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Note

Determination of 2′,3′-dideoxyadenosine, 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine in biological samples

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Several purine and pyrimidine 2′,3′-dideoxynucleosides suppress the in vitro infectivity and cytopathic effect of the HIV-1 (HTLV-III) retrovirus [1–3]. As the triphosphate derivatives, they are potent inhibitors of retroviral reverse transcriptases [3,4]. Since the activity of these compounds is observed at concentrations that do not affect the growth and immune functions of T cells, they are being considered for clinical trials in the treatment of acquired immunodeficiency syndrome (AIDS).

Several methods have been reported for the analysis of 2′,3′-dideoxyadenoside (ddA) and 2′,3′-dideoxycytidine (ddC) and their phosphorylated dideoxynucleotide anabolites in cellular systems [5–7]. These methods employed ion-exchange high-performance liquid chromatography (HPLC); detection was by measurement of eluted radioactivity. The current report describes the development of quantitative analytical methods that employ isocratic, reversed-phase HPLC coupled with detection by UV absorbance or radioassay. These methods have been applied toward the measurement of ddC in plasma from cats and ddA in plasma from mice and cats. Since ddA is rapidly deaminated to 2′,3′-dideoxyinoside (ddI) by adenosine deaminase [6,8], we have also measured levels of ddI in plasma following administration of ddA. An additional method, appropriate for the analysis of the more polar potential metabolites of ddA (hypoxanthine, xanthine and uric acid), was developed, in view of the conversion of ddI to hypoxanthine by purine nucleoside phosphorylase [9]. This system, incorporating paired-ion gradient chromatography, was adapted from the work of Perrone and

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Brown [10] and was applied to the quantification of these metabolites in plasma and urine from mice administered [^{3}H]ddA.

EXPERIMENTAL

Materials

The compounds ddA, N-methyl-2′-deoxyadenosine (MdA), and tetrabutylammonium phosphate (TBAP) were purchased from Aldrich (Milwaukee, WI, U.S.A.), ddI from Calbiochem-Behring (LaJolla, CA, U.S.A.), 2′-deoxycoformycin (dCF) from Parke-Davis Division of Warner Lambert (Ann Arbor, MI, U.S.A.) and hypoxanthine, xanthine and uric acid from Sigma (St. Louis, MO, U.S.A.). ddC was provided by Dr. Ken Paul, National Cancer Institute. [G-^{3}H]ddA, with a stated specific activity of 3.24 Ci/mmol (13.8 mCi/mg), was provided by Research Triangle Institute (Research Triangle Park, NC, U.S.A.). Spectral-grade N,N-dimethylformamide was purchased from Mallinckrodt (Paris, KY, U.S.A.), and glass-distilled methanol and acetonitrile from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Equipment

Two Waters liquid chromatographs (Waters Chromatography Division, Milford, MA, U.S.A.) were used. Analyses of single samples were performed with a system consisting of two Model 510 pumps, a Model 680 solvent gradient programmer, a U6K universal injector, a Model 490 programmable, multi-wavelength UV–visible absorbance detector, and a Model 740 data module. Unattended analyses of multiple samples were performed with a system consisting of Model 6000A and M45 pumps, a Model 720 system controller, a Model 710 WISP autoinjector, a Model 440 UV–visible absorbance detector and a Model 730 data module.

Chromatographic conditions

Analyses of ddA were carried out with either a 250 mm×4.6 mm I.D. Ultrasphere ODS 5-μm column (Beckman Instruments, San Ramon, CA, U.S.A.) or a 250 mm×4.6 mm I.D. Ultremex 5-μm C18IP analytical column (Phenomenex, Rancho Palos Verdes, CA, U.S.A.). Each column was fitted with a 25 mm×2.0 mm I.D. SGP ODS guard column (Phase Separations, Norwalk, CT, U.S.A.). Resolution of ddA and ddI was achieved isocratically with 10 mmol/l ammonium dihydrogenphosphate, pH 6.8, in 7.5% aqueous N,N-dimethylformamide at a flow-rate of 1.0 ml/min. Detection was by UV absorbance at 254 nm. Resolution of additional potential metabolites (hypoxanthine, xanthine and uric acid) as well as ddA and ddI required gradient elution with an ion-pair system consisting of 10 min of isocratic elution with 100% solvent A, followed by a 10-min linear (Waters curve No. 6) gradient to 100% solvent B and 10 min of isocratic elution with solvent B. The column was re-equilibrated with 100% solvent A for 30 min prior to each subsequent injection; flow-rate was 1.0 ml/min throughout. Solvent A contained 20 mmol/l ammonium dihydrogenphosphate, pH 6.8, in 5 mmol/l TBAP; solvent B was the same as solvent A, except for addition of 10% (v/v)

N,N-dimethylformamide. Detection was by UV absorbance at 254 nm or by collection of 1-min fractions for radioassay. Baseline UV absorbance drift caused by N,N-dimethylformamide was corrected with the Model 490 programmable UV–visible absorbance detector. A blank gradient served as reference.

Analyses of ddC were accomplished isocratically with a 250 mm × 4.6 mm I.D. Spherisorb ODS-2 5-μm analytical column (Phase Separations) fitted with a 25 mm × 2.0 mm I.D. SGP ODS guard column. Samples were eluted with 20 mmol/l ammonium dihydrogenphosphate, pH 6.8, in 2.5% aqueous acetonitrile at a flow-rate of 1.0 ml/min. Detection was by UV absorbance at 280 nm.

For identification of unlabeled ddA and ddI in biological samples, spectral analyses were accomplished with a diode-array Hewlett-Packard 1040A spectrophotometric detector connected to the HPLC system. Quantification of all UV-absorbing components was by peak-area integration and external standardization. Quantification of radiolabeled compounds was by dilution of collected fractions with scintillator (Safety Solve, Research Products International, Mt. Prospect, IL, U.S.A.) and radioassay by liquid scintillation counting (Packard Instruments, Downers Grove, IL, U.S.A.).

Sample preparation

For analysis of ddA and ddI, plasma was processed by two methods. The first involved mixing plasma with a solution containing an internal standard, MdA, in N,N-dimethylformamide. The final sample, containing plasma, 2 μg of MdA and 7% N,N-dimethylformamide in a final volume of 60 μl, was analyzed directly by HPLC.

The second method employed solid-phase extraction for sample clean-up prior to HPLC analysis. Portions (0.25 ml) of plasma were diluted with 2.25 ml of an aqueous solution containing 1 μmol/l dCF (to block deamination during sample processing, see ref. 11) and applied to Bond-Elut C_{18} solid-phase extraction cartridges (Analytichem International, Harbor City, CA, U.S.A.) previously activated with 10 ml of methanol followed by 5 ml of water. The sample-loaded cartridges were washed with 2 ml of water, and the adsorbed analytes were eluted with 2 ml of methanol. Eluates were concentrated under a stream of nitrogen, reconstituted with 0.5 ml of an aqueous solution containing 2.5 μg/ml MdA and analyzed by HPLC.

Plasma was prepared for analysis of ddC by modification of previously reported methods involving perchloric acid extraction [12,13]. Samples (0.25 ml) were diluted with 0.25 ml of water and 0.50 ml of ice-cold 1.0 mol/l perchloric acid, mixed by vortex and allowed to stand on ice for 10–15 min. These acidified samples were neutralized by dropwise addition of 0.50 ml of ice-cold 1.0 mol/l potassium bicarbonate with constant vortex-mixing. Precipitated protein and potassium perchlorate were removed by centrifugation at 5 °C, and 0.15 ml of each supernatant was analyzed for ddC by HPLC. Cloudy supernatants were clarified by passage through 0.2-μm filters (Acro LC13, Gelman Sciences, Ann Arbor, MI, U.S.A.) prior to analysis.

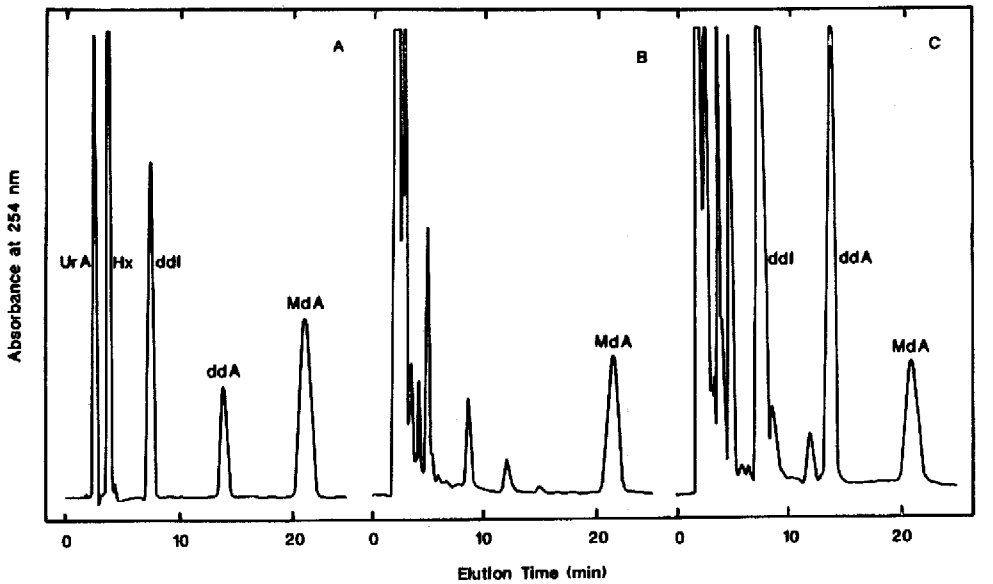

Fig. 1. Isocratic chromatography of (A) a mixture containing 500 ng each of the standards ddA, ddI, hypoxanthine (Hx) and uric acid (UrA) and the internal standard MdA, (B) blank CD2F1 mouse plasma and (C) plasma collected from a CD2F1 mouse 5 min following i.p. administration of ddA, 478 mg/kg.

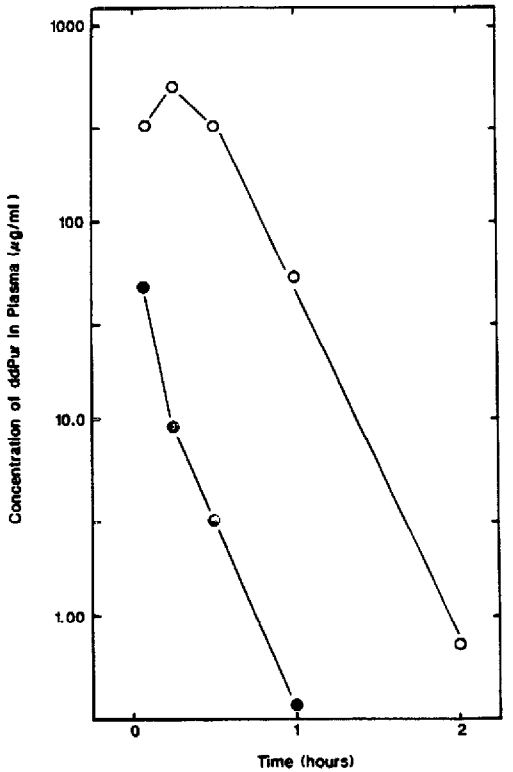

Fig. 2. Disposition of dideoxypurine nucleosides (ddPur) ddA (●) and ddI (○) in CD2F1 mouse plasma following i.p. administration of ddA, 478 mg/kg.

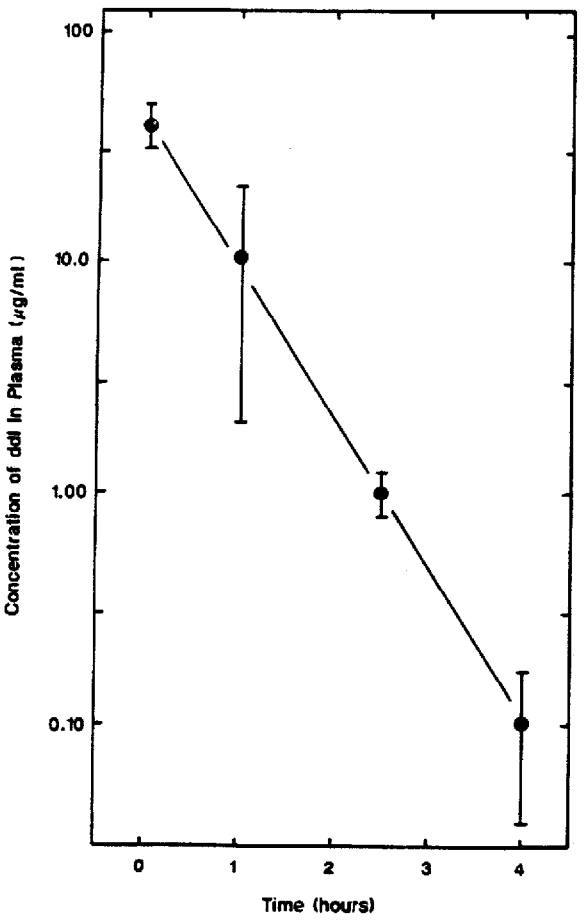

Fig. 3. Disposition of ddI in cat plasma following i.v. administration of ddA, 20 mg/kg. Represented are the means ± S.D. of four individual analyses at each time point.

RESULTS AND DISCUSSION

Typical isocratic chromatography of ddA, ddI, hypoxanthine, uric acid and the internal standard, MdA, is illustrated in Fig. 1. Also illustrated is the chromatography of control CD2F1 mouse plasma and plasma collected from a CD2F1 mouse 5 min following intraperitoneal (i.p.) administration of ddA, 478 mg/kg. Plasma was prepared for analysis by mixing with MdA and N,N-dimethylformamide as described in Experimental. The minimum limit of quantification for ddA and ddI was 10 ng per injection or 0.20 μg/ml in plasma. This method was used to follow the disposition of ddA in plasma collected from CD2F1 mice following i.p. administration of ddA, 478 mg/kg (Fig. 2). The administered drug was rapidly deaminated to ddI, after which both nucleosides were eliminated exponentially, ddA with a half-life of 10 min and ddI with a half-life of 12 min.

An alternative method, employing solid-phase extraction, was investigated as a means of sample clean-up prior to HPLC analysis. Recovery of ddA and ddI

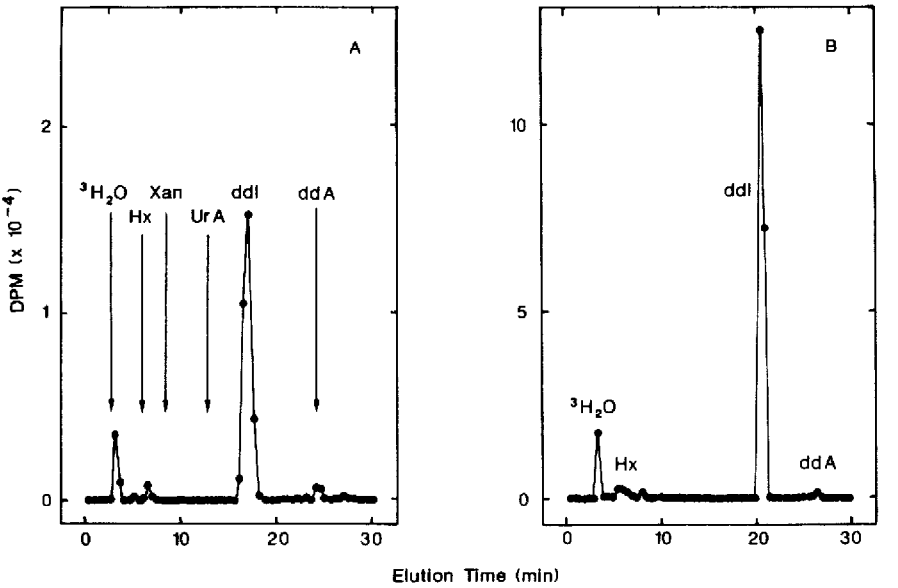

Fig. 4. Paired-ion gradient chromatography of (A) plasma collected from a CD2F1 mouse 15 min following i.p. administration of [^{3}H]ddA, 478 mg/kg, and (B) urine collected from CD2F1 mice 24 h following i.v. administration of [^{3}H]ddA, 29.8 mg/kg. The points for elution of ^3H_2O, hypoxanthine (Hx), xanthine (Xan), uric acid (UrA), ddI, and ddA are indicated in panel A.

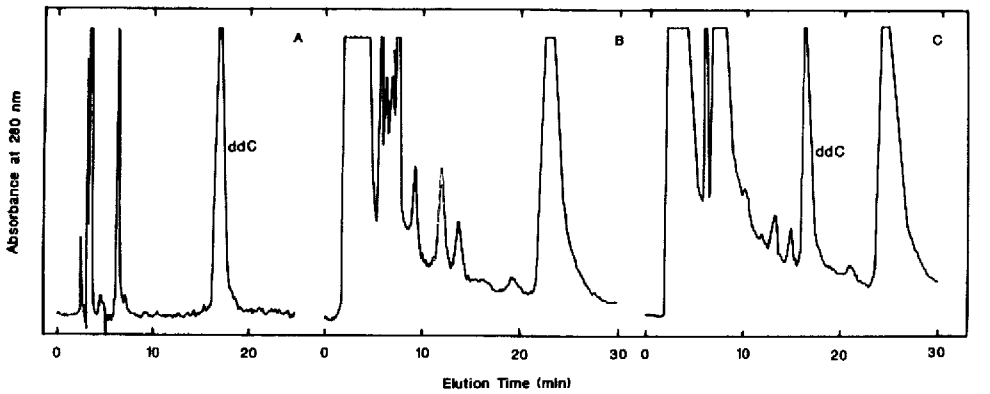

Fig. 5. Isocratic chromatography of (A) 164 ng of a ddC standard, (B) blank cat plasma and (C) plasma collected from a cat 2 h following i.v. administration of ddC, 20 mg/kg.

was determined by supplementing duplicate portions of control plasma from Fischer 344 female rats with concentrations of ddA and ddI over the range 0.3–10 μg/ml and processing as described in Experimental. At and above 1 μg/ml, recovery in plasma was 106%. Below 1 μg/ml, recovery was 135–155%, indicating some interference at these low levels by endogenous components. This method was used to follow the disposition of ddA in plasma collected from four cats administered ddA, 20 mg/kg, intravenously (i.v.). Unchanged ddA was not detected. The deamination product, ddI, however, was quantified and found to be

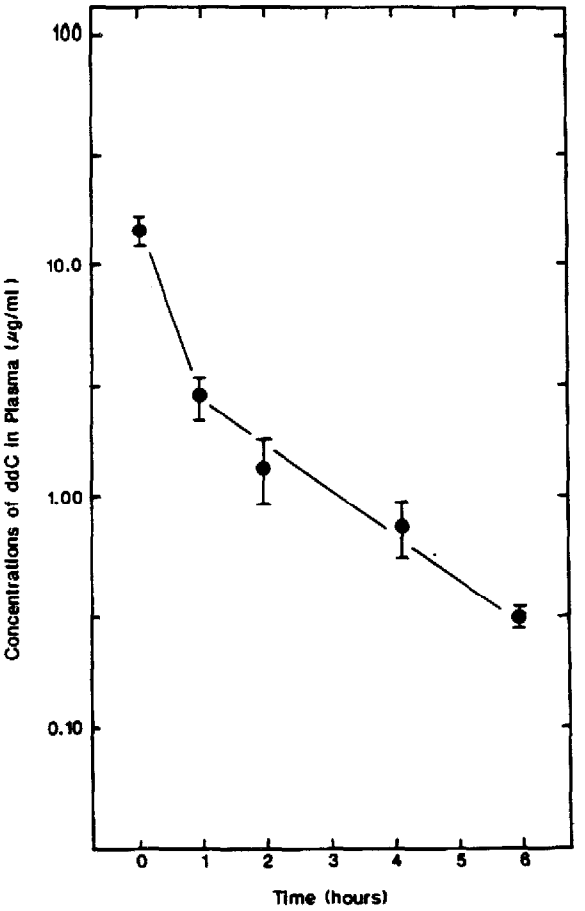

Fig. 6. Disposition of ddC in cat plasma following i.v. administration of ddC, 20 mg/kg. Represented are the means ± S.D. of four individual analyses at each time point.

eliminated exponentially with a half-life of 27 min (Fig. 3). Chromatography was similar to that illustrated in Fig. 1.

Analysis of the other ddA metabolites required development of a paired-ion gradient system. This system was used to quantify the metabolites of ddA in plasma collected from mice 15 min following i.p. administration of [^{3}H]ddA, 478 mg/kg, and in urine collected from mice for 24 h following i.v. administration of [^{3}H]ddA, 29.8 mg/kg. Radiochromatograms of plasma and urine are illustrated in Fig. 4. As anticipated from previous observations, ddA was extensively and rapidly deaminated to ddI. The presence of 10–12% of the radiolabel in urine as ^3H_2O, representing tritium exchange, was confirmed by its disappearance following evaporation and reconstitution of the urine sample prior to HPLC analysis.

Recovery of ddC from plasma was determined by supplementing duplicate blank samples of plasma from Fischer 344 female rats with concentrations of ddC over the range 0.3–10 μg/ml and processing as described in Experimental. Under these conditions, recovery was 104 ± 19.0%. The minimum limit of quantification for

ddC was 5 ng per injection or 0.20 μg/ml in plasma. This method was used to follow the disposition of ddC in plasma collected from four cats following i.v. administration of ddC, 20 mg/kg. Typical chromatograms of a ddC standard, blank cat plasma and plasma collected 2 h following administration of ddC are illustrated in Fig. 5. The administered drug was eliminated exponentially with a terminal half-life of 72 min (Fig. 6). This value is comparable to that for mice (67 min) and that for monkeys (109 min) dosed intravenously [14].

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