Column-Switching High-Performance Liquid Chromatographic Method for Determination of a New Antiviral Agent, Cyclaradine, in Serum

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Cyclaradine is a novel carbocyclic nucleoside with good activity against the viruses of the herpes group. To facilitate pharmacokinetic studies on cyclaradine, an automated column-switching high-performance liquid chromatographic (HPLC) method was developed for the determination of cyclaradine in serum. Deproteinized serum containing cyclaradine was directed to an on-line extraction column which retained cyclaradine while many potentially interfering components were eluted to waste. Fourteen minutes later, the switching valve was automatically rotated, permitting an appropriate mobile phase to elute cyclaradine from the extraction column onto an analytical C18 column for further separation and UV detection. The method showed excellent linearity (r = 0.9995) for cyclaradine concentrations ranging from 0.05 to 5 μg/ml. It also provided good sensitivity (0.05 μg/ml). The assay was precise, with within-run and between-run coefficients of variation of <1.90%. The accuracy, expressed as differences between observed values and theoretical values, ranged from −4.12 to 4.80%. The assay involves a simple deproteinization of serum followed by a fully automated sample cleanup, eliminating long and tedious manual extraction prior to HPLC. The column-switching HPLC method has been successfully used for the determination of cyclaradine serum levels in squirrel monkeys following a single oral dose of 20 mg/kg.

Cyclaradine, (+)-9-(α-(2α, 3β-dihydroxy-4α-(hydroxy-methyl)cyclopentyl)adene (Fig. 1), is a carbocyclic nucleoside which inhibits the replication of herpes simplex virus type 1 (strain HF) and type 2 (strain MS) in tissue culture at noncytotoxic concentrations (2). It is highly efficacious in the treatment of herpes simplex virus type 1 encephalitis in mice (1) and is relatively resistant to the action of adenosine deaminase, a serum enzyme that inactivates 9-β-D-arabinofuranosyladenine (Ara-A), a well-known antiviral agent (2).

To facilitate the pharmacokinetic evaluation of cyclaradine in humans and other animals, a simple and sensitive column-switching high-performance liquid chromatographic (HPLC) method was developed. It involves simple protein precipitation followed by a fully automated sample cleanup. This paper describes the method and its application in determining the serum levels of cyclaradine in squirrel monkeys following oral administration of 20 mg/kg.

MATERIALS AND METHODS

Compounds. Acetonitrile (HPLC grade) and trichloroacetic acid (reagent grade) were obtained from MCB Manufacturing Chemists, Inc., Cincinnati, Ohio. Sodium acetate (analytical grade) was obtained from Mallinckrodt, Inc., Paris, Ky. Cyclaradine was obtained from Schering Corp., Bloomfield, N.J.

Preparation of standards. A stock solution of cyclaradine (100 μg/ml) was prepared in distilled water. Further dilutions were made to prepare lower working drug concentrations. Serum standards were prepared by spiking 0.2 ml of blank squirrel monkey serum with appropriate volumes (10 to 100 μl) of the working drug solutions. For routine analysis, calibration standards containing 2.5 μg of cyclaradine per ml of serum were prepared in triplicate.

Sample pretreatment. The spiked serum sample or 0.2 ml of unknown serum sample was diluted to 0.4 ml with distilled water. The sample was then deproteinized by adding 10% trichloroacetic acid (0.2 ml) followed by centrifugation for 10 min at 1,000 × g. A sample (0.4 ml) of the clear supernatant was neutralized with 1 N sodium acetate (0.2 ml) and transferred to a 300-μl limited volume insert for injection.

Calculations. Peak heights, retention times, and concentrations were calculated by an HP-3357 Lab Automation System (Hewlett-Packard Co., Palo Alto, Calif.). Calibration by an external standard method was done with triplicate single-point serum standards (2.5 μg/ml) weighted equally on the average.

Drug administration and sample collection. Four male squirrel monkeys (about 1 kg in body weight) were fasted overnight and given single oral dose of 20 mg of cyclaradine per kg. The dose was prepared as a solution in water. Before dosing and at 0.5, 1, 2, 3, 4, 6, and 8 h after dosing, blood samples were obtained via the saphenous vein. Samples were allowed to clot at room temperature for 30 min. After centrifugation, serum was removed and kept frozen until analyzed.

Chromatographic conditions. The HPLC system consisted of a WISP injector (Waters Associates, Inc., Milford, Mass.), two model 6000A solvent delivery systems (Waters), an extraction column (Lichrosorb RP-18 cartridge, 10 μm; 10 by 0.46 cm; Brownlee Labs), an analytical column (Partisil 10 ODS-3, 10 μm; 25 by 0.46 cm; Whatman, Inc., Clifton, N.J.), and a model 440 UV detector (Waters). The switching device was a 10-port valve module connected to a Solenoid Interface and controlled by a Chromtol CD programmable timer (Autochorm, Inc.). Figure 2 shows the schematic of the column-switching system. In position I, samples (50 to 200 μl) were injected onto the extraction column and eluted with acetate buffer, 0.05 M (pH 5.5), and acetonitrile (98:2, vol/vol) delivered by pump A. Under this condition,
Cyclaradine was retained while potentially interfering components were eluted to waste. Fourteen minutes after sample injection, the switching valve was automatically rotated to position II. This allowed a stronger mobile phase consisting of acetate buffer, 0.05 M (pH 5.5), and acetonitrile (94:6, vol/vol) delivered by pump B to elute cyclaradine from the extraction column onto the analytical column for further separation. Thirty minutes after injection, the switching valve was automatically rotated back to position I to allow a 5-min re-equilibration of the extraction column for the next injection. The eluent from the analytical column was continuously monitored by the detector operated at 254 nm and 0.05 absorbance unit, full scale. The overall run time for each injection was 35 min.

RESULTS AND DISCUSSION

The column-switching HPLC methodology described here proved to be a simple, sensitive, and efficient technique for the determination of cyclaradine in serum. Figure 3 shows chromatograms of blank squirrel monkey serum, blank squirrel monkey serum spiked with 1.25 μg of cyclaradine per ml, and serum obtained from squirrel monkey 4 h after oral administration of cyclaradine. These chromatograms demonstrate the lack of interference from the serum matrix. The retention time of cyclaradine (27 min) was reproducible. On an injection-to-injection and on a day-to-day basis, a coefficient of variation of <0.4% was obtained for retention time. This retention time was clearly different from those of several analogs (acyclovir, Ara-A, Ara-Hx, caffeine, theophylline, theobromine, 1-methylxanthine, xanthine, and adenine). It was also different from those of possible coadministered drugs such as aspirin and acetaminophen.

To determine the recovery, aqueous solutions of cyclaradine (2.5 μg/ml) and serum samples spiked with cyclaradine (2.5 μg/ml) were analyzed, and their peak heights were compared. The recovery of cyclaradine was 101.6% (coefficient of variation [CV] = 2.00%; n = 4).

A series of blank squirrel monkey serum spiked with cyclaradine to obtain concentrations of 0.05, 0.5, 1.0, 2.5, and 5.0 μg/ml (n = 4) was analyzed to determine the linearity of the method. The linear regression analysis for observed concentrations (y) and added concentrations (x) yielded the

FIG. 1. Chemical structure of cyclaradine.

FIG. 2. Flow scheme of the column-switching HPLC system.

FIG. 3. Typical HPLC chromatograms of (A) blank squirrel monkey serum, (B) blank squirrel monkey serum spiked with 1.25 μg of cyclaradine per ml, and (C) serum obtained from a squirrel monkey 4 h after oral administration of 20 mg of cyclaradine per kg.
equation $y = 0.96x + 0.06$, with a correlation coefficient of 0.9995.

To determine the accuracy of the assay, three concentrations of cyclaradine in serum ($n = 5$ to 6) were prepared and analyzed. The differences between observed and theoretical values were 4.80% at 0.05 μg/ml, 3.84% at 1.25 μg/ml, and −4.12% at 2.5 μg/ml, with CVs ranging from 0.91 to 4.18%. The sensitivity of the HPLC method was estimated to be 0.05 μg/ml, the lowest concentration tested with a small CV. To evaluate the precision of the HPLC method, six serum samples containing 2.5 μg of cyclaradine per ml were analyzed on five separate days. There was good within-day reproducibility of the assay (Table 1), with CVs ranging from 0.76 to 1.87%. The day-to-day precision was also excellent, with a CV of only 1.33%.

To investigate the feasibility of using the HPLC method to measure cyclaradine levels in animals, we analyzed serum samples from squirrel monkeys after oral administration of 20 mg of the drug per kg. Figure 4 shows the mean concentration-time profile of cyclaradine with a maximum concentration of 2.15 μg/ml reached at 2 h after drug administration. The concentrations of cyclaradine in the serum samples were well within the linear range of the HPLC method. In addition, the method had adequate sensitivity to detect concentrations of cyclaradine in serum 8 h following administration of the drug.

The results of this study demonstrated that the method offers a high level of precision and accuracy. It is linear, specific, and sufficiently sensitive. The method has been successfully used for determining serum levels of cyclaradine in squirrel monkeys following oral administration of the drug. The assay features a column-switching approach to sample cleanup which greatly simplifies sample pretreatment with deproteinization of serum. It eliminates long and tedious procedures such as multiple extractions with organic solvents, phase separation, evaporation, and reconstitution prior to HPLC. The system is fully automated and capable of overnight runs. Approximately 40 samples can be analyzed per day on a routine basis. Since samples were deproteinized prior to injection, the lifetime of the column was prolonged and pressure buildup was avoided. For routine maintenance, columns were simply cleaned once a week with water followed by methanol. Approximately 200 samples can be analyzed without changing the column.

**TABLE 1. Precision of the HPLC assay**

<table>
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<th>Day of</th>
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<th>Observed concn (μg/ml)</th>
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</table>

* Between-day variation: mean, 2.52; CV, 1.33%.

**FIG. 4. Serum levels of cyclaradine in squirrel monkeys after an oral dose of 20 mg/kg. Each point represents mean serum concentration (n = 4).**

**LITERATURE CITED**
