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Note

High-performance liquid chromatographic determination of 2′,3′-dideoxycytidine and 3′-azido-3′-deoxythymidine in plasma using a column-switching technique

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2′,3′-Dideoxycytidine (DDC) is one of a family of nucleoside analogues which have strong anti-retrovirus activity [1–4]. Several of these compounds, including DDC and 3′-azido-3′-deoxythymidine (AZT), are promising as therapeutic agents for treating the human retrovirus disease, acquired immune deficiency syndrome (AIDS) [5]. Animals that suffer from retrovirus diseases, such as cats infected with feline leukemia virus (FeLV) [6], may also benefit from this group of drugs.

To properly evaluate the antiviral activity of DDC, drug plasma concentrations should be monitored so that therapy can be optimized. In this report, we describe a sensitive and reproducible high-performance liquid chromatographic (HPLC) procedure to measure DDC directly from plasma of FeLV-infected cats on drug therapy, using a valve switching technique. We also demonstrate the successful use of this procedure for plasma to which AZT has been added.

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EXPERIMENTAL

Materials

DDC was provided by The National Institute of Allergy and Infectious Disease, AIDS Program (Bethesda, MD, U.S.A.) AZT was available commercially (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.). Ethanol (HPLC grade) was purchased from Aldrich (Milwaukee, WI, U.S.A.). A 1 *M* stock solution of phosphate buffer, pH 7.1, was prepared by mixing 162 ml of solution A (1 *M* K_2HPO_4) with 38 ml of solution B (1 *M* KH_2PO_4). The pH was adjusted to 7.1 by adding either solution A or solution B. After preparation, the 1 *M* phosphate buffer stock solution was filtered through a 0.2-μm membrane filter. Phosphate buffer stock solution was diluted 1:10 with HPLC-grade water (Fisher Scientific, Cincinnati, OH, U.S.A.) just prior to use.

Instrumentation

The HPLC system was a Hewlett-Packard Model 1090M equipped with a binary pump, a variable-volume autoinjector, a six-port switching valve, an analytical work station with a Winchester 20-megabyte disc storage device and a diode-array detector. Chromatograms were stored and analyzed for DDC or AZT concentrations using work station programming to calculate an area percentage and an external calibration.

Chromatography

The instrument arrangement for the chromatography is shown in Fig. 1. The chromatography time table is shown in Table I. Sample volume was 10 μl and

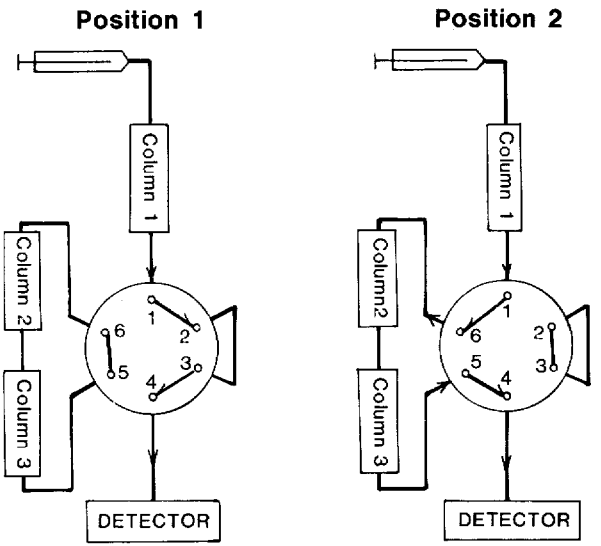

Fig. 1. Arrangement of column-switching valve for position 1 and position 2. Column 1 is a 150 mm×4.6 mm Pinkerton ISRP. Columns 2 and 3 are 100 mm×4.6 mm and 250 mm×4.6 mm Cyclobond I columns, respectively, Flow direction is indicated by arrows.

TABLE I

LIQUID CHROMATOGRAPHY TIME TABLE FOR DDC AND AZT

Solvent A = 0.1 *M* phosphate buffer pH 7.1; solvent B = 100% ethanol.

Chromatographic event	Time table (min)	
	DDC	AZT
Column-switching valve to position 1 Solvent A = 100% Solvent B = 0% Sample injection	0	0
Column-switching valve to position 2	2.6	2.8
Column-switching valve to position 1	3.45	4.39
Solvent A = 95% Solvent B = 5%	3.45	4.40
Solvent A = 100% Solvent B = 0%	11.00	12.00
Column-switching valve to position 2	15.00	16.00
End of run Column-switching valve to position 1	25.00	25.00

flow-rate was 1 ml/min. AZT and DDC were detected at 265 and 275 nm, respectively. A Pinkerton internal-surface reverses-phase (ISRP) 150 mm×4.6 mm I.D. column packed with 5-μm tripeptide glycine-phenylalanine-phenylalanine silica particles (Regis, Morton Grove, IL, U.S.A.) was used as a precolumn for separating serum drug from plasma proteins. The elution of DDC and AZT was retarded on the ISRP column allowing plasma proteins to pass while retaining the drugs. The switching valve was used to capture either drug from the eluent of the ISRP column for analysis by chromatography on the Cyclobond I (100 mm×4.6 mm I.D. and 250 mm×4.6 mm I.D. columns packed with 5-μm β-cyclodextrin-bonded silica particles (Advanced Separation Technologies, Whippany, NY, U.S.A.) and connected in tandem (Fig. 1). As indicated in Table I, the switching valve was activated (position 2) at 2.6 and 2.8 min and inactivated (position 1) at 3.45 and 4.39 min into the analysis of DDC and AZT, respectively. The ISRP column was then washed with phosphate buffer containing 5% ethanol for 7.54 or 7.6 min to remove the remaining serum components. At 15 min for DDC and 16 min for AZT, the switching valve was again activated (position 2) and the captured drug chromatographed on the Cyclobond I columns.

Preparation of standards

External standards were used to determine unknown drug concentrations in plasma samples. DDC or AZT were dissolved in prefiltered feline plasma at a concentration between 10 and 15 μg/ml. Serial two-fold dilutions of the drug plasma preparation were made using plasma as diluent. All samples were filtered through 0.2-μm membrane filters prior to chromatographic analysis.

Plasma samples

Feline plasma was from normal, specific pathogen-free cats or from cats given DDC therapy. Known amounts of either DDC or AZT were added to normal feline plasma samples to prepare HPLC standards. Plasma samples were passed through 0.2-μm membrne filters prior to analysis.

RESULTS AND DISCUSSION

Analysis of DDC and AZT on the ISRP–Cyclobond I column sequence

Using the column configuration shown in Fig. 1, an optimized chromatographic procedure was developed to determine DDC or AZT concentrations in plasma samples from cats on drug therapy. Initially, DDC and AZT were separately chromatographed on the ISRP column to determine their retention times (Fig. 2). This information was later used in the chromatography time table (Table I) as the center of the time window for drug capture. The retention times for DDC and AZT were 2.853 and 3.029 min, respectively. Feline plasma samples spiked with phosphate buffer, DDC or AZT were also analyzed on the ISRP column (not shown). The large amounts of absorbing material in plasma overshadowed the drug peaks.

The chromatographic separation of DDC and AZT using the protocol defined

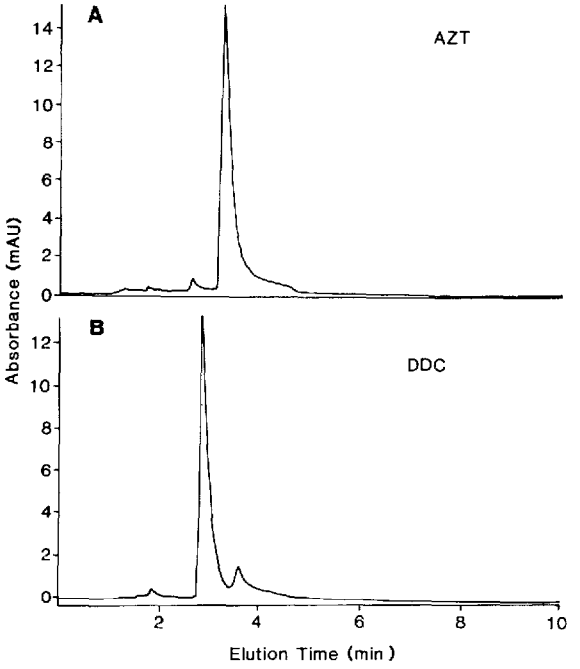

Fig. 2. Chromatographic elution profile of (A) AZT and (B) DDC from a 15-cm Pinkerton ISRP column. AZT (15 μg/ml) or DDC (10 μg/ml) were dissolved in 0.1 *M* phosphate buffer, pH 7.1. The sample volume was 10 μl. The retention times for AZT and DDC were 3.029 and 2.853 min, respectively. Chromatographic conditions are described in the text.

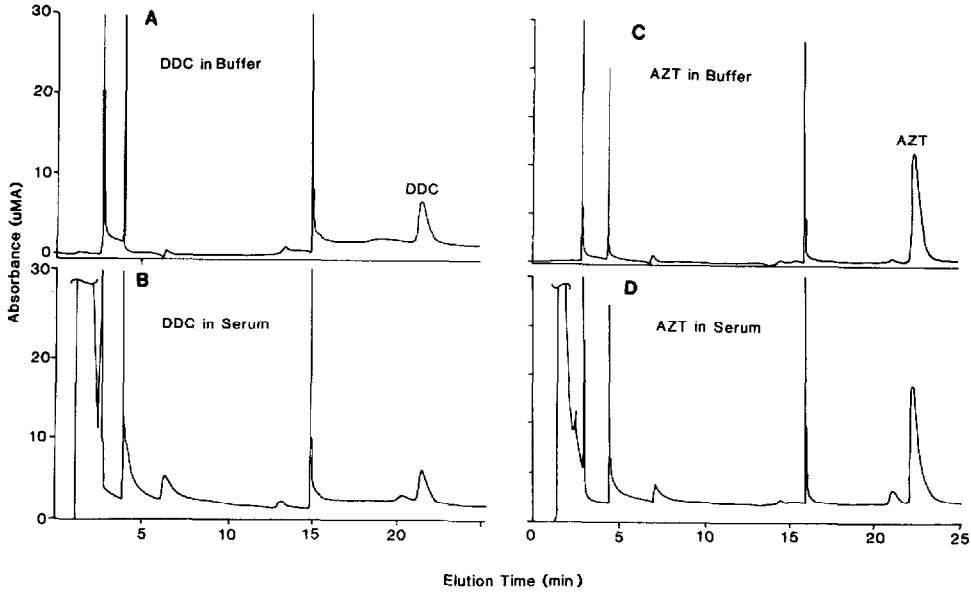

Fig. 3. Chromatographic separation of (A and B) DDC (10 μg/ml) and (C and D) AZT (15 μg/ml) on the ISRP-Cyclobond I column combination following the appropriate chromatographic time table described in Table I. In A and C drug is dissolved in 0.1 *M* phosphate buffer, pH 7.1. In B and D drug is dissolved in feline plasma. Sample volume was 10 μl. Chromatographic conditions are described in the text.

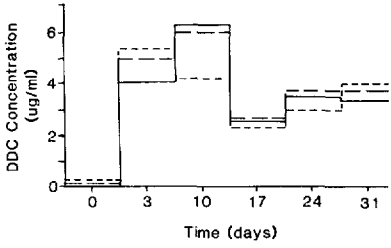

Fig. 4. Plasma concentration of DDC for three cats receiving DDC therapy by continuous infusion (2 mg/h) for a period of five weeks. DDC plasma concentrations were determined as described in the text.

in Table I is shown in Fig. 3. DDC and AZT eluted as separate distinct peaks from serum components.

The calibration curve derived from the analysis of plasma samples spiked with DDC was linear with a correlation coefficient of 1.000 over the concentration range 0.3125–50.0 μg/ml. The coefficients of variation for eight replicate analyses of spiked plasma samples at 0.625 and 0.3125 μg/ml were 4.8 and 10.9%, respectively. The day-to-day coefficients of variation were 7.0% for identical samples analyzed on eight separate days. The signal-to-noise ratio for the 0.3125 μg/ml DDC spiked plasma sample was 14.1 at the drug elution time. Using the calibration curve, a series of plasma samples taken from three cats given DDC by continuous intravenous infusion were analyzed for DDC content. Fig. 4 shows these

data. DDC plasma concentration stabilized to between 2.5 and 4 μg/ml after the first ten days.

In summary, a method of analysis of AZT and DDC in plasma based on a direct injection column-switching technique was developed. This procedure is advantageous because it does not require an internal standard, has higher sensitivity, is less time-consuming and requires small plasma volume.

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