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Determination of cicletanine enantiomers in plasma by high-performance capillary electrophoresis

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ABSTRACT

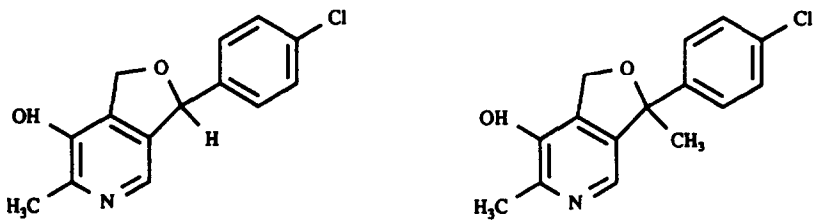
A sensitive and selective high-performance capillary electrophoresis procedure was developed for the determination of *S*(+) and *R*(−) enantiomers of cicletanine in human plasma. The procedure consisted in extraction of the drug with diethyl ether and analysis by micellar electrokinetic capillary chromatography in a fused-silica capillary using γ-cyclodextrins in the run buffers and ultraviolet detection. The method was linear from 10 to 500 ng/ml and the limit of detection was 10 ng/ml for each enantiomer in plasma samples. The within-run precision of the method, expressed as relative standard deviation, was 10.4 and 9.6% at 25 ng/ml for *S*(+) and *R*(−) cicletanine, and 4.2 and 4.6% at 500 ng/ml, respectively. This method has been used to follow the time course of the concentrations of the cicletanine enantiomers in human plasma after a single therapeutic dose of cicletanine given by mouth.

INTRODUCTION

Cicletanine hydrochloride (Tenstaten, IPSEN; 2-methyl-3-hydroxy-4H,5H-5-(4′-chlorophenyl) isofuropyridine hydrochloride) is a member of a class of antihypertensive drugs, the furopyridines. It shows a direct vascular antihypertensive effect and, at higher doses, a natriuretic effect that can be determined separately in clinical and pharmacological models [1]. Its structure is unusual for an antihypertensive molecule and is characterized by the presence of a furopyridine nucleus, as shown in Fig. 1. The molecule has a chiral carbon and therefore exists as two non-superimposable mirror images, or enantiomers [*S*(+) and *R*(−) forms]. Although enantiomers have essentially identical physicochemical properties in a non-chiral environment, they may behave differently when exposed to an optically discriminating enrivonment such as the human body. Consequently, they may differ in either their pharmacodynamic or pharmacokinetic properties, or both.

Cicletanine is given as racemic mixture so it is rational to develop methodologies to evaluate the potential pharmacokinetic differences between the enantiomers. High-performance liquid chromatography (HPLC) has until now been one of the most important techniques for the separation of optical isomers. However, high-performance capillary electrophoresis (HPCE) is also a powerful technique for the determination of enantiomers [2–6].

This paper describes a selective and sensitive



(a) (b)

Fig. 1. Chemical structures of (a) cicletanine and (b) internal standard.

HPCE method using γ-cyclodextrins (γ-CDs) for the separation and quantitation of *S*(+)- and *R*(−)-cicletanine enantiomers in human plasma after the administration of therapeutic doses of cicletanine.

EXPERIMENTAL

Chemicals and reagents

S(+)-, *R*(−)- and (±)-cicletanine and (±)-2-methyl-3-hydroxy-4H,5H-5-methyl-(4′-chlorophenyl)isofuropyridine hydrochloride, used as an internal standard, were supplied by Expansia (Aramon, France). Boric acid and sodium hydroxide solutions (analytical-reagent grade) and sodium dodecyl sulphate (SDS, for biochemistry) were obtained from Merck (Darmstadt, Germany). γ-CDs were purchased from Fluka (Buchs, Switzerland). Diethyl ether, used without further purification, acetonitrile and methanol (all HPLC grade) were from Romil Chemicals (Leicester, UK). The run buffers were filtered through a 0.5-μm Millipore filter and thoroughly degassed in an ultrasonic bath before use. The water was doubly distilled and purified through a Milli-Q system (18 MΩ cm resistivity).

Stock solutions (100 μg/ml) of cicletanine and the internal standard racemates were prepared in a mixture of methanol–water (1:9) and were stable at 4°C for at least 6 months. Working solutions were prepared daily by diluting the stock solution with the same solvent.

Plasma preparation and sampling of human plasma

Heparinized blood (0.75 mg sodium heparinate per ml of blood) was obtained from healthy donors by venepuncture. Blood was centrifuged at 1000 *g* (4°C) for 10 min using Merck's "separating agent for the preparation of erythrocyte-free plasma", according the instructions supplied by the manufacturer. The plasma obtained was separated with a pipette and stored frozen at −22°C in polypropylene tubes. This plasma was used for method validation.

The method described here was applied to follow the time course of cicletanine enantiomers in the plasma of two healthy subjects. For this purpose they received, while fasting, one Tenstaten capsule containing 50 mg of cicletanine racemate hydrochloride. Before giving the drug, a 30-ml sample of blood was taken to prepare the blank plasma and the calibration graph. Blood samples were taken at 0.167, 0.25, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 7, 10, 18, 24 and 30 h after the oral administration of cicletanine. Plasma for calibration and analysis samples was prepared in the same manner.

Apparatus

Electrophoretic separations were carried out on a Beckman P/ACE 2000 system (Beckman, Palo Alto, CA, USA). Separations were performed at 15 kV with a capillary temperature of 35°C and a detection wavelength of 214 nm. Injections were made by pressure (10 s) and the volumes injected, calculated by Poiseuille's equation, were 46 nl.

Electrophoresis

The capillary cartridge (Beckman) contained a 75 μm I.D. capillary that was 57 cm in total length and 50 cm to the detector. The run buffers consisted of 100 m*M* sodium borate buffer (pH

8.6)–110 m*M* SDS–25 m*M* γ-CDs containing 10% acetonitrile as an organic modifier. A washing capillary programme of water–0.1 *M* sodium hydroxide–water (1 min each) was used at the end of each injection to give inter-run reproducibility. Before sample injection, the run buffer was passed through the capillary for 1 min.

Standard and sample preparations

(±)-Cicletanine standards in the concentration range 0.4–20 μg/ml were prepared by dilution of the stock solution. To 2.0-ml aliquots of mixed heparinized plasma samples from healthy donors placed in 15-ml glass centrifuge tubes with PTFE-lined screw-caps were added 0.1-ml volumes of various standards of cicletanine to obtain calibration graphs in the range 10–500 ng cicletanine per ml of plasma for each enantiomer, and 0.1 ml of internal standard solution (500 ng racemate per ml plasma final concentration). Repeated calibration graphs were used to validate the analytical method. Then, 7 ml of diethyl ether were added to the tubes, which were placed on a mechanical shaker for 10 min and centrifuged. The organic phases (5 ml) were transferred into clean, labelled 6-ml conical glass tubes. The solvent was evaporated under a stream of nitrogen at 40°C and the extraction step was applied again to the biological residue by adding 5 ml of diethyl ether and then transferring 6 ml of the organic phases to the previous evaporation residues. The solvent was evaporated again and the residues were redissolved in 200 μl of acetonitrile–water (1:9). Volumes around 46 nl of different samples were injected into the capillary. Calibration and analysis samples from subjects receiving the drug were prepared in the same way.

Quantitation

Quantitation was by the internal standard method with a calibration graph using peak-height ratios. Data points were acquired at a rate of five per second and integrated using the Chromatography Software System Gold V. 3.11. Data were fitted by weighted least-squares linear regression using the reciprocal of the squared concentration values as a weighting factor.

RESULTS AND DISCUSSION

Linearity

Linearity was assessed in the concentration range 10–500 ng/ml for each cicletanine enantiomer. For six calibration graphs, the *S*(+)- and *R*(−)-cicletanine regression coefficients obtained were $r^2 = 0.9925$ [0.71% relative standard deviation (R.S.D.)] and $r^2 = 0.9934$ (0.77% R.S.D.), respectively. The equation parameters were: slope [*S*(+)] = 453.78 (6.6% R.S.D.), intercept [*S*(+)] = − 3.88 (74% R.S.D.), slope [*R*(−)] = 454.75 (3.5% R.S.D.) and intercept [*R*(−)] = − 2.75 (130% R.S.D.).

Recovery

The recovery of the analytical procedure for total cicletanine determined by an HPLC method (unpublished) was 78.8, 77.5 and 71.8% at (±)-cicletanine concentrations of 10, 500 and 1000 ng/ml (samples in triplicate).

Precision

Repeated calibration graphs in the concentration range 10–500 ng/ml *S*(+)- and *R*(−)-cicletanine were prepared as described under Experimental. The within-run precision of the assay was determined by analysing six calibration graphs and comparing the ratio of the response of the cicletanine and internal standard peak heights. The R.S.D. values are given in Table I. The between-day precision was evaluated by analysing calibration graphs obtained on four different days. The R.S.D. values are given in Table I. The variability of the back-calculated concentrations (*i.e.* concentration values estimated from the corresponding standard curve equations) for each theoretical concentration are summarized in Table II [*S*(+)-cicletanine] and Table III [*R*(−)-cicletanine]. Mean concentrations, standard deviations, R.S.D. values and relative errors from twelve standard graphs obtained on four different days are shown.

Selectivity and limit of detection

Fig. 2 shows electropherograms from blank human plasma and plasma spiked with 500 ng/ml (±)-cicletanine and 500 ng/ml (±)-internal standard. Irrelevant interfering peaks are visible in

TABLE I

MEANS, STANDARD DEVIATIONS (S.D.) AND RELATIVE STANDARD DEVIATIONS (R.S.D.) DERIVED FROM WITHIN-RUN AND BETWEEN-DAY PRECISION STUDIES OF THE DETERMINATION OF *S*(+)- AND *R*(−)-CICLETANINE ENANTIOMERS

Effective concentration (ng/ml)	Mean ratio of peak heights		S.D.		R.S.D. (%)	
	S(+)	*R*(−)	*S*(+)	*R*(−)	*S*(+)	*R*(−)
Within-run (n = 6):						
10	0.03196	0.02993	0.007	0.008	20.8	27.4
25	0.06374	0.05768	0.007	0.005	10.4	9.0
50	0.11743	0.11838	0.01	0.008	8.6	6.9
250	0.54221	0.55496	0.04	0.02	6.7	3.5
500	1.09250	1.11369	0.05	0.05	4.2	4.6
Between-day (n = 12):						
10	0.03044	0.02932	0.007	0.008	23.3	26.9
25	0.06378	0.06051	0.007	0.006	11.0	9.6
50	0.11722	0.11491	0.009	0.007	7.2	6.4
250	0.57005	0.57509	0.05	0.04	8.7	6.3
500	1.11798	1.15531	0.08	0.09	6.7	8.0

TABLE II

MEANS, STANDARD DEVIATIONS (S.D.) AND RELATIVE STANDARD DEVIATIONS (R.S.D.) OF BACK-CALCULATED VALUES OF STANDARD CURVES FOR *S*-(+) CICLETANINE DETERMINATION IN HUMAN PLASMA (n = 12)

Effective concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Relative error (%)
10	10.001 ± 0.27	2.70	0.01
25	25.010 ± 2.22	8.87	0.04
50	50.056 ± 4.22	8.43	0.11
250	249.019 ± 10.92	4.39	0.39
500	492.318 ± 18.98	3.85	1.54

TABLE III

MEANS, STANDARD DEVIATIONS (S.D.) AND RELATIVE STANDARD DEVIATIONS (R.S.D.) OF BACK-CALCULATED VALUES OF STANDARD CURVES FOR *R*(−)-CICLETANINE DETERMINATION IN HUMAN PLASMA (n = 12)

Effective concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Relative error (%)
10	10.204 ± 0.37	3.66	2.04
25	24.197 ± 2.43	10.05	3.21
50	48.355 ± 3.59	7.42	3.29
250	252.181 ± 10.87	4.31	0.87
500	508.505 ± 19.35	3.81	1.70

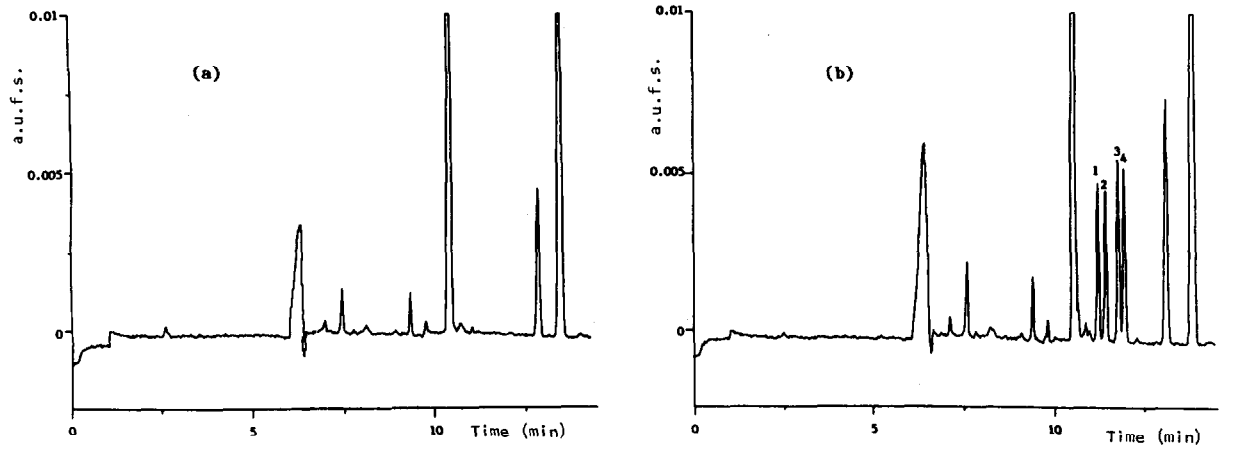


Fig. 2. Electropherograms of cicletanine enantiomers in human plasma. (a) Blank plasma; (b) plasma spiked with 500 ng/ml of cicletanine and 500 ng/ml of internal standard racemates. Peaks: 1 = *S* or *R* internal standard; 2 = *R* or *S* internal standard; 3 = *S*(+)-cicletanine; 4 = *R*(−)-cicletanine.

blank plasma samples at the migration time of the compounds of interest.

The limit of detection, expressed as the concentration of compound that produces a detector response equal to the mean blank value plus three standard deviations, was 10 ng/ml for the two cicletanine enantiomers.

Time course of cicletanine enantiomers concentration in human plasma

The method provided a selective and sensitive procedure for the determination of cicletanine enantiomers in human plasma. Tables IV and V show the time course of cicletanine enantiomers in plasma of two human volunteers receiving a single 50 mg dose of (±)-cicletanine hydrochloride by mouth while fasting. The results are interesting because no detectable concentrations of *R*(−)-cicletanine are observed in the two volunteers.

Cicletanine is primarily eliminated by conjugation with glucuronic and sulphuric acids; that excreted as the free form is negligible. The method described here can be used for the determination of free cicletanine in urine and for cicletanine determinations after urine hydrolysis with β-glucuronidase. The analytical procedure is basically the same as that described for plasma but uses a mixture of diethyl ether–*n*-hexane (20:80) as the extraction solvent instead of diethyl ether. Tables IV and V also show the amounts of cicletanine enantiomers obtained after urine hydrolysis with β-glucuronidase and the percentage of the total administered cicletanine hydrochloride recovered in urine. It is noteworthy that cicletanine is mainly recovered as the *R*(−) form, which is not seen in plasma samples.

In this method, a racemic methylated derivative of cicletanine was used as the internal standard. Two resolved electrophoretic peaks were obtained for this molecule. The use of either a (+)- or (−)-internal standard could be sufficient for quantitation purposes; however, the purified enantiomers were not available. The sum of both peak heights was used for quantitation. The non-availability of the purified enantiomers for the internal standard meant that the identification of the corresponding peaks was not possible and the purified *S*(+)- and *R*(−)-cicletanine enantiomers were used to identify the corresponding peaks. The use of β-CD instead of γ-CD resulted in two overlapping peaks which could not be resolved; one was from the internal standard with the *S*(+)-cicletanine enantiomer peak. The addition of SDS to the run buffers was very important to obtain the separation showed in Fig. 2. The ionic micelle generally migrates slower than the bulk solution in the capillary and consequently the analyte which is incorporated into the micelle migrates slower than the analyte that is in the surrounding aqueous phase [7]. The use of CDs in the run buffers promotes inclusion

TABLE IV

CICLETANINE CONCENTRATIONS FROM SUBJECT 1 AFTER A SINGLE DOSE OF 50 mg CICLETANINE BY MOUTH

Time after dose (h)	Plasma (ng/ml)		Interval after dose (h)	Urine[a] (μg)	
	S(+)	*R*(−)		*S*(+)	*R*(−)
0.17	ND[b]	ND	0–2	ND	ND
0.25	ND	ND	2–4	1114.7	1601.0
0.33	ND	ND	4–6	810.6	588.1
0.50	ND	ND	6–8	352.6	154.9
0.75	ND	ND	8–12	351.1	612.6
1	ND	ND	12–24	184.4	1421.8
1.5	63.1	ND	24–36	87.4	559.5
2	366.9	ND	36–48	ND	594.7
3	816.9	ND	48–60	75.3	466.9
4	798.1	ND			
5	456.0	ND	Total	2976.1	5999.5
7	246.6	ND			
10	95.5	ND	Dose recovered (%)	5.9	12.0
18	41.2	ND			
24	27.2	ND			
30	17.4	ND			

[a] After hydrolysis with β-glucuronidase.
[b] ND = Not detected.

TABLE V

CICLETANINE CONCENTRATIONS FROM SUBJECT 2 AFTER A SINGLE DOSE OF 50 mg CICLETANINE BY MOUTH

Time after dose (h)	Plasma (ng/ml)		Interval after dose (h)	Urine[a] (μg)	
	S(+)	*R*(−)		*S*(+)	*R*(−)
0.17	ND[b]	ND	0–2	592.1	957.5
0.25	ND	ND	2–4	2192.9	1988.7
0.33	ND	ND	4–6	1204.2	482.3
0.50	93.5	ND	6–8	548.4	200.2
0.75	238.6	ND	8–12	523.3	793.4
1	519.6	ND	12–24	769.9	2510.8
1.5	854.9	ND	24–36	416.6	1614.5
2	1204.9	ND	36–48	106.1	615.0
3	1096.1	ND	48–60	292.3	263.6
4	692.8	ND			
5	609.0	ND	Total	6645.8	9426.0
7	242.6	ND			
10	–	ND			
18	–	ND	Dose recovered (%)	13.3	18.9
24	21.4	ND			
30	ND	ND			

[a] After hydrolysis with β-glucuronidase.
[b] ND = Not detected.

complexes and the enantiomers fit into the hydrophobic cavities of these complexes; chiral selectivity is obtained when one enantiomer is more strongly included in this cavity than the other. Thus, the *S*(+) enantiomer migrated faster than the *R*(−) enantiomer, indicating that the *S*(+) enantiomer is included more strongly by γ-CDs than the *R*(−)-enantiomer.

As shown in Tables II and III, the analytical method is very accurate (relative error 0.01–1.54% for *S*(+)-cicletanine and 0.87–3.29% for *R*(−)-cicletanine. The high R.S.D. values (greater than 20%) at the lower concentration (10 ng/ml) shown in Table I could be explained by the proximity to the limit of detection of the analytical technique.

The experimental results for the time course of cicletanine enantiomers in human plasma (Tables IV and V) did not show the presence of *R*(−)-cicletanine at any time. However, when urine was hydrolysed with β-glucuronidase and then analysed, *R*(−)-cicletanine had the highest concentration. The total percentage of cicletanine recovered in urine (as the free form and after hydrolysis with β-glucuronidase) from subject 1 was 5.9 and 12.0% for the *S*-(+) and *R*(−) enantiomers, respectively. For subject 2 these percentages were 13.3 and 18.9%, respectively. These results suggest a faster metabolism of the *R*(−)-cicletanine form.

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