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Comparison of high-performance liquid chromatography and high-performance capillary electrophoresis for the determination of cicletanine in plasma

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ABSTRACT

A sensitive and selective high-performance capillary electrophoresis (HPCE) procedure was developed for the determination of total 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 sodium dodecyl sulphate in the run buffers and ultraviolet detection. The concentrations of cicletanine obtained by this method were compared with those obtained by a high-performance liquid chromatographic (HPLC) method used routinely. The within-run precision of the methods, expressed as relative standard deviation, ranged from 1.6 to 7.8% for HPLC and from 6.4 to 11.1% for HPCE. Both methods showed an adequate level of accuracy; the relative errors ranged from 0.02 to 3.25% for HPLC and from 0.21 to 2.90% for HPCE. The HPCE method required less than half the time taken by the HPLC method, making HPCE a useful alternative technique for the routine determination of cicletanine in plasma. Both methods were used to follow the time course of total cicletanine in human plasma after a single oral therapeutic dose of the drug.

INTRODUCTION

Cicletanine hydrochloride [Tenstaten, IPSEN; 2-methyl-3-hydroxy-4*H*,5*H*-5-(4′-chlorophenyl)-isofuropyridine hydrochloride] is a member of the furopyridine antihypertensive drugs. 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]. The molecule (Fig. 1) has a chiral carbon and therefore has enantiomeric forms [(*S*)-(+) and (*R*)-(−)]. Recently, a sensitive and selective high-performance capillary electrophoresis (HPCE) procedure for the determination of (*S*)-(+) and (*R*)-(−) enantiomers of cicletanine in human plasma was reported [2].

Several high-performance liquid chromatographic (HPLC) methods for the determination of total (racemate) cicletanine in plasma have

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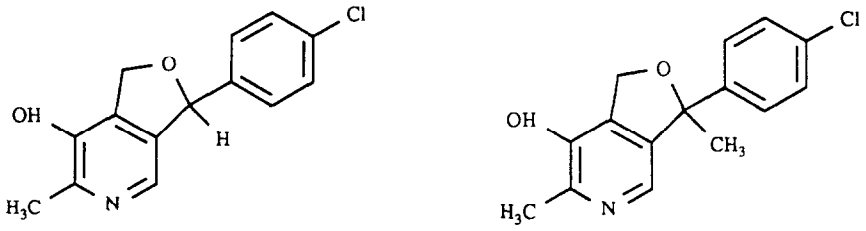

(a) (b)

Fig. 1. Structures of (a) cicletanine and (b) the internal standard.

been reported [3,4]. This paper describes a selective and sensitive HPCE method using micellar electrokinetic capillary chromatography (MECC) for the determination of cicletanine racemate in human plasma after the administration of therapeutic doses of cicletanine. The results obtained were compared with those obtained by a sensitive HPLC method (unpublished) which was developed with the aim to elucidating the pharmacokinetics of cicletanine at therapeutic dose levels (50 mg).

EXPERIMENTAL

Chemicals and reagents

(±)-Cicletanine and (±)-2-methyl-3-hydroxy-4*H*,5*H*-5-methyl-(4′-chlorophenyl)isofuropyridine hydrochloride, used as an internal standard, were supplied by Expansia (Aramon, France). Boric and phosphoric acid, isopropylamine, sodium hydroxide (analytical-reagent grade) and sodium dodecyl sulphate (SDS) (biochemistry grade) were obtained from Merck (Darmstadt, Germany). Diethyl ether, used without further purification, acetonitrile and methanol (all of HPLC grade) were supplied by 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. Water was doubly distilled and purified through a Milli-Q system (18 MΩ cm resistivity).

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

Plasma preparation and human plasma sampling

Blood and plasma from healthy donors were obtained and prepared as described elsewhere [2].

The method described here was applied to follow the time course of total cicletanine in the plasma from 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 individual 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, 30 and 36 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 P/ACE System 2000 (Beckman, Palo Alto, CA, USA). Chromatographic separations were performed using Waters HPLC equipment (Waters Assoc., Milford, MA, USA) consisting of an M-510 pump, a WISP 700 automatic injector, an M-480 UV detector and an M-840 data and chromatography control station.

Electrophoresis and column liquid chromatography

The capillary cartridge (Beckman) for the

HPCE technique contained a 75 μm I.D. fused-silica 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)–25 m*M* SDS containing 10% acetonitrile as an organic modifier. Separations were performed at 20 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 60 nl. A capillary washing programme with water–0.1 *M* sodium hydroxide–water (1 min each) was used at the end of each injection to ensure inter-run reproducibility. Before sample injection, run buffer was passed through the capillary for 1 min.

The column for the HPLC techniques was a Nova-Pak C_{18} (4 μm particle diameter) Radial Pak cartridge (Waters) of 10 × 0.8 cm I.D., inserted in a Radial Compression Module RCM 8 × 10. A precolumn (0.5 × 0.4 cm I.D.) of the same filling material was also used. The mobile phase consisted of acetonitrile–water (22:78) containing 0.02% isopropylamine (pH 2.4) with 0.085% phosphoric acid. Separations were performed at a flow-rate of 2.5 ml/min and a detection wavelength of 220 nm. The volumes injected were 100 μl. A column washing programme with acetonitrile (3 min) was used at the end of each injection.

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 different standards of cicletanine to obtain calibration graphs in the range 10–1000 ng of cicletanine per ml of plasma, and 0.1 ml of internal standard solution (500 ng/ml of plasma, final concentration). Repeated calibration graphs were used to validate the analytical methods. Then, 7 ml of diethyl ether were added to the tubes, which were placed on a mechanical shaker for 10 min and centrifuged (1000 *g*, 5 min). 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 dissolved in 200 μl of acetonitrile–water (1:9). Volumes of *ca.* 60 nl of different samples were injected into the capillary for the HPCE technique and 100 μl into the column for the HPLC technique.

Calibration and analysis samples from subjects receiving the drug were prepared in the same way and aliquots of the same sample were analysed by both the HPLC and HPCE methods.

Quantification

Quantification was effected by the internal standard method with calibration graphs using peak-height ratios. Data points for HPCE were acquired at five per second and integrated using the Chromatography Software System Gold V.3.11. For HPLC, data points were acquired at a rate of two per second and integrated using the Waters Expert System V.6.21. Data were fitted by weighted least-squares linear regression using the reciprocal of the squared concentration values as weighting factor.

RESULTS AND DISCUSSION

Linearity

Linearity was assessed in the concentration range 10–1000 ng/ml for the determination of cicletanine by HPLC and 20 – 1000 ng/ml for the determination by HPCE. For twelve calibration graphs, the regression coefficients obtained for the determination of cicletanine by HPLC and HPCE were $r^2 = 0.9980$ [relative standard deviation (R.S.D.) 0.13%] and $r^2 = 0.9951$ (R.S.D. 0.20%), respectively. The equation parameters were slope (HPLC) = 216.99 (R.S.D. 4.9%), intercept (HPLC) = 0.58 (R.S.D. 194%), slope (HPCE) = 324.44 (R.S.D. 8.6%) and intercept (HPCE) = −3.28 (R.S.D. 104%).

Recovery

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

Precision

Repeated calibration graphs in the cicletanine concentration range 10–1000 ng/ml were prepared as described under Experimetal. The within-run precision of the assays was determined by analysing six calibration graphs and comparing the ratio of the response of the cicletanine and internal standard peak heights. The between-day precision was evaluated by analysing calibration graphs obtained on three different days. The R.S.D.s for both within-run and between-day precision studies are given in Table I, where comparative values for the HPLC and HPCE techniques are shown. The variability of the back-calculated concentrations (*i.e.*, concentration values calculated from the corresponding calibration graph equations) for each theoretical concentration are summarized in Table II. Mean concentrations, S.D.s, R.S.D.s and relative errors from six standard graphs obtained in a day are given.

A graph of regression line of back-calculated concentrations (Table II) obtained by HPLC *versus* HPCE is shown in Fig. 2. The parameters for the corresponding equation expressed as the value ± confidence limit were slope = 1.016 ± 0.0286 and intercept = −5.164 ± 14.328 (r = 0.9975, n = 30, $p < 0.001$). The cicletanine concentration values obtained by HPLC and HPCE correlate well, as indicated by the slope of the linear graph not being different from the theoretical slope of unity (t = 1.148, p = 0.26) and the intercept not being different from zero (t = 0.74, p = 0.46).

Selectivity and limit of detection

Figs. 3 and 4 show chromatograms and electropherograms, respectively, from blank human plasma and plasma spiked with 500 ng/ml cicleta-

TABLE I

MEANS, STANDARD DEVIATIONS AND RELATIVE STANDARD DEVIATIONS OF PEAK-HEIGHT RATIOS DERIVED FROM WITHIN-RUN AND BETWEEN-DAY PRECISION STUDIES FOR THE DETERMINATION OF CICLETANINE IN HUMAN PLASMA BY HPLC AND HPCE

Effective concentration (ng/ml)	Mean		S.D.		R.S.D. (%)	
	HPLC	HPCE	HPLC	HPCE	HPLC	HPCE
Within-run (n = 6)						
10	0.0441	–	0.00345	–	7.8	–
20	–	0.0793	–	0.00701	–	8.8
50	0.2173	0.1724	0.00806	0.01916	3.7	11.1
100	0.4505	0.3277	0.00928	0.03295	2.1	9.7
500	2.2199	1.4579	0.05645	0.09251	2.5	6.4
1000	4.2801	2.9650	0.06799	0.26191	1.6	8.8
Between-day (n = 12)						
10	0.0434	–	0.00413	–	9.5	–
20	–	0.0727	–	0.00826	–	11.4
50	0.2204	0.1699	0.01204	0.01800	5.5	10.6
100	0.4539	0.3202	0.00846	0.03260	1.9	10.2
500	2.3227	1.5273	0.09869	0.13053	4.2	8.5
1000	4.7747	3.1644	0.41630	0.32602	8.7	10.3

TABLE II

MEANS, STANDARD DEVIATIONS AND RELATIVE STANDARD DEVIATIONS OF BACK-CALCULATED VALUES OF CALIBRATION GRAPHS FOR THE DETERMINATION OF CICLETANINE IN HUMAN PLASMA BY HPLC AND HPCE (n = 6)

Effective concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)		R.S.D. (%)		Relative error (%)	
	HPLC	HPCE	HPLC	HPCE	HPLC	HPCE
20	19.97 ± 0.256	19.88 ± 0.548	1.3	2.8	0.16	0.58
50	50.13 ± 1.571	50.11 ± 2.790	3.1	5.6	0.27	0.21
100	100.02 ± 4.767	102.50 ± 6.402	4.8	6.2	0.02	2.50
500	512.34 ± 16.968	485.51 ± 31.180	3.3	6.4	2.47	2.90
1000	967.48 ± 32.713	993.23 ± 42.027	3.4	4.2	3.25	0.68

nine and 500 ng/ml internal standard. The limit of detection, expressed as the concentration of compound that produces a detector response equal to the mean blank plus three standard deviations, was 10 ng/ml for HPLC and 20 ng/ml for HPCE.

Time course of cicletanine concentration in plasma of humans

The methods described provided selective and sensitive procedures for the determination of total cicletanine in human plasma. Table III shows the time course of cicletanine in plasma, determined by HPLC and HPCE, for two human volunteers receiving a single 50-mg oral dose of cicletanine hydrochloride while fasting. Good agreement between plasma cicletanine values obtained by HPLC and HPCE was observed. For subject 1 the differences between the two methods were < 10% except for samples corresponding to 18 and 24 h after dose, where large discrepancies were manifest. For subject 2, all the differences were < 15%.

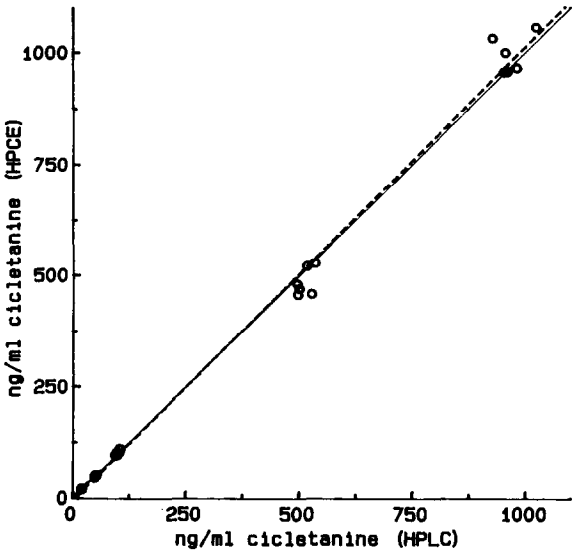

Fig. 2. Correlation graph (dashed line) for plasma cicletanine concentration values obtained by HPLC and HPCE. The continuous line represents the theoretical line of slope = 1 and intercept = 0.

As shown in Table I, the R.S.D.s for the HPLC technique (1.6–7.8% for within-run and 1.9–9.5% for between-day precision) are lower than those for the HPCE method (6.4–11.1% and 8.5–11.4%, respectively). The same is observed for accuracy (Table II), both techniques being very accurate (relative error 0.02–3.25% for HPLC and 0.21–2.90% for HPCE). Although the accuracy and precision are slightly better for HPLC it should be noted that the HPCE technique required less than half the time for the HPLC method. The run time of analysis plus the washing and equilibration times for the capillary or the column were 12 and 27 min for HPCE and HPLC, respectively. When the HPCE technique described here is compared with a published HPLC method with a similar limit of detection [3], the HPCE technique also resulted in a slightly shorter analysis time (12 *versus* 15 min). Further, the

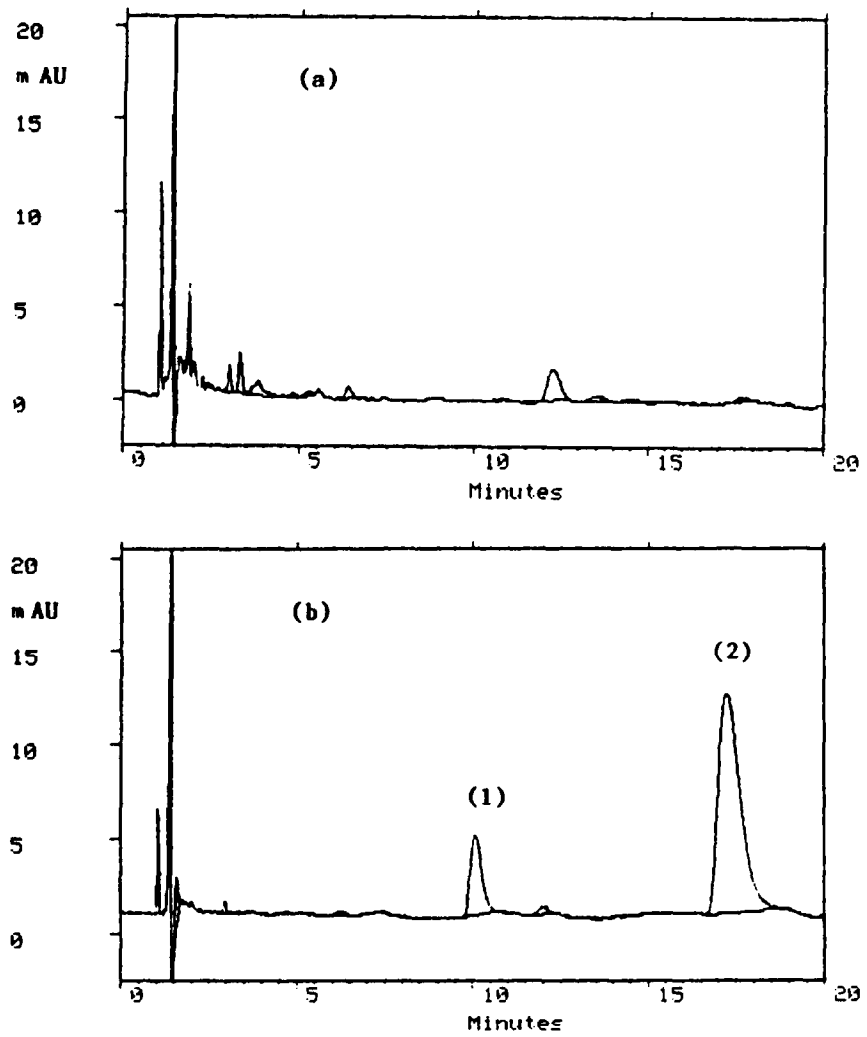

Fig. 3. Chromatograms of cicletanine in human plasma. (a) Plasma blank; (b) plasma sample from a subject 15 min after receiving a single oral dose of 50 mg of cicletanine. Peaks: 1 = cicletanine; 2 = internal standard. The cicletanine concentration was found to be 371.5 ng/ml.

low volumes of sample injected in HPCE would permit several replicate injections whereas in HPLC this would not be possible. Both techniques could easily be automated, making HPCE a useful alternative technique to HPLC for the routine determination of cicletanine in plasma (pharmacokinetic studies). The fact that the separation principles of HPLC and HPCE techniques are different opens up new perspectives for such separations.

The limits of detection with the HPLC and HPCE techniques were 10 and 20 ng/ml, respectively, the injection volumes into the column and capillary being 100 μl and 60 nl, respectively. The detection limit for HPLC is only a factor of 2 lower while the injection volume is more than 1000 times higher, and the dimensions of the UV detector cell are *ca.* 1000 times higher than in HPCE with the intruments used. The extremely low injection volumes used in HPCE suggest the possibility of using smaller volumes of starting plasma. This would lead to an important consid-

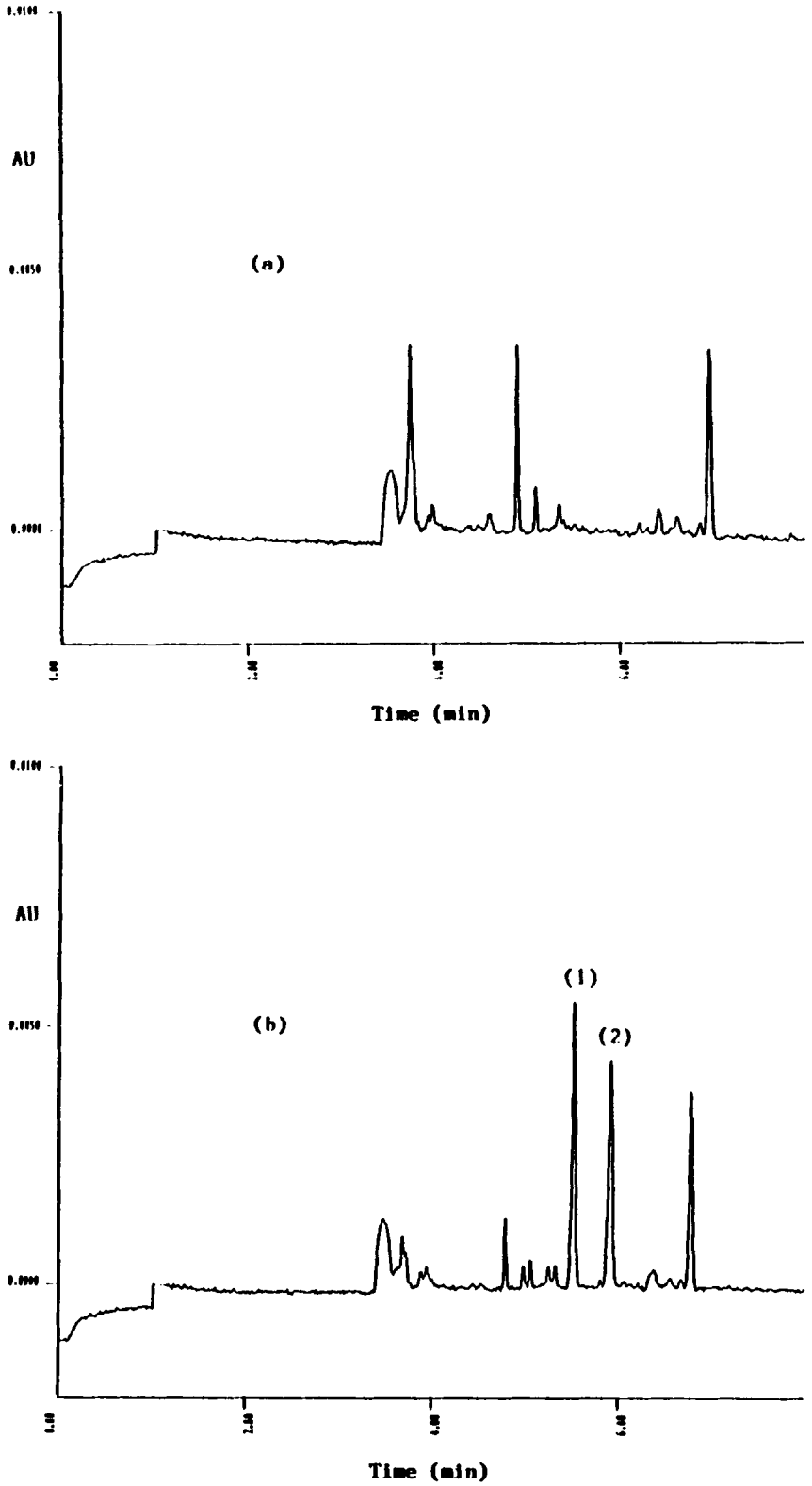

Fig. 4. Electropherograms of cicletanine in human plasma. (a) Plasma blank; (b) plasma sample from a subject 5 h after receiving a single oral dose of 50 mg of cicletanine. Peaks: 1 = cicletanine; 2 = internal standard. The cicletanine concentration was found to be 447 ng/ml.

TABLE III

CICLETANINE PLASMA CONCENTRATIONS DETERMINED BY HPLC AND HPCE FOR TWO SUBJECTS AFTER A SINGLE ORAL DOSE OF 50 mg OF CICLETANINE

At 0.167, 0.25 and 0.33 h after dose no cicletanine was detected.

Time after dose (h)	Subject 1				Subject 2			
	Plasma cicletanine (ng/ml)			Difference (%)[a]	Plasma cicletanine (ng/ml)			Difference (%)[a]
	HPLC	HPCE	Difference		HPLC	HPCE	Difference	
0.50	N.D.[b]	N.D.	–	–	76.0	N.A.[c]	–	–
0.75	N.D.	N.D.	–	–	180.7	N.A.	–	–
1	N.D.	N.D.	–	–	458.6	525.8	67.2	14.6
1.5	67.5	70.4	2.9	4.3	865.5	909.2	43.7	5.0
2	353.3	366.2	12.9	3.6	1220.1	1268.1	48.0	3.9
3	861.0	832.6	28.4	3.3	1228.7	1174.0	54.7	4.4
4	798.2	756.1	42.1	5.3	872.1	779.3	92.8	10.6
5	489.5	447.0	42.5	8.7	627.8	630.2	2.4	0.4
7	207.9	196.1	11.8	5.7	278.2	261.0	17.2	6.2
10	94.7	103.5	8.8	9.3	141.0	154.0	13.0	9.2
18	35.6	58.9	23.3	65	30.3	30.7	0.4	1.3
24	23.3	65.6	42.3	181	18.1.	N.A.	–	–
30	N.D.	28.6	–	–	15.5	16.2	0.7	4.5
36	N.D.	N.D.	–	–	N.D.	N.D.	N.D.	–

[a] Percentage difference with respect to the HPLC values.
[b] N.D. = not detected.
[c] N.A. = sample not available.

eration in experimental pharmacokinetics with small animals with the possibility that the pharmacokinetic profile of a drug could be assayed with the same animal. This could therefore mean an important saving in the number of animals that need to be killed and also the possibility of following individual pharmacokinetc parameters for these small animals, improving the information and the quality of the studies.

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