel, Tokyo, Japan) for separation of pirmenol enantiomers. Linearity, precision and accuracy were evaluated following analysis of calibration standards and quality control samples prepared in dog plasma. Possible differences in the pharmacokinetics and pharmacodynamics of each enantiomer are currently being evaluated using this high-performance liquid chromatographic (HPLC) method for quantitation of (+)-pirmenol and (−)-pirmenol in plasma.

EXPERIMENTAL

Chemicals

Racemic pirmenol hydrochloride and pirmenol free base were synthesized, and pirmenol enantiomers were separated at the Parke-Davis Pharmaceutical Research Division, Warner Lambert (Ann Arbor, MI, USA). Internal standard used for the assay was (+)-propranolol hydrochloride (Sigma, St. Louis, MO, USA). Mobile phase consisted of HPLC-grade hexane (EM Science, Gibbstown, NJ, USA), HPLC-grade isopropanol (Baxter, Burdick and Jackson Division, Muskegon, MI, USA) and diethylamine (Sigma). Glassware was silanized using a 10% solution of dichlorodimethylsilane (Sigma) in HPLC-grade toluene (Baxter, Burdick and Jackson Division). Hydrochloric acid and sodium hydroxide solutions were prepared using standard volumetric solutions purchased from J. T. Baker (Phillipsburg, NJ, USA) with HPLC-grade water (EM Science). Control beagle dog plasma was obtained at the Parke-Davis Pharmaceutical Research Division, Warner Lambert. Control human plasma was purchased from Interstate Blood Bank (Memphis, TN, USA).

Preparation of solutions

A 250 μg/ml stock solution of racemic pirmenol and a 100 μg/ml stock solution of (+)-propranolol were prepared in 0.01 *M* hydrochloric acid using silanized volumetric flasks. A 25 μg/ml racemic pirmenol stock solution was prepared by dilution (1:10) of the 250 μg/ml stock solution. Concentrations represent free base equivalents. Stock solutions were diluted with 0.01 *M* hydrochloric acid for

preparation of calibration standards in silanized volumetric flasks. Concentrations of pirmenol enantiomers in calibration standard solutions ranged from 0.100 to 25.0 μg/ml. Internal standard solution (2.50 μg/ml) was prepared by dilution (1:40) of internal standard stock solution with 0.01 *M* hydrochloric acid.

Quality control samples containing racemic pirmenol were prepared at 0.0600, 0.300 and 1.50 μg/ml of each enantiomer. Appropriate volumes of 25 or 250 μg/ml stock solutions were added to silanized 25-ml volumetric flasks and brought to volume with control beagle dog plasma. Aliquots of quality control samples were stored at −15°C in silanized glass tubes.

Extraction procedure

For calibration standards, 0.5 ml of control dog plasma and 0.1 ml of calibration standard solution containing racemic pirmenol were combined in a 100 mm × 16 mm disposable glass culture tube. A 0.5-ml aliquot of dog plasma and 0.1 ml of 0.01 *M* hydrochloric acid were used for quality controls and study samples. A 0.1-ml aliquot of 2.5 μg/ml internal standard solution and 0.2 ml of 1 *M* sodium hydroxide were added to each tube. The mixture was extracted with 5 ml of toluene by shaking for 10 min, centrifuged at 750 *g* for 5 min, and the aqueous layer was frozen in a dry ice–acetone bath. The remaining toluene was transferred to a 15-ml disposable glass centrifuge tube, back-extracted with 0.5 ml of 0.1 *M* hydrochloric acid by shaking for 10 min and centrifuged at 420 *g* for 5 min. The aqueous layer was frozen in a dry ice–acetone bath and toluene was discarded. After thawing, 0.2 ml of 1 *M* sodium hydroxide was added and the mixture was extracted with hexane by shaking for 10 min followed by centrifugation at 420 *g* for 5 min. The aqueous layer was frozen in a dry ice–acetone bath and hexane was transferred to a silanized 75 mm × 12 mm disposable glass culture tube. Hexane was evaporated under nitrogen gas and the residue was dissolved in 200 μl of mobile phase. A 175-μl aliquot was injected into the HPLC system.

Chromatography

The HPLC system consisted of a Model 590 solvent delivery module, a Model 712 autosampler, Models CHM and TCM column temperature-controlling system (Waters, Milford, MA, USA), and a Model 8490 ultraviolet absorbance detector and Chromjet integrator (Spectra-Physics, San Jose, CA, USA).

Pirmenol enantiomers and internal standard were separated using a Chiralcel OJ column with 10 μm particle size and dimensions of 25 cm × 4.6 mm I.D. (J. T. Baker) and a mobile phase of hexane–isopropanol–diethylamine (98.9:1.0:0.1, v/v). Flow-rate was 1.0 ml/min, column temperature was maintained at 65°C, and absorbance was monitored at 262 nm.

Assay validation

Calibration standards and quality control samples were analyzed in triplicate

on three separate days for a total of nine determinations at each concentration. Calibration standard concentrations ranged from 0.0200 to 5.00 μg/ml for (+)-pirmenol and (−)-pirmenol (0.0400–10.0 μg/ml racemic pirmenol). Pirmenol enantiomer concentrations in quality control samples of dog plasma were 0.0600, 0.300 and 1.50 μg/ml (0.120, 0.600 and 3.00 μg/ml racemic pirmenol). Least-squares linear regression with a weighting factor of 1/concentration was used to construct calibration curves for peak-height ratio as a function of pirmenol enantiomer concentration. Regression parameters (slope; y-intercept) were used to calculate (+)-pirmenol and (−)-pirmenol concentrations in calibration standards, quality control samples and *in vivo* plasma samples.

Recovery

Recovery of pirmenol enantiomers from dog plasma was determined at calibration standard concentrations of 0.0500, 0.250 and 2.50 μg/ml (+)-pirmenol and (−)-pirmenol (0.100, 0.500 and 5.00 μg/ml racemic pirmenol). Nine determinations were performed at each concentration. Peak heights of (+)-pirmenol, (−)-pirmenol and internal standard following extraction from dog plasma were compared with mean peak heights from nine injections of reference (non-extracted) standards. For reference standards, stock solutions of racemic pirmenol free base in isopropanol and (+)-propranolol hydrochloride (internal standard) in methanol were prepared. Aliquots of these stock solutions were pipetted into silanized volumetric flasks, solvents were evaporated under nitrogen, and residue was dissolved in hexane–isopropanol–diethylamine (99.1:0.8:0.1, v/v).

Dog plasma samples

A single 5 mg/kg intravenous dose of racemic pirmenol was administered to two male beagle dogs. The dose was prepared in 0.9% saline and given as a 5-min infusion. Serial blood samples were obtained for 8 h following the end of the infusion. After centrifugation, plasma was separated and stored frozen at −15°C until assayed. Pirmenol enantiomer concentrations in dog plasma were determined using the method described. Pharmacokinetic parameters were calculated using non-compartmental methods [9].

RESULTS AND DISCUSSION

Chromatography

Representative chromatograms of pirmenol enantiomers in dog plasma are shown in Fig. 2, where retention times of (+)-pirmenol, (−)-pirmenol and internal standard are 7.4, 9.2 and 20.9 min, respectively. No chromatographic peaks at retention times of pirmenol enantiomers or internal standard were observed in control dog plasma. In addition, control human plasma did not show any interfering peaks, suggesting that this method may also be applicable to human plasma samples.

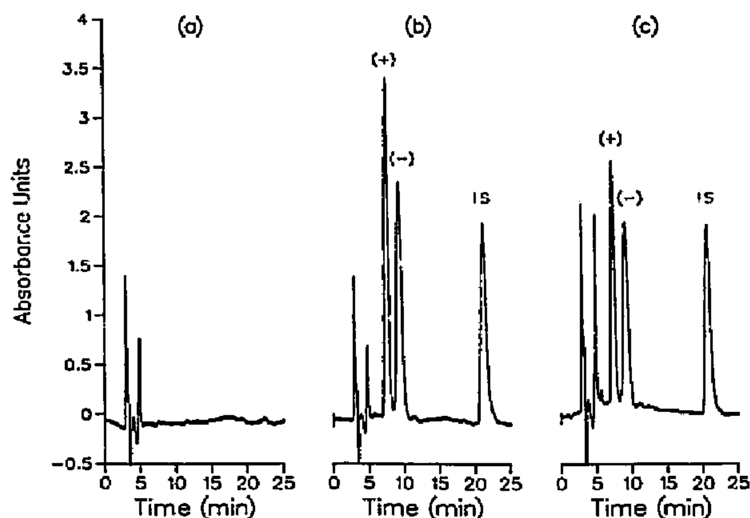

Fig. 2. Chromatograms of (a) control dog plasma with no pirmenol or internal standard added, (b) a calibration standard containing 0.500 μg/ml (+)-pirmenol and (−)-pirmenol (1.00 μg/ml racemic pirmenol) and internal standard, and (c) a dog plasma sample obtained 45 min after the end of a 5-min infusion of 5 mg/kg racemic pirmenol. Peaks: (+) = (+)-pirmenol; (−) = (−)-pirmenol; IS = internal standard, (+)-propranolol.

Elution order of enantiomers was identified by comparing retention times of (+)-pirmenol and (−)-pirmenol reference standards to retention from racemic pirmenol.

Calibration standards

Calibration curves were linear over the concentration range 0.0200–5.00 μg/ml for (+)-pirmenol and (−)-pirmenol in dog plasma. Regression parameters (slope; *y*-intercept; coefficient of determination, r^2) calculated following analysis of nine sets of calibration standards are shown in Table I. Variability of slope, as judged by coefficient of variation, was less than 3.0% for both enantiomers.

Mean calculated concentrations of calibration standards analyzed in triplicate on three separate days are shown in Table II. The coefficient of variation (C.V.) for nine determinations was 5.9% or less for (+)-pirmenol and 7.9% or less for (−)-pirmenol. The bias, calculated as the percentage difference from theoretical concentration, was ±3.5% for (+)-pirmenol and ±5.5% for (−)-pirmenol.

Quality controls

Table III contains mean calculated concentrations for quality control samples containing racemic pirmenol in dog plasma. Precision was at least 7.1% for each enantiomer in quality control samples, based on C.V. values for (+)-pirmenol and (−)-pirmenol. Calculated concentrations were within 2.2% of actual concentrations for both pirmenol enantiomers. Precision and accuracy were also evaluated for the three determinations performed on each day (intra-day) and for

TABLE I

INDIVIDUAL AND MEAN (n = 9) REGRESSION PARAMETERS FOR PIRMENOL ENANTIOMERS IN DOG PLASMA FOLLOWING ASSAY OF CALIBRATION STANDARDS IN TRIPLICATE ON THREE SEPARATE DAYS

Calibration concentrations ranged from 0.0200 to 5.00 μg/ml (+)-pirmenol and (−)-pirmenol; r^2 = coefficient of determination.

Day	Curve No.	(+)-Pirmenol			(−)-Pirmenol		
		Slope	y-Intercept	r^2	Slope	y-Intercept	r^2
1	1	3.16	0.0174	0.9985	2.21	0.0051	0.9978
	2	3.21	0.0169	0.9998	2.24	0.0017	0.9997
	3	3.28	0.0141	0.9995	2.30	0.0028	0.9990
2	4	3.29	0.0294	0.9987	2.27	0.0127	0.9989
	5[a]	3.30	0.0343	0.9994	2.27	0.0096	0.9991
	6	3.16	0.0268	0.9997	2.20	0.0094	0.9997
3	7	3.26	0.0063	0.9998	2.26	−0.0034	0.9998
	8	3.33	0.0041	0.9995	2.30	−0.0020	0.9996
	9	3.46	0.0078	0.9997	2.39	−0.0030	0.9996
Mean		3.27	0.0175	0.9994	2.27	0.0037	0.9992
S.D.		0.09	0.0107	0.0005	0.06	0.0059	0.0006

[a] Calibration standard concentrations ranged from 0.0250 to 5.00 μg/ml pirmenol enantiomers.

TABLE II

MEAN (n = 9) CALCULATED CONCENTRATIONS OF (+)-PIRMENOL AND (−)-PIRMENOL IN CALIBRATION STANDARDS ANALYZED IN TRIPLICATE ON THREE SEPARATE DAYS

Concentration added (μg/ml)	(+)-Pirmenol			(−)-Pirmenol		
	Mean concentration found (μg/ml)	C.V. (%)	Bias (%)	Mean concentration found (μg/ml)	C.V. (%)	Bias (%)
0.0200	0.0193[a]	5.85	−3.50	0.0189	5.77	−5.50
0.0250	0.0243	5.43	−2.80	0.0238	7.94	−4.80
0.0500	0.0509	4.99	1.80	0.0512	7.21	2.40
0.100	0.102	3.02	2.00	0.105	3.64	5.00
0.250	0.253	2.75	1.20	0.255	3.84	2.00
0.500	0.508	2.13	1.60	0.509	2.24	1.80
1.00	1.01	3.33	1.00	1.01	3.61	1.00
2.50	2.48	2.94	−0.80	2.48	3.30	−0.80
5.00	5.01	1.53	0.20	5.00	1.51	0.00

[a] n = 8.

TABLE III

MEAN (n = 9) CALCULATED CONCENTRATIONS OF (+)-PIRMENOL AND (−)-PIRMENOL IN QUALITY CONTROL SAMPLES ANALYZED IN TRIPLICATE ON THREE SEPARATE DAYS

Concentration added (μg/ml)	(+)-Pirmenol			(−)-Pirmenol		
	Mean concentration found (μg/ml)	C.V. (%)	Bias (%)	Mean concentration found (μg/ml)	C.V. (%)	Bias (%)
0.0600	0.0587	7.12	−2.17	0.0609	6.44	1.50
0.300	0.297	3.47	−1.00	0.300	2.18	0.00
1.50	1.53	4.33	2.00	1.52	4.22	1.33

calibration standards analyzed on each of three days (inter-day). Intra-day assay precision (C.V.) ranged from 1.0 to 8.5% for (+)-pirmenol and from 0.7 to 8.9% for (−)-pirmenol. Intra-day assay accuracy (bias) was ±9.3% for (+)-pirmenol and ±4.7% for (−)-pirmenol. Inter-day assay precision (C.V.) ranged from 2.5 to 10.5% and inter-day assay accuracy (bias) was within 4.2% for (+)-pirmenol. Inter-day precision (C.V.) ranged from 1.5 to 9.5% and inter-day accuracy (bias) was within 4.7% for (−)-pirmenol.

Recovery

Recovery of pirmenol enantiomers and internal standard from dog plasma is shown in Table IV. Mean (n = 9) recovery was approximately 80% for both pirmenol enantiomers over the concentration range 0.0500–2.50 μg/ml, with C.V.

TABLE IV

RECOVERY OF PIRMENOL ENANTIOMERS AND INTERNAL STANDARD FROM DOG PLASMA

Recovery is expressed as ratio of peak height for (+)-pirmenol, (−)-pirmenol or internal standard after extraction from dog plasma to mean peak height after nine injections of non-extracted standards.

Compound	n	Concentration (μg/ml)	Mean recovery (%)	C.V. (%)
(+)-Pirmenol	9	0.0500	79.6	7.21
		0.250	79.8	3.22
		2.50	77.9	3.81
(−)-Pirmenol	9	0.0500	83.9	6.83
		0.250	79.6	3.38
		2.50	77.4	3.94
Internal standard	27	0.500	73.5	3.76

TABLE V

STABILITY OF PIRMENOL ENANTIOMERS IN DOG PLASMA

Values are expressed as percent of added (+)-pirmenol or (−)-pirmenol concentration in dog plasma quality control samples stored at −15°C until analysis.

Concentration added (μg/ml)	Percentage remaining					
	(+)-Pirmenol			(−)-Pirmenol		
	Two months	Three months	Four months	Two months	Three months	Four months
0.0600	93.3	93.0	96.7	103	94.0	97.3
0.300	96.7	106	100	115	107	99.3
1.50	96.7	101	96.0	96.7	102	96.7

values of 7.2% or less. Pirmenol enantiomers showed a slight decrease in recovery with increasing concentration. Mean (n = 27) internal standard recovery was 74%.

Stability

Stability data for pirmenol enantiomers in quality control samples are listed in Table V. These values represent single determinations of pirmenol enantiomer concentrations in quality controls which were analyzed with study samples. Pirmenol enantiomers showed no degradation and are considered stable in dog plasma stored at −15°C for at least four months.

Pirmenol enantiomer pharmacokinetics in beagle dogs

Following intravenous administration of racemic pirmenol (5 mg/kg) to two beagle dogs, plasma (−)-pirmenol concentrations were higher than (+)-pirmenol

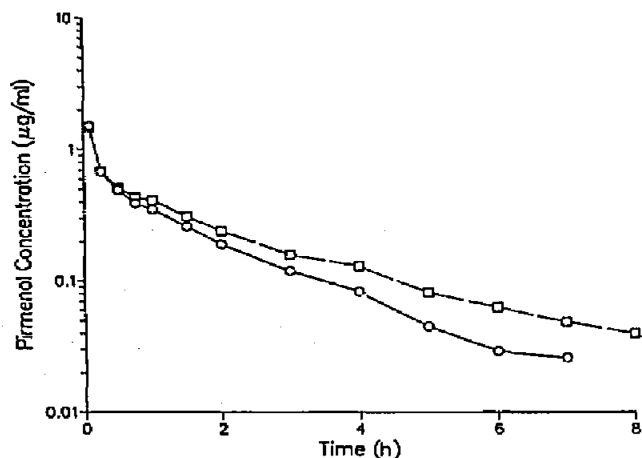

Fig. 3. Plasma concentration–time profile for (+)-pirmenol (○) and (−)-pirmenol (□) following a 5-min intravenous infusion of 5 mg/kg racemic pirmenol to a beagle dog.

concentrations. Fig. 3 shows a plasma concentration–time profile of pirmenol enantiomers in a representative dog. In this animal, terminal-phase half-life was 1.7 and 2.3 h for (+)-pirmenol and (−)-pirmenol, respectively. Plasma clearance was 33.3 ml/min/kg for (+)-pirmenol and 25.6 ml/min/kg for (−)-pirmenol.

CONCLUSION

A normal-phase HPLC assay for pirmenol enantiomers has been developed and validated in dog plasma. Direct separation is achieved on a chiral stationary phase (Chiralcel OJ) without derivatization. Calibration curves are linear in the concentration range 0.0200–5.00 μg/ml for (+)-pirmenol and (−)-pirmenol. This method can be used for the pharmacokinetic evaluation of pirmenol enantiomers in dogs following administration of racemic pirmenol.

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