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Short communication

Sensitive high-performance liquid chromatographic determination of cyclizine and its demethylated metabolite, norcyclizine, in biological fluids using coulometric detection

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Abstract

An accurate, sensitive, selective and reproducible high-performance liquid chromatographic method with coulometric detection for the determination of cyclizine and its inactive demethylated metabolite, norcyclizine, in biological fluids has been developed. The drugs were separated using a custom packed reversed-phase C_{18} analytical column and phosphate buffer (0.05 *M*, pH 3)–acetonitrile (7:3) as mobile phase. The dual electrode coulometric detector was operated in the "oxidative-screen" mode with the upstream electrode (detector 1) set at 0.55 V and the downstream electrode (detector 2) set at 0.90 V. Serum and urine samples were prepared for analysis by solid-phase extraction, followed by a simple phase-separation step. The limit of quantitation was 1 ng/ml for both cyclizine and norcyclizine in serum and urine.

1. Introduction

Cyclizine is a piperazine derivative that has been used effectively for the prevention and treatment of nausea and vomiting associated with motion sickness. Despite widespread use of the drug, particularly for paediatric application, little is known about the pharmacokinetics of this compound. This paucity of information is more than likely a result of the lack of a suitable analytical method required to accurately quantitate the low levels of cyclizine obtained following therapeutic doses of the drug.

Methods for the determination of cyclizine in biological fluids using methyl orange [1] or derivatization with tritiated acetic anhydride [2] lacked specificity and proved to be relatively inaccurate for the determination of low concentrations of cyclizine found following administration. A gas–liquid chromatographic method utilizing nitrogen-selective detection has been used to determine plasma and urine concentrations of the drug [3] and a similar system has been used to determine blood and urine concentrations of the drug [4]. More recently, a high-performance liquid chromatographic (HPLC) method employing ultraviolet detection at 200 nm was applied to determine serum and urine concentrations of both cyclizine and its inactive demethylated metabolite norcyclizine following administration of a single oral dose (50 mg) of cyclizine to a single male volunteer [5].

HPLC with electrochemical detection provides an alternate method for the selective, accurate

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and precise quantitation of compounds in biological fluids and has been used successfully for the determination of drugs that ordinarily require monitoring at 200 nm or specialized equipment to achieve the desired sensitivity and selectivity for application in pharmacokinetic studies [6–8].

The analytical method reported in this note utilizes reversed-phase HPLC with coulometric detection (ECD) in the "oxidative-screen" mode for the accurate, precise, sensitive and selective determination of both cyclizine and norcyclizine in biological fluids using a modified solid-phase extraction procedure of our previously reported method [5], followed by a simple phase-separation step.

2. Experimental

2.1. Reagents and chemicals

Cyclizine hydrochloride (CYC) was obtained from Wellcome (Pty), South Africa, norcyclizine (NCYC) and the internal standard, chlorcyclizine hydrochloride (CLCYC) were obtained from the Wellcome Foundation, UK. As there are no solvents designed specifically for ECD, the acetonitrile was distilled-in-glass UV grade (Burdick and Jackson Labs., Muskegon, WI, USA). The 0.05 *M* phosphate buffer was prepared by adding 3.2 ml of phosphoric acid to 1000 ml of HPLC grade water, that had been purified through a Milli-Q system (Millipore, Bedford, MA, USA) and adjusting to a pH of 3.0 with AnalaR grade sodium hydroxide pellets (BDH Chemicals, Poole, Dorset, UK).

2.2. Chromatographic system and conditions

The modular HPLC system consisted of Waters Model M 6000A dual piston constant flow pump (Waters Assoc., Milford, MA, USA), an automated Waters Intelligent Sample Processor Model 710B (Waters Assoc.), a Model 5100A Coulochem dual electrode electrochemical detector with a Model 5010 analytical cell (Environmental Sciences Associates, Bedford, MA, USA) preceded by a carbon filter and a dual-pen Model 561 strip chart recorder (Hitachi, Tokyo, Japan) to record data. The mobile phase was constantly degassed using an in-line degasser Model ERC-3510 (Erma Optical Works, Tokyo, Japan). In addition, the system included a Model 5020 guard cell (Environmental Sciences Associates) preceded by a carbon filter. The analytical column was a custom packed 15 cm × 4.1 mm I.D. stainless-steel column, packed with Techsil 5 μm octadecylsilane (C_{18}) material (HPLC Technology, Cheshire, UK). The analytical column was preceded by an Uptight Precolumn Kit (Upchurch Scientific, Oak Harbor, WA, USA) packed with glass beads. Both the guard and analytical columns were maintained at 30°C with a Model LC-22 temperature controller (Bioanalytical Systems, West Lafayette, IN, USA).

The mobile phase was 0.05 *M* phosphate buffer (pH 3)–acetonitrile (7:3) which had been degassed and filtered through a 0.45-μm membrane filter (type HVLP, Millipore). The flow-rate was 1.0 ml min^{-1} with a column back pressure of 100 bar. Under these conditions, the retention times were approximately 5 min for NCYC, 6 min for CYC and 12 min for the internal standard. The guard cell potential was set at +1.00 V and the analytical cell potentials at +0.55 V for the first electrode and +0.90 V for the second electrode with a gain of 80. The chart speed was 2.5 mm min^{-1} and the chart recorder input 10 mV full scale.

2.3. Extraction procedure

The previously described extraction procedure [5] initially involved deproteinization of a 1-ml sample of serum or urine by mixing with 1 ml of acetonitrile, vortexing for 30 s and then centrifuging for 5 min at 1600 *g*. The resultant supernatant was transferred to a 15-ml culture tube (Kimble, Vineland, NJ, USA) containing 4 ml of water and 100 ng of internal standard that had been added as a solution (100 μl) with the aid of a Micro LabP automated dispenser (Hamilton Bonaduz AG, Bonaduz, Switzerland). The diluted supernatant was vortexed for 30 s and loaded onto a pre-washed 1-ml Bond Elut C_{18} disposable extraction column (Analytichem

International, Harbor City, CA, USA) with the aid of a custom-made glass reservoir. The pre-wash sequence involved wetting the column with 5 ml acetonitrile and then washing with 5 ml acetonitrile–0.05 *M* (pH = 3) phosphate buffer (70:30) followed by a 5-ml water wash. On completion of sample loading the columns were washed with 5 ml water followed by a 5-ml acetonitrile–water wash (70:30). Washing, sample loading and clean-up of the columns and samples were achieved by allowing the respective solutions to drain through the columns under gravity. Care was taken to ensure that drying out of the column did not occur at any stage of sample preparation. Following the final wash, columns were dried under vacuum using a modified Vac-Elut system (Analytichem International). Samples were eluted into 3-ml Kimax tapered collection tubes (Kimble), with three 500-μl aliquots of acetonitrile–0.05 *M* (pH = 3) phosphate buffer (70:30). The samples were then evaporated to dryness at 40°C in a rotary vacuum centrifuge (Savant Instruments, Hicksville, NY, USA). The residue was reconstituted in 20 μl of water and vortexed for 1 min. On addition of 30 μl acetonitrile two layers formed. The samples were then vortexed for a further minute and centrifuged at 1600 *g* for 30 s. An aliquot (25–30 μl) of clean supernatant was transferred to a WISP limited-volume insert (Waters Associates) using a 100-μl micro-syringe (Hamilton Company, Reno, NA, USA). Aliquots (2–10 μl) of the supernatant were injected onto the column for analysis. Modification of this method for use with electrochemical detection was achieved by removal of the "elution buffer" conditioning step and utilizing a 20-ml water wash after sample loading. Both CYC and NCYC were extracted from 1-ml aliquots of serum (Fig. 1) and from 500-μl aliquots of urine (Fig. 2). Following reconstitution, aliquots of 5–15 μl of the acetonitrile layer were injected onto the chromatographic system for analysis.

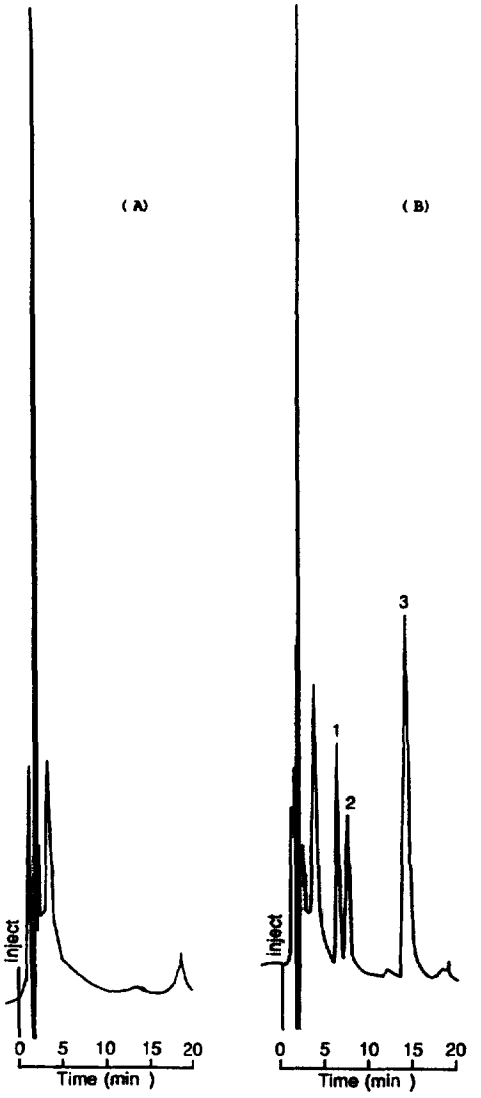

Fig. 1. HPLC chromatograms of blank human serum (A) and human serum (B) spiked with (1) norcyclizine (20.02 ng/ml), (2) cyclizine (20.30 ng/ml), and (3) 100 ng/ml of internal standard (chlorcyclizine).

3. Results and discussion

Calibration curves for both CYC and NCYC were constructed after extraction of serum and urine samples, by linear regression of plots of peak height ratios of either NCYC or CYC and internal standard versus concentration of NCYC or CYC, respectively, and were found to be linear over the calibration range of 0–100 ng/ml for serum and 0–400 ng/ml of urine. The standard curves were linear for both NCYC and CYC in both matrices with linear regression equations for NCYC defined by $y = 0.0329x + 0.0501$ ($r^2 = 0.9995$) and $y = 0.00538x - 0.00194$

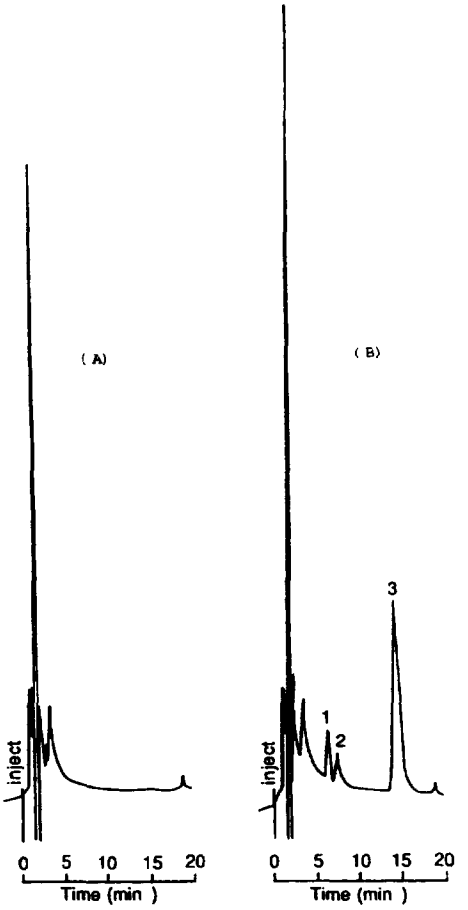

Fig. 2. HPLC chromatograms of blank human urine (A) and human urine (B) spiked with (1) norcyclizine (8.01 ng/ml), (2) cyclizine (8.22 ng/ml) and (3) 200 ng/ml of internal standard (chlorcyclizine).

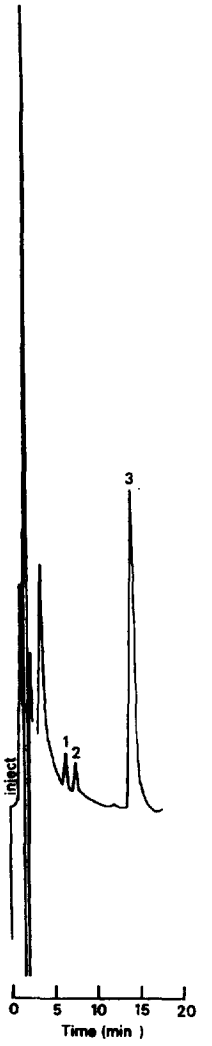

Fig. 3. HPLC chromatogram of human serum showing (1) norcyclizine (3.79 ng/ml), (2) cyclizine (3.59 ng/ml) and 100 ng/ml of (3) internal standard (chlorcyclizine) after oral administration (50 mg) of cyclizine hydrochloride to a human volunteer.

($r^2 = 0.9993$) for serum and urine, respectively, and for CYC, $y = 0.0251x + 0.0505$ ($r^2 = 0.9999$) and $y = 0.00414x + 0.0251$ ($r^2 = 0.9998$) for serum and urine, respectively.

The limit of quantitation (LOQ) based on a relative standard deviation and accuracy of $\leq 10\%$ was found to be 1 ng/ml. This corresponds to an on-column load of 0.5 ng of either NCYC or CYC in a 15-μl injection using 50% of the final reconstitution. Fig. 3 shows a typical chromatogram, in the region of the limit of quantitation, obtained following administration of 50 mg cyclizine hydrochloride to a human volunteer as a single oral dose.

Accuracy, defined as the closeness in agreement between the test result and the accepted reference value and precision defined as the closeness of agreement between independent test results obtained under prescribed conditions [9] were determined for both CYC and NCYC in serum (Table 1) and urine (Table 2) over the concentration ranges studied. Inter-day precision for NCYC and CYC in serum samples at high,

Table 1
Precision and accuracy data for the extraction of norcyclizine and cyclizine from serum

Spiked concentration (ng/ml)	n	Assayed concentration (mean ± S.D.) (ng/ml)	Precision (R.S.D., %)	Accuracy (% error)
Norcyclizine				
1.001	6	1.02 ± 0.14	5.58	+1.89
2.002	6	2.31 ± 0.37	9.53	+15.38
5.005	6	4.89 ± 0.40	6.29	−2.30
10.10	6	10.56 ± 0.57	4.71	+5.49
20.02	6	20.18 ± 1.04	4.80	+0.80
50.05	6	48.39 ± 1.44	2.89	−4.18
100.10	6	100.84 ± 3.03	2.93	+0.74
Cyclizine				
1.02	6	0.959 ± 0.18	6.78	−5.47
2.03	6	2.21 ± 0.16	3.46	+8.62
5.08	6	4.80 ± 0.35	4.96	−5.47
10.15	6	9.96 ± 0.53	4.34	−1.88
20.30	6	20.68 ± 0.76	3.33	+1.85
50.75	6	50.89 ± 1.67	3.15	+0.27
101.50	6	101.36 ± 2.40	2.31	−0.13

middle and low concentrations extracted on three consecutive days are depicted in Table 3.

Mean extraction efficiency values ($n = 5$) determined at low (5 ng/ml), middle (50 ng/ml) and high (100 ng/ml) concentrations for NCYC and CYC from serum were 65% and 67%, respectively. Mean recovery values ($n = 5$) for CYC and NCYC at low (40 ng/ml) and high (400 ng/ml) from urine were 70% and 67%, respectively. Whilst a high recovery is desired for sensitivity, consistent recovery ensured precision and accuracy of the method.

Table 2
Precision and accuracy data for the extraction of norcyclizine and cyclizine from urine

Spiked concentration (ng/ml)	n	Assayed concentration (mean ± S.D.) (ng/ml)	Precision (R.S.D., %)	Accuracy (% error)
Norcyclizine				
8.008	3	8.39 ± 0.15	1.90	+4.78
20.02	3	19.00 ± 0.78	4.20	−5.08
80.08	3	82.10 ± 2.92	3.80	−2.52
120.12	3	122.63 ± 6.92	5.70	+2.09
200.20	3	193.25 ± 9.31	4.80	−3.47
400.40	3	402.42 ± 15.66	3.90	+0.50
Cyclizine				
8.22	3	8.04 ± 0.07	0.82	−2.23
20.56	3	19.46 ± 0.64	3.20	−5.33
82.24	3	84.44 ± 1.12	1.30	+2.67
123.36	3	124.91 ± 4.27	3.40	+1.25
205.60	3	202.55 ± 4.25	2.10	−1.49
411.20	3	411.98 ± 13.40	3.30	+0.19

Table 3
Inter-day precision data for the extraction of norcyclizine and cyclizine from serum on three consecutive days

Calibrator ($n = 3$)	Concentration (ng/ml)				Precision (R.S.D., %)
	Day 1	Day 2	Day 3	Mean ± S.D.	
Norcyclizine					
A	102.90	103.03	102.28	102.74 ± 0.327	0.32
B	18.73	19.09	19.71	19.18 ± 0.405	2.11
C	1.84	1.98	1.61	1.81 ± 0.153	8.43
Cyclizine					
A	100.83	100.71	100.95	100.83 ± 0.097	0.097
B	19.45	19.64	19.59	19.56 ± 0.080	0.44
C	1.09	1.15	1.29	1.18 ± 0.084	7.12

4. Conclusions

A rapid and sensitive assay with the precision and accuracy necessary for pharmacokinetic studies has been developed for the analysis of both cyclizine and its demethylated metabolite, norcyclizine, in both serum and urine. Previously reported studies were unable to isolate norcyclizine from these biological matrices with any confidence. The use of a dual electrode electrochemical detector operated in the "oxidative-screen" mode allowed the selective quantitation of norcyclizine and cyclizine from both serum and urine. SPE provided a suitable means to extract between 80 and 100 samples within 6–8 h despite the large water wash that was necessary to improve both the cleanliness and precision of the extraction process. One important aspect of electrochemical detector use in the analysis of cyclizine is that the choice of internal standard appears to be critical. Despite similarities in retention properties, the internal standard must have similar oxidation characteristics. The use of an electrochemical detector, more specifically a coulometric detector with a dual electrode analytical cell, allowed for selective quantitation of both CYC and NCYC in the biological fluids collected. Selectivity was enhanced by operation of the cell in the "oxidative-screen" mode, in which the upstream electrode effectively acts as a filter, oxidizing compounds that may interfere with the chromatography of the compounds of interest. In addition, the LOQ of both NCYC and CYC in serum and urine was 1 ng/ml as opposed to the 5 ng/ml using ultraviolet detection.

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