Journal of Chromatography, 573 (1992) 93–98
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6095

Determination of cicletanine in human plasma by high-performance liquid chromatography using automated column switching

Susan M. Antoniewicz*

Department of Drug Metabolism and Pharmacokinetics, Sterling Research Group, 81 Columbia Turnpike, Rensselaer, NY 12144 (USA)

Jack A. Cook

Pharmacokinetics/Drug Metabolism Department, Parke-Davis Research Center, 2800 Plymouth Road, P.O. Box 1047, Ann Arbor, MI 48106-1047 (USA)

Richard R. Brown

Department of Drug Metabolism and Pharmacokinetics, Sterling Research Group, 81 Columbia Turnpike, Rensselaer, NY 12144 (USA)

(First received April 16th, 1991; revised manuscript received August 6th, 1991)

ABSTRACT

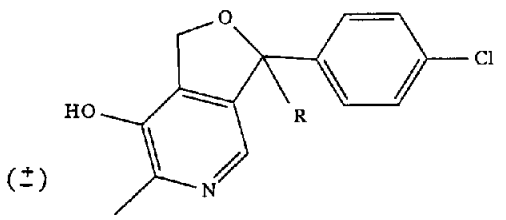
The automated determination of cicletanine in human plasma by column-switching high-performance liquid chromatography with fluorescence detection is described. A linear response was observed for the plasma calibration curve in the range 0.05–10 μg base/ml. The minimum quantifiable level of the assay was 0.05 μg base/ml plasma. The extraction efficiency was approximately 98% for both cicletanine and the internal standard. Precision (coefficient of variation) ranged from 0.9 to 6.3% and the accuracy ranged from −8.5 to 5.3%. Freezing samples up to three months had no apparent effect on sample integrity. The method has been used to analyze rat and dog plasma samples from toxicology studies as well as human plasma from phase I and II clinical trials. This method has several advantages over previously published methods including reduced sample volume requirement and minimal sample preparation.

INTRODUCTION

Cicletanine, (±)-1,3-dihydro-3-(4-chlorophenyl)-7-hydroxy-6-methylfuro[3,4-*c*]pyridine hydrochloride, is a novel antihypertensive agent with a chemical structure (Fig. 1) characterized by the presence of a furopyridine group [1]. It exhibits vasorelaxant, natriuretic, and diuretic actions in preclinical and clinical studies [1–4]. However, the mechanism(s) responsible for cicletanine's antihypertensive activity has not been clearly established. Cicletanine stimulates prostacyclin synthesis and appears to antagonize intracellular Ca^{2+} mobilization which may contribute to vascular smooth muscle relaxations. Recently, it has been reported that cicletanine inhibits cGMP phosphodiesterases with low Michaelis constants (K_M) which may also contribute to the vasorelaxant and antihypertensive actions of cicletanine [5]. Cicletanine has also been reported to reduce cardiac and renal hypertrophy in animal studies [4].

Previous studies used analytical methods [6,7] that required 1.0 ml of plasma for the quantita-

0378-4347/92/$05.00



Compound	R	Mol. Wt.
Cicletanine Hydrochloride	H	298.2
Internal Standard	CH_3	275.8

Fig. 1. Structures of cicletanine and the internal standard.

tion of cicletanine concentrations. However, the prospect of human pharmacokinetic studies requiring multiple blood samples or preclinical studies involving rodent as test animals necessitated the development of an assay which utilizes a smaller volume of plasma. This report describes a method for the quantitation of cicletanine using a reversed-phase column-switching high-performance liquid chromatography (HPLC) procedure [8,9] which is automated, sensitive, and utilizes 0.10 ml of plasma.

EXPERIMENTAL

Reagents

The hydrochloride salt of cicletanine and the internal standard (Fig. 1) were obtained from Biomeasure (Hopkinton, MA, USA) and used without further purification.

Monobasic potassium phosphate (J. T. Baker, Phillipsburg, NJ, USA), citric acid (Mallinckrodt, Paris, KT, USA), sodium citrate (Mallinckrodt), and orthophosphoric acid (85%, Fisher, Fairlawn, NJ, USA) were all ACS grade or better. Acetonitrile, methanol, and water were all HPLC grade from J. T. Baker.

Citrate buffer was prepared by the gradual addition of 0.5 *M* sodium citrate to 0.5 *M* citric acid until the resultant pH equalled 2.5, then diluted 1:1 (v/v) with water resulting in a final molar concentration of 0.25 *M*. Monobasic potassium phosphate (0.1 *M*) was adjusted to pH 2.5 with orthophosphoric acid.

Preparation of plasma calibration curve standards

A 200 μg base/ml solution of cicletanine in methanol was prepared. From this stock solution, dilutions were made in methanol to obtain concentrations of 1.0, 2.0, 10.0, 20.0, 50.0, 100 and 150 μg base/ml. Portions (50 μl) of the appropriate diluted stock solutions were pipetted into round-bottom tubes and evaporated to dryness under a nitrogen stream. To each residue was added 1.0 ml of control human plasma (potassium oxalate anticoagulant), and the mixture vortexed, thus creating a nine-point calibration curve with concentrations of 0 (methanol compensate), 0.05, 0.10, 0.50, 1.0, 2.5, 5.0, 7.5, and 10 μg base/ml.

Preparation of spiked plasma samples

The spiked plasma samples were prepared by pipetting appropriate aliquots of the stock standard solutions into round-bottom tubes and evaporating to dryness under a nitrogen stream. Each residue was dissolved with 1.0 ml of control human plasma (potassium oxalate anticoagulant) to give final concentrations of 0 (methanol compensate), 0.11, 0.90, 3.0, and 8.0 μg base/ml. Three sets of five replicates of each concentration were randomized and prepared for analysis under single-blind conditions.

The first set was analyzed upon preparation (fresh set); the second set was analyzed after eight days of storage in the laboratory freezer (frozen set 1) by a second analyst; the third set was analyzed after eleven days of storage in the laboratory freezer (frozen set 2) by the original analyst.

Sample preparation procedure

A 100-μl portion was taken from each plasma standard or spiked sample and placed in a clean test tube. To each 100-μl portion were added 200 μl of the internal standard solution (0.50 μg/ml in 0.25 *M* citrate buffer, pH 2.5), followed by vortex-mixing for approximately 10 s. For analysis, 50 μl were injected onto the chromatographic system.

Instrumentation and chromatographic conditions

The HPLC system was operated isocratically at ambient temperature. The system included two columns; a customized ion-exchange column (Bondapak Cx/Corasil, 37–50 μm, 5 cm × 2 mm I.D., Waters Assoc., Milford, MA, USA) was employed for sample clean-up and concentration, and a chemically bonded phase of the C_{18} type (reversed-phase) was used for analytical separation [Nova-Pak C_{18} 4 μm Radial-Pak cartridge (10 cm × 8 mm) in a RCM 8 × 10, Waters Assoc.]. The pre-column for the analytical column was a Bondapak C_{18}/Corasil column (37–50 μm, 23 mm × 3.9 mm I.D., Waters Assoc.).

Samples were injected onto the ion-exchange column and washed with water (1.0 ml/min) for 1.0 min. The column eluent passed through a Rheodyne 7000 switching valve (Fig. 2) to waste. Concurrently, the analytical mobile phase (0.1 *M* monobasic potassium phosphate adjusted to pH 2.5 with orthophosphoric acid–acetonitrile, 75:25, v/v) was directed through the analytical column at a flow-rate of 2.0 ml/min. At 1 min, the switching valve re-directed the flow of analytical mobile phase such that it passed through the ion-exchange column in a backflush mode eluting the sample onto the analytical column. This was continued for 2 min at which time the switching valve completed its cycle and the ion-exchange column was re-equilibrated with water in preparation for the injection of the next sample. This process was automated using a Hewlett Packard sampler-event control module interfaced with an HP 3357 LAS computer system (Hewlett Packard, Palo Alto, CA, USA).

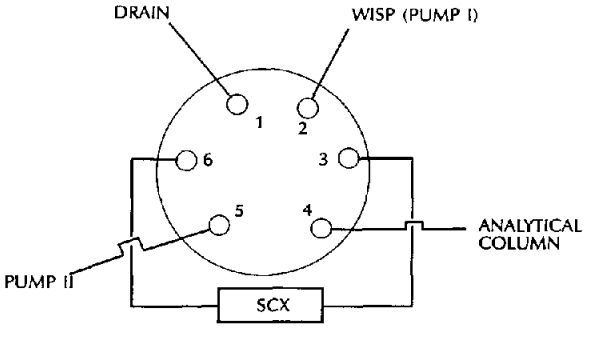


Fig. 2. Schematic of the switching column interfaced to the chromatographic system.

The analytical system also included a Waters intelligent sample processor (WISP, Waters Assoc.), two Varian 2510 solvent-delivery pumps (Varian Assoc., Walnut Creek, CA, USA), and a Kratos Spectroflow 980 fluorescence detector [excitation wavelength 290 nm, emission wavelength > 370 nm (filter), Applied Biosystems, Ramsey, NJ, USA].

Data processing

The detector was interfaced with an HP 3357 LAS computer system (Hewlett Packard) for data acquisition and processing. Drug concentrations were determined by inverse prediction from a linear regression of standard peak-height ratios as a function of calibration curve concentrations. The observed concentrations for the spiked samples were expressed as percentage differences from the nominal values. The range of these percentage differences was used to define the accuracy of the assay. Precision was estimated from the coefficient of variation (C.V.).

Extraction efficiency

In a separate experiment, the extraction efficiency was determined by comparing standards dissolved in 0.25 *M* citrate buffer pH 2.5 (1.0 ml) and direclty injected (direct standards) onto the analytical column with standards prepared in plasma using the sample preparation procedure described above (processed standards) and injected onto the two-column HPLC system described above. Direct standards were prepared in triplicate at concentrations of 0, 0.10, 1.0, and 7.5 μg base/ml. Internal standard (200 μl per sample at a concentration of 0.5 μg/ml) was added to each standard. Processed standards were prepared in triplicate at concentrations equal to those used in the direct standards.

The recovery of cicletanine and the internal standard were calculated at each concentration by dividing mean peak areas obtained from the processed standards by the mean peak areas obtained from the direct standards.

RESULTS AND DISCUSSION

Chromatography

Fig. 3A shows a representative chromatogram

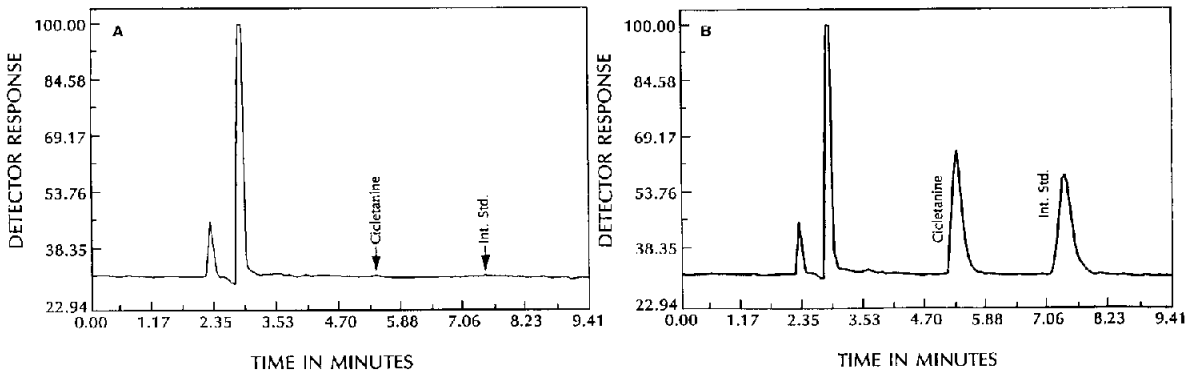


Fig. 3. Computer-generated chromatograms of plasma analysis. (A) Processed control human blank. (B) Plasma sample from a healthy volunteer taken 1 h after a 50-mg oral dose of cicletanine.

of a processed human plasma sample (blank). The chromatogram of this blank sample indicates that there are no apparent interferences from endogenous compounds. Blank plasma samples from rat, dog, and monkey similarly suggested the absence of endogenous interferences. Fig. 3B shows a plasma sample from a healthy volunteer taken 1 h after a 50-mg oral dose of cicletanine. Approximate retention times were 5.3 min for cicletanine and 7.3 min for the internal standard.

It should also be noted that sodium heparin may also be used as an anticoagulant with no apparent chromatographic interference.

Linearity and limit of detection

A regression analysis of the peak-height ratios *versus* concentration demonstrated linearity over the range 0.05–10 μg base/ml. Calibration curves were truncated to 1.0 μg base/ml. Truncation was used to allow for more accurate quantitation of samples with low concentrations. Data for those samples having concentrations less than or equal to the nominal maximum standard concentration on the truncated calibration curve were obtained from the truncated curve; data for all other samples were obtained from the full calibration curve. The minimum quantifiable level (MQL) was defined as the lowest standard which gave a regression-estimated concentration within 15% of the nominal concentration, 0.05 μg base/ml.

Precision, accuracy and recovery

Table I summarizes the validation data for fresh and frozen sets of spiked plasma samples. Analysis of one of the sets of spiked plasma samples (frozen set 1) was performed by a second analyst to demonstrate that the assay was analyst-independent. All samples of 0 μg base/ml concentrations gave values of < MQL. The accuracy of the assay, defined as the mean percentage difference from the nominal value, ranged from −8.5 to 5.3%. The precision, as defined by the C.V. of observed concentrations of the validation samples, ranged from 0.9 to 6.3%. The extraction efficiency of the assay was approximatley 98% for both cicletanine and the internal standard.

Affect of freezing on sample integrity

There was no apparent freezing affect (Table I) as assayed concentrations of samples frozen for up to eleven days at −20°C were not markedly different from nominal concentrations. Nor were they markedly different from samples analyzed immediately after preparation. The performance characteristics of the analytical method have been investigated over a three-month period. The mean (±S.D.) concentrations for the frozen quality control samples of cicletanine was 0.60 ± 0.03 μg/ml (nominal concentration was 0.60 μg base/ml), indicating no apparent degradation of cicletanine in frozen plasma samples.

Utility

The utility of the method has been demonstrated by the analysis of plasma samples from mice, rats, dogs, and humans. The time course of plasma concentrations of cicletanine in a healthy volunteer following a single 50-mg oral dose of cicletanine is presented in Fig. 4. The maximum plas-

TABLE I

CONCENTRATION DATA FOR VALIDATION SAMPLES OF CICLETANINE IN PLASMA

Samples with nominal concentrations of 0.0 μg/ml gave values < MQL.

Nominal concentration (μg/ml)	Fresh set concentration (μg/ml)	Frozen set 1 concentration (μg/ml)	Frozen set 2 concentration (μg/ml)
0.11	0.097	0.118	0.107
	0.103	0.100	0.108
	0.100	0.107	0.109
	0.104	0.104	0.121
	0.099	0.110	0.107
Mean	0.101	0.108	0.110
S.D.	0.003	0.007	0.006
C.V. (%)	2.864	6.305	5.420
0.90	0.909	0.935	0.917
	0.932	0.923	0.927
	0.922	0.947	0.921
	0.952	0.935	0.937
	0.950	0.934	0.946
Mean	0.933	0.935	0.930
S.D.	0.018	0.008	0.012
C.V. (%)	1.968	0.909	1.276
3.0	3.095	3.102	3.151
	3.055	3.232	3.098
	2.865	3.165	3.187
	3.060	3.031	3.172
	2.857	3.127	3.182
Mean	2.986	3.131	3.158
S.D.	0.116	0.075	0.036
C.V. (%)	3.869	2.380	1.149
8.0	8.219	8.495	8.076
	8.638	8.386	8.097
	8.368	7.946	8.188
	8.261	8.343	8.042
	7.822	7.938	8.239
Mean	8.262	8.222	8.128
S.D.	0.295	0.261	0.082
C.V. (%)	3.571	3.177	1.010

ma cicletanine concentration observed in this individual was 2.52 μg/ml; the terminal half-life was 3.63 h.

The performance characteristics of the analytical method were investigated over a three-month period. The coefficient of determination (r^2) from each calibration curve was 0.999 or greater. The C.V. for the mean values of the calibration curve slope was 6% ($n = 18$). The lowest calibration standard 0.05 μg base/ml was found to have met the criteria for MQL for each analytical run. The mean (± S.D.) observed concentration for the standard was 0.049 ± 0.004 μg base/ml ($n = 18$). The analytical column performed for approximately 600–800 injections before routine replacement occurred. To prevent pressure build-up on the ion-exchange column, the end fittings were routinely replaced every 200–300 injections, with replacement of the entire column occurring every 500–600 injections.

The analytical method presented in this paper offers the following advantages over previously reported methods [6,7]:

(1) The method is automated.

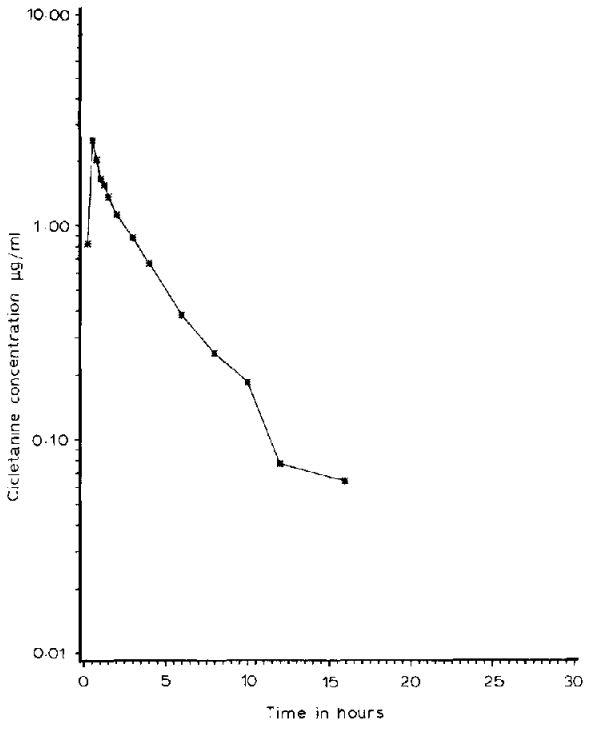


Fig. 4. Time course of plasma concentrations of cicletanine in a healthy volunteer following a 50-mg oral dose of cicletanine.

(2) The method utilizes a reduced volume of plasma, 0.10 ml.

(3) The method requires a minimal amount of sample preparation and minimized analyst exposure to potentially hazardous or infectious plasma.

(4) The method offers high sample throughput (100 samples or more can be processed in a 24-h period). The rate-limiting step is the time required for chromatographic analysis (approximately 10 min).

The validation of an HPLC method for the quantitation of cicletanine in urine has been performed in our laboratories and will be presented in a separate publication.

CONCLUSION

An automated method for the determination of cicletanine in human plasma by column-switching HPLC has been developed. The method has several advantages over previously published methods and has been used to analyze both animal and human plasma samples.

ACKNOWLEDGEMENT

The authors wish to thank Dr. R. Allen Buchholz for his assistance in the preparation of this manuscript.

REFERENCES

1 P. E. Chabrier, P. Guinot, T. Tarrade, M. Auguet, M. Cabanie, F. Clostr, A. Etienne, A. Esanu and P. Braquet, *Cardiovasc. Drug Rev.*, 6 (1988) 166.
2 G. P. Fodor and P. Guinot, *Drug Exp. Clin. Res.*, 14 (1988) 195.
3 T. Tarrade and P. Guinot, *Drug Exp. Clin. Res.*, 14 (1988) 205.
4 E. Malherbe, M. LeHegarat, F. Clostre and P. Braquet, *Drug Exp. Clin. Res.*, 14 (1988) 83.
5 P. Silver, R. A. Buchholz, R. L. Dundore, A. L. Harris and E. D. Pagani, *J. Cardiovasc. Pharmacol.*, 16 (1990) 501.
6 G. Cusinaud, M. Terrier, N. Ferry, S. Proust and J. Sassard, *J. Chromatogr.*, 341 (1985) 97.
7 F. Clostre and A. Etienne, *Drug Exp. Clin. Res.*, 14 (1988) 73.
8 W. Voelter and T. Kronbach, *J. Chromatogr.*, 239 (1982) 475.
9 W. Roth and K. Beschke, *J. Pharm. Biomed. Anal.*, 2 (1984) 289.