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Short Communication

High-performance liquid chromatographic analysis of 2′,3′-dideoxyinosine in biological samples

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ABSTRACT

A high-performance liquid chromatographic analysis for the anti-AIDS drug 2′,3′-dideoxyinosine (ddI) in rat plasma and urine, with a limit of detection of 0.2 μg/ml and requiring a sample size of 100 μl is described. Diluted plasma or urine samples were extracted using a C_{18} solid-phase extraction column. Retention of ddI on more polar solid-phase extraction columns was insufficient for sample clean-up. This method is useful for pharmacokinetic studies of ddI in small rodents.

INTRODUCTION

2′,3′-Dideoxynucleosides (ddNS) are effective against human immunodeficiency virus (HIV) *in vitro*. The triphosphate nucleotides of ddNS inhibit the viral enzyme reverse transcriptase which is necessary for viral replication and infectivity. These compounds, including 3′-azido-3′-deoxythymidine, 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyinosine (ddI), are currently the most promising and vigorously pursued drugs for the treatment of patients afflicted with HIV [1]. Of the ddNS studied to date using the human T-cells, ddC is the most potent agent on a molar basis [2]. But the therapeutic index of ddI is superior to ddC [2]. ddI is currently in phase I and II clinical trials [3].

The chronicity of HIV infection requires continuous therapy to maintain an effective inhibitory drug concentration in plasma. There are very limited pharmacokinetic studies in experimental animals and patients. The analysis of ddI in mouse plasma has been performed using high-performance liquid chromatography (HPLC) with UV detection. The assay used for ddI analysis has a detection

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limit of 400 ng/ml using a 0.25-ml sample [4]. In this study, several mice were used for each concentration–time point. The large sample size excludes the possibility of studying the pharmacokinetics of ddI in the same rodent. The present report describes an HPLC assay for ddI in plasma and urine samples, which has an improved sensitivity and requires a smaller sample size. Preliminary disposition data obtained from a rat given an intravenous dose of ddI are shown here to demonstrate the applicability of the assay.

EXPERIMENTAL

Chemicals

All HPLC solvents and reagents were of analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). ddI (Lot No. 234-B-1) was obtained from Dr. K. Flora, Pharmaceutical Research Branch of the National Cancer Institute (Bethesda, MD, U.S.A.). N^6-Methyladenosine (MeAdo, Lot No. 58F4013) was purchased from Sigma (St. Louis, MO, U.S.A.). 5′-Deoxy-5-fluorouridine (dFUR, Lot No. 305001) was a gift from Hoffmann LaRoche (Nutley, NJ, U.S.A.). HPLC analysis showed that ddI and dFUR were >98% pure. Both chemicals were used as received. ddI and dFUR solutions can be stored at −20°C for up to five months with negligible degradation (< 5%) as determined by chromatographic analysis.

Animal study

A six-month-old female Fischer rat (Charles River Breeding Lab., Kingston, NJ, U.S.A.) weighing 194 g was used for the pharmacokinetic study. One day prior to the experiment, a permanent catheter was inserted into the right external jugular vein of the rat under light ether anesthesia. On the day of the experiment, a dose (8 or 40 mg/kg) of ddI was given intravenously over 15 s. Serial blood samples (200 μl each) were withdrawn through the venous catheter and collected for a period of 6 h. Urine samples were collected for 24 h. Blood samples were placed in heparinized tubes and kept on ice to prevent clotting and/or ddI and dFUR decomposition. After each sampling, the lost blood volume was replaced with an equal volume of saline. Blood samples were centrifuged at 1100 *g* for 10 min at 0°C, and the plasma fraction was transferred to a separate tube.

Sample extraction

We compared the extraction of ddI using liquid–liquid extraction and solid–liquid extraction. In the liquid–liquid extraction, 1 ml of 100 m*M* phosphate buffer of pH 6.9 which contained 4.7 μg of ddI was mixed with ethyl acetate (10 ml), acetonitrile (1 ml) or a mixture of ethyl acetate and acetonitrile (90:10, v/v, 10 ml). The mixture was vortex-mixed for 30 s. The organic layer was transferred to a second tube and dried under a flow of nitrogen. The phosphate buffer and acetonitrile mixtures were cooled to 4°C to obtain phase separation. In the solid–

liquid extraction, sample clean up was done using solid-phase extraction (SPE) columns (Supelco, Bellefonte, PA, U.S.A.). In the final method, SPE-C_{18} columns and phosphate wash were used to optimize the recovery of ddI and dFUR. The SPE-C_{18} columns were preconditioned with 2 × 1 ml methanol followed by 2 × 1 ml of 100 m*M* phosphate buffer of pH 6.9. Each fraction of the preconditioning solvents was allowed to equilibrate with the SPE column for 1 min and pulled through the column under suction. Care was exercised that the solid-phase material was immersed in solvent at all times.

Two compounds were used as the internal standard, *i.e.* MeAdo (12.5 μg/ml in 100 m*M* phosphate buffer, pH 6.9) and dFUR (10 μg/ml in 100 m*M* phosphate buffer, pH 6.9). Plasma (100 μl) was mixed with 400 μl of the internal standard solution. To the preconditioned SPE-C_{18} column, the plasma and internal standard mixture was added, allowed to equilibrate for 1 min and pulled through under suction. The column was then washed with two fractions of 1 ml of 100 m*M* phosphate buffer, pH 6.9. The washes were discarded. After the washes, ddI and the internal standard were eluted with 1 ml of a 75:25 (v/v) mixture of methanol and 5 m*M* phosphate buffer, pH 6.9. The eluent was concentrated under a stream of nitrogen to about 150 μl. The extract was analyzed by HPLC. For analysis of ddI in rat urine, the samples were diluted ten-fold with water. Diluted urine sample (100 μl) was mixed with 400 μl internal solution and a 100-μl aliquot was injected on the HPLC system. Standard curves were prepared for ddI in rat plasma and urine.

HPLC analysis

Similar HPLC techniques were used for plasma and urine samples. The HPLC system consisted of a solvent delivery system (Spectroflow 400, Kratos Analytical Instruments, Ramsey, NJ, U.S.A.), an automated injector (WISP 710B, Waters Assoc., Milford, MA, U.S.A.) or a manual injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.). During the assay developmental stage, a diode array detector (Model 990, Waters Assoc.) with scanning capability was used. Routine assays used a fixed-wavelength detector at 254 and 280 nm (Model 440, Waters Assoc.), and an HP 3390A integrator (Hewlett-Packard, Menlo Park, CA, U.SA.). The column was a reversed-phase μBondapak C_{18} (300 mm × 3.9 mm I.D., 10 μm particle size, Waters Assoc.). The aqueous mobile phase consisted of 4% (v/v) acetonitrile in 10 m*M* phosphate buffer adjusted to pH 6.9 with dilute phosphoric acid. The solvent flow was maintained at 2.0 ml/min. All analyses were carried out at ambient temperature.

RESULTS AND DISCUSSION

Sample extraction

We compared the recoveries of ddI using liquid–liquid and solid–liquid extractions. In the liquid–liquid extractions, the fractions of ddI extracted by the organ-

ic solvents from water were 10% for ethyl acetate, 23% for the ethyl acetate–acetonitrile mixture, and 33% for acetonitrile. These low recoveries made the liquid–liquid extraction an unacceptable method for sample clean-up.

The solid–liquid extraction gave better results. We compared the retention of ddI and MeAdo on several SPE columns, *i.e.* SPE-CN, SPE-C_8, SPE-phenyl, and SPE-C_{18}. After washing with three fractions of 1 ml of water, over 80% of MeAdo was retained on the SPE-phenyl, -C_8, and -C_{18} columns. The retention of MeAdo on a CN column was only 20%. The more polar ddI was less well retained with recoveries of 0% on a CN column, 6% on a phenyl column and 60% on a C_8 column, after three water washes. Using the SPE-C_{18} column, the three water washes removed 1, 2 and 5% of ddI, respectively, and about 90% of ddI was retained. For dFUR, two water washes removed 72% of the amount applied to the SPE-C_{18} column. Washing the SPE-C_{18} column with 100 m*M* phosphate buffer (pH 6.9) instead of water reduced the loss of dFUR to 58%. The loss of ddI by the two phosphate washes was $<10\%$. Hence the 100 m*M* phosphate buffer was selected for the final method. The analyte recovery from plasma was determined for this final method. Using two washes with phosphate buffer and eluting with a 75:25 (v/v) mixture of methanol–5 m*M* phosphate buffer, the recoveries of ddI, MeAdo, and dFUR were >90, >90, and 41%, respectively. The recovery of dFUR was reproducible at 41.1% with a 4.5% coefficient of variation ($n=7$). The recovery of MeAdo was higher than that of dFUR. However, dFUR had a shorter HPLC retention time and was therefore preferred. The assay time using dFUR as the internal standard was 12 min. In comparison, the assay time with MeAdo was 38 min. The intra-day (six samples each day) and inter-day (over five days) assay precisions of ddI analysis were 5.1 and 10.1%, respectively.

HPLC analysis

We used the UV scanning technique to identify interfering endogenous plasma

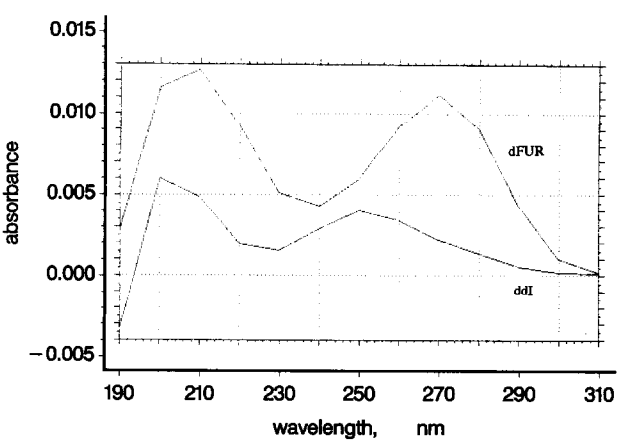


Fig. 1. UV spectra of authentic ddI and dFUR eluted from the HPLC system.

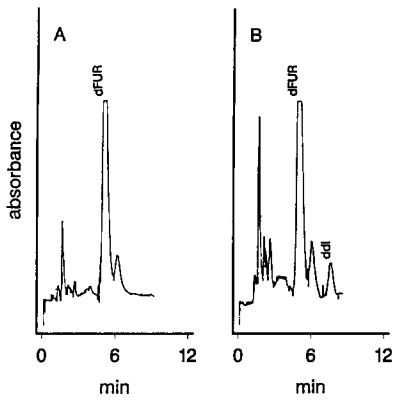


Fig. 2. Chromatograms of extracts of rat plasma samples obtained (A) before the administration of ddI (blank) and (B) 20 min after administration of 8 mg/kg ddI intravenously. The internal standard, dFUR, was added to both samples. The stationary phase was a μBondapak C_{18} column (300 mm × 3.9 mm I.D., 10 μm particle size). The mobile phase consisted of 4% (v/v) acetonitrile and 10 m*M* phosphate buffer, pH 6.9.

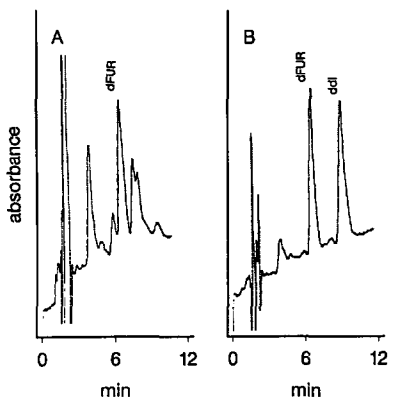
components and to develop the final assay conditions. The interfering plasma peaks were characterized by their UV spectra, and the differential effects of changes in mobile phase on the retention of ddI and plasma constituents were determined. Furthermore, the optimal detection wavelength was verified by comparison of UV spectra of ddI and adjacent plasma peaks. Fig. 1 shows the UV scans of authentic samples of ddI and dFUR. The elution volumes of ddI and dFUR were 10.1 and 15.5 ml, respectively. The coefficients of variation of the elution volumes were 0.8% ($n = 10$) and 1.8% ($n = 5$) for intra-day and inter-day analyses using a single HPLC column. Retention times on different columns varied up to 20%. Representative chromatograms of the HPLC analysis of rat plasma samples obtained before and 20 min after an intravenous ddI dose (8 mg/kg) are shown in Fig. 2. The concentration of ddI in the post-treatment sample was 438 ng/ml, or 44 ng per 100 μl of plasma assayed.

The known metabolites of ddI, uric acid and hypoxanthine, eluted with retention volumes of 3.2 and 5.0 ml. These compounds were poorly retained on the SPE-C_{18} columns and were not analyzed using the current assay. A separate assay is needed for these compounds.

Fig. 3 shows the HPLC profile of the urine samples taken before treatment and 24 h after treatment. There were no interferences in the urine samples.

Standard curves

The standard curve of ddI obtained from the extraction of spiked rat plasma was linear over the concentration range 0.2–200 μg/ml. The regression equation for this line was (concentration) = 13.4(peak-height ratio) − 0.002, with a corre-



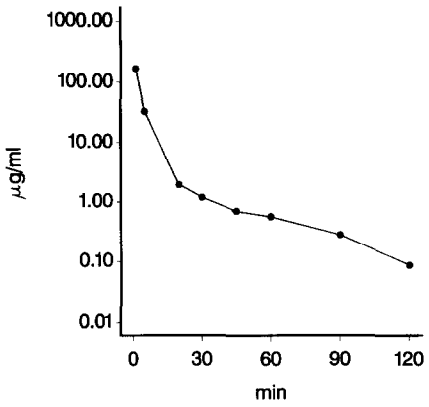


Fig. 3. Chromatograms of rat urine samples obtained (A) before and (B) 24 h after the administration of 40 mg/kg ddI. The internal standard, dFUR, was added to both samples. The ten-fold diluted samples were analyzed, using the same conditions as described in Fig. 2.

Fig. 4. Concentration–time profile of ddI in a rat given an intravenous dose of 40 mg/kg.

lation coefficient of 0.996. The lower detection limit of this assay was 200 ng/ml, using a plasma sample of 100 μl.

The standard curve obtained from the extraction of spiked rat urine was linear over the concentration range 0.5–200 μg/ml. The regression equation for this line was (concentration) = 9.38(peak-height ratio) − 0.009, with a correlation coefficient of 0.9995. The sensitivity of the urine assay could be increased by using 1 ml of the diluted urine sample instead of the 100-μl sample assay described. For high ddI concentrations, the extraction step could be omitted.

Application to rat samples

Fig. 4 shows the plasma concentration–time profile of ddI in a rat given an intravenous dose of 40 mg/kg. In this rat, the terminal half-life of ddI was 24 min, the total body clearance was 57 ml/min/kg, and the fraction of the dose excreted unchanged in the urine was 14%.

CONCLUSIONS

We have described here an analytical method for ddI utilizing a solid–liquid extraction and reversed-phase HPLC. This assay has a lower limit of detection of 20 ng per sample or 200 ng/ml using a 100-μl sample. As shown in this report, this assay is useful for pharmacokinetic studies of ddI in small rodents.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

A new internal standard, ftorafur [*R*,S-1-(tetrahydro-2-furanyl)-5-fluorouracil], is now being used. Compared to dFUR, ftorafur has a better extraction recovery (90%) and a similar HPLC retention time.

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