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# Note

## Quantitation of didanosine in human plasma and urine by high-performance liquid chromatography

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Didanosine (2′,3′-dideoxyinosine, BMY-40900, ddI) is a nucleoside analogue currently under development for the treatment of acquired immune deficiency syndrome (AIDS) [1]. ddI inhibits the infectivity of the human immunodeficiency virus (HIV) *in vitro* at concentrations greater than 10 $\mu M$ and possesses a high therapeutic index in human cell lines [2]. It is proposed that ddI, through a series of intracellular conversions, generates the triphosphate form of dideoxyadenosine (ddATP) [2]. Incorporation of ddATP into viral DNA is thought to terminate chain elongation, leading to the inhibition of DNA synthesis.

Several methods have been reported for the analysis of ddI in biological samples [3–6]. These methods employ reversed-phase high-performance liquid chromatography (HPLC) with detection by UV absorbance or measurement of eluted radioactivity. Limitations in assay sensitivity or suitability for processing large numbers of samples from multi-center clinical trials necessitated the development of other analytical methods. This paper describes the validation of HPLC methods for the quantitation of ddI in plasma and urine samples obtained from AIDS patients. All samples are heated at 57°C prior to extraction to inactivate the HIV [7,8].

## EXPERIMENTAL

### *Materials*

Didanosine and internal standards [2′,3′-didehydro-3′-deoxythymidine (d4T) or 3′,5′-anhydrothymidine (d4T oxetane)] were used as received at Bristol-Myers Squibb (Syracuse, NY, U.S.A.). The purity of all reference compounds was greater than 98%. Human plasma, containing sodium heparin, was purchased from Cocalico Biologicals (Reamstown, PA, U.S.A.) or obtained from volunteers on site. Human urine was collected from volunteers and pooled prior to use. The

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following were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.): HPLC-grade methanol, potassium phosphate monobasic and dibasic, sodium phosphate monobasic and dibasic and phosphoric acid. 2-Methoxyethanol was obtained from Burdick and Jackson (Muskegon, MI, U.S.A ) and triethylamine (TEA) from Kodak (Rochester, NY, U.S.A ) All mobile phases and buffers were prepared using water purified through a Milli-Q system (Millipore, Milford, MA, U.S.A.).

*Equipment*

The chromatographic system consisted of a Waters (Waters, Division of Millipore, Milford, MA, U.S.A.) 590 solvent delivery pump, a 710B WISP autoinjector and a Kratos Spectroflow 783 ultraviolet (UV)–visible variable-wavelength detector (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.) operated at 254 nm. The analog output of the UV detector was digitized by an analog-to-digital converter and analyzed using the Hewlett-Packard Model 3357 laboratory automation system (Hewlett-Packard, Avondale, PA, U.S.A.) according to previously described procedures [9]. A Brown Type WK water bath with orbital shaker (B. Braun Instruments, Burlingame, CA, U.S.A.) was used to heat samples prior to processing. Solid-phase extraction (SPE) procedures were conducted using a Baker vacuum manifold (J. T. Baker Research Products, Phillipsburg, NJ, U.S.A.) Extracted plasma samples were evaporated in an N-EVAP evaporator (Organomation Assoc., South Berlin, MA, U.S.A.).

*Sample preparation*

Standards and quality control samples (QCs) were prepared directly in the appropriate matrix. Urine samples were buffered with 200 m*M* potassium phosphate buffer, pH 8.0 (1 part urine to 2 parts buffer) at the time of preparation to improve the stability of ddI, which hydrolyzes rapidly under even mildly acidic conditions [10]. Samples not processed immediately were stored at −20°C. All clinical samples and QCs were heated at 57°C for 3 h to inactivate the HIV [7,8] prior to extraction. Frozen standard solutions were thawed at room temperature but were not heated.

Plasma samples were extracted using $C_{18}$ SPE columns (500 mg) from J. T. Baker Research Products. SPE columns were activated with methanol, followed by 10 m*M* sodium phosphate buffer, pH 8.0 A 1.0-ml aliquot of internal standard solution (d4T oxetane, 3 μg/ml in sodium phosphate buffer) was placed on the cartridge, followed by 0.5 ml of plasma. The samples were aspirated slowly. Columns were washed with 3 ml of the conditioning buffer and 3 ml of water, dried, then eluted using two 0.5-ml aliquots of methanol. Plasma eluates were evaporated under a stream of nitrogen at 40°C, followed by reconstitution in 0.20 ml mobile phase (pH adjusted to 7.0).

Urine samples were extracted using Baker 500-mg phenylsilane columns. Prior to sample extraction, columns were washed with methanol, followed by 20 m*M*

potassium phosphate buffer, pH 8.0. Buffered sample (0 5 ml) was pipetted on the column, followed by 0 1 ml of a 400 μg/ml d4T internal standard solution prepared in water The columns were washed sequentially with 3 ml of each of the following 20 m*M* monobasic potassium phosphate, 20 m*M* potassium phosphate buffer (pH 8.0) and water. Columns were dried under vacuum, then eluted with two 0.5-ml aliquots of aqueous 70% methanol containing 0.02% TEA. The eluate was mixed with 1.0 ml potassium phosphate buffer (20 m*M*, pH 7.2) prior to transfer to autosampler vials.

*Chromatographic conditions*

Plasma samples were analyzed using an Ultrasphere ODS column (5 μm particle diameter, 250 mm × 4.6 mm I.D.) (Beckman Instruments, San Ramon, CA, U S.A.), preceded by a Waters guard column (2.3 cm × 0.4 cm I.D.) packed with Whatman Pellicular ODS (Whatman International, Maidstone, U.K.). The mobile phase consisted of 15% methanol in 50 m*M* potasssium phosphate buffer containing 0.05% TEA, pH 4.0, and was delivered at a flow-rate of 0.7 ml/min The injection volume of sample extract was 50 μl

Urine samples were chromatographed on a Zorbax $C_8$ 5-μm column (250 mm × 4.6 mm I.D., Dupont, Wilmington, DE, U.S.A.). The guard column was identical to the type used for plasma analysis. The mobile phase consisted of 3.9% methoxyethanol in 18.3 m*M* potassium phosphate buffer, pH 7.2. The flow-rate was 1.0 ml/min. A 20-μl aliquot of processed sample was injected.

*Validation procedure*

Validation of the analytical methods included documentation of assay linearity, accuracy, precision, lower limit of quantitation (LLQ), specificity, recovery and stability of ddI during sample processing and storage. A minimum of six standard concentrations, processed in duplicate, comprised each standard curve. Peak-height ratios (ddI to internal standard) were calculated for each chromatogram. A least-squares regression line, weighted by the inverse of the concentration of each standard, was used to predict the concentration of ddI in QCs and samples. The accuracy and precision of the method were assessed at concentrations in the upper and lower quartile of the standard curve range. Accuracy was defined as the deviation of the mean observed concentration from nominal, expressed as a percentage of the nominal concentration. Intra-assay precision was expressed as the percentage relative standard deviation (R.S.D.) of the predicted concentrations of the replicate samples analyzed from each pool. The data from three different runs was evaluated by a one-way ANOVA. The inter-assay precision (R.S.D.) was calculated according to the following equation:

$$100(\mathrm{TrMS} - \mathrm{EMS}/n)^{0.5}/\mathrm{GM}$$

where TrMS, EMS and GM refer to treatment and error mean squares and grand mean, respectively LLQ and specificity were evaluated concurrently using matrix

obtained from ten different volunteers. A spiked sample containing the lowest concentration of ddI used in the standard curve was assayed along with a blank sample from each volunteer. Chromatograms were inspected to assess the degree of interference by endogenous constituents with ddI and the internal standard. Recovery of ddI was determined by comparing the slope of a matrix standard curve to the slope obtained when aqueous standards were injected directly onto the HPLC system. Stability of ddI in matrix was assessed during the heating step, after processing and during storage at −20°C for up to one year.

*Collection and processing of clinical samples*

The pharmacokinetics and extent of urinary excretion of ddI in patients with AIDS or AIDS-related complex (ARC) have been investigated as part of a Phase I safety and efficacy study Patients received intravenous doses ranging from 0 4 to 16.5 mg/kg for two weeks at 12-h intervals, followed by oral therapy at twice the intravenous dose. Serial blood samples and the total urinary output were obtained after the first intravenous or oral dose and at steady state. Plasma and urine samples were analyzed as previously described.

## RESULTS AND DISCUSSION

Representative chromatograms obtained from blank plasma, a plasma sample

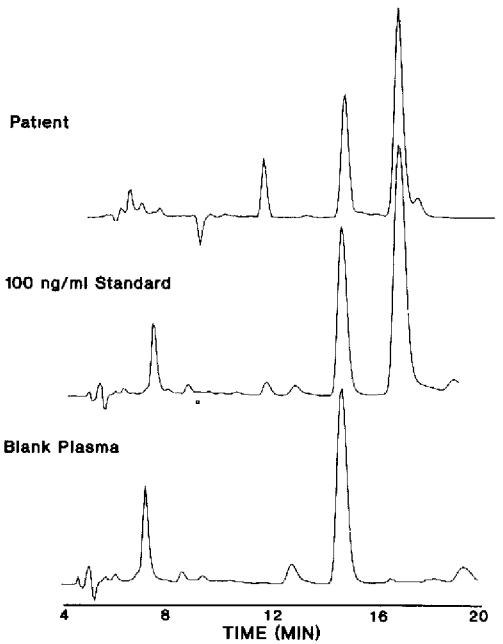


Fig 1. Representative chromatograms of blank extracted human plasma, plasma spiked with 100 ng/ml ddI and a sample from a patient after administration of an intravenous dose of ddI

TABLE I

ACCURACY AND PRECISION DATA FOR THE ANALYSIS OF ddI IN HUMAN PLASMA AND URINE

| Matrix[a] | ddI concentration (μg/ml) | | | R.S.D (%) | Deviation from nominal (%) |
|---|---|---|---|---|---|
| | Nominal | Predicted | S D | | |
| Plasma | 0.10 | 0 09 | 0 0009 | 0 9 | −9.0 |
| | 8 40 | 8 0 | 0 03 | 0 4 | −4.8 |
| Urine | 6 9 | 7 0 | 0 67 | 8 1 | +1 5 |
| | 350 0 | 344.9 | 18 97 | 5 1 | −1 5 |

[a] A total of ten replicates of each concentration were assayed

spiked with ddI and a sample from a patient receiving ddI are shown in Fig. 1 The retention times for ddI and internal standard were approximately 11.2 and 16.8 min, respectively There was no evidence of any endogenous substance causing interference at the retention time of ddI. The LLQ in plasma was 0.025 μg/ml. Accuracy at the LLQ was 9.6% and the precision was 7.1% R.S.D. The detector

TABLE II

STABILITY OF ddI IN PLASMA AND URINE SAMPLES STORED AT −20°C

| Duration of storage (days) | Nominal concentration (μg/ml) | Mean predicted concentration (μg/ml) | Deviation from nominal (%) | R S D (%) |
|---|---|---|---|---|
| *Plasma* | | | | |
| 0 | 0 10 | 0 09 | −9 0 | 0 9 |
| | 8 40 | 8 00 | −4.8 | 0 4 |
| 60 | 0.19 | 0 18 | −7 9 | 1 4 |
| | 8 00 | 8 35 | 4 4 | 1 8 |
| 261 | 0.20 | 0 24 | 19 1 | 0 2 |
| | 20 00 | 23 86 | 19 3 | 1 6 |
| 369 | 0.20 | 0 21 | 6 4 | 1 4 |
| | 20.00 | 21 38 | 6 9 | 2 0 |
| *Urine* | | | | |
| 0 | 17.6 | 16 8 | 4.4 | 5 3 |
| | 293.0 | 314 4 | 7 3 | 13.1 |
| 97 | 15.0 | 14 7 | −1 9 | 0 8 |
| | 300 0 | 287 5 | −4 2 | 0.9 |
| 205 | 15 0 | 16.3 | 8 5 | 4 5 |
| | 300 0 | 332 0 | 10.7 | 7 4 |
| 530 | 17 6 | 19 5 | 11 0 | 4 9 |
| | 293 0 | 340 4 | 16.2 | 1 8 |

response was linear over the range 0.025–10 μg/ml The intra-assay (within-day) accuracy and precision data for ddI extracted from plasma are shown in Table I. The inter-assay (between-day) precision was 1.4% R.S.D. at a concentration of 0 1 μg/ml and 3.8% R.S D. at a concentration of 8.4 μg/ml ddI in plasma. The recovery of ddI from extracted plasma samples was 85%. Stability of ddI at 56°C for 3 h and in the injection vehicle for 44 h was documented. Stability studies also demonstrated that samples could be frozen, heated, refrozen and then assayed without affecting the accuracy or precision of the analytical data. Periodic analysis of sample aliquots stored for up to one year at −20°C revealed no evidence of ddI degradation, as shown in Table II

Typical urine chromatograms obtained from blank and spiked samples and a specimen from a patient are shown in Fig. 2. The retention times for ddI and internal standard were approximately 15.3 and 19.4 min, respectively. The urine assay was linear over the range 1.0–400 μg/ml Accuracy at the LLQ, established as 1.0 μg/ml, was 10% with a precision of 9.1% R.S.D. The within-day accuracy and precision data are shown in Table I. Between-day precision values at concentrations of 6 9 and 350 μg/ml were 3.6 and 2.9% R S D , respectively Extraction recovery averaged 97%. The stability of ddI in buffered urine or processed urine extracts was equivalent to that observed in plasma (Table II).

Analyses of ddI in large numbers of plasma and urine samples collected during

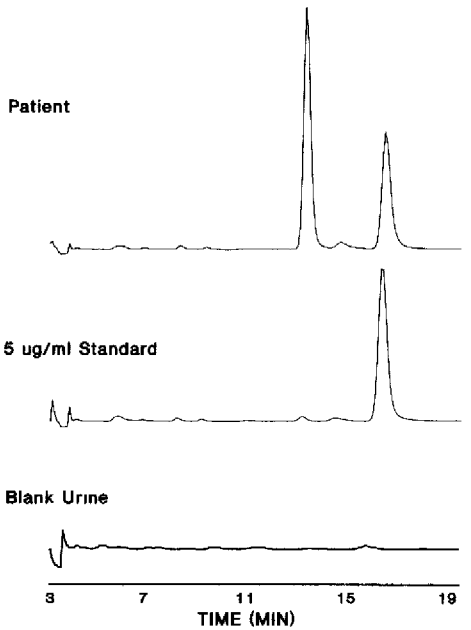


Fig. 2 Representative chromatograms of blank extracted human urine, urine spiked with 5 0 μg/ml ddI and a sample from a patient after administration of an intravenous dose of ddI

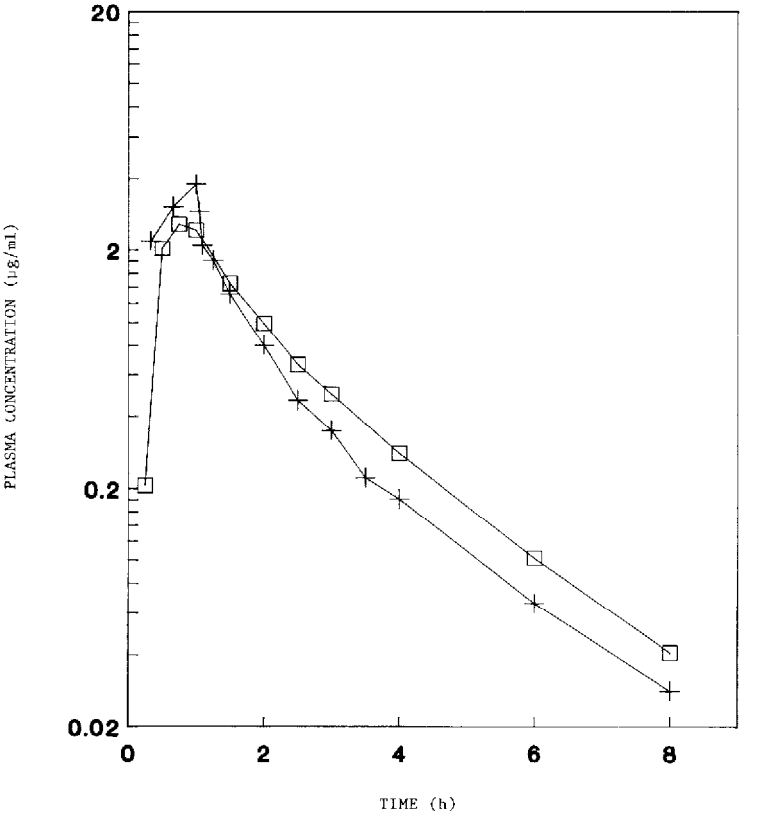


Fig 3 Typical plasma concentration *versus* time profile in an AIDS patient receiving either 3 0 mg/kg ddI intravenously (+) or 6 0 mg/kg ddI orally (□).

Phase I clinical trials have been conducted. Fig. 3 is a representative plasma concentration *versus* time profile for ddI in a patient receiving 3 mg/kg intravenously or 6 mg/kg orally Quantifiable levels of ddI were detected for up to 8 h after both routes of administration. The maximum plasma concentration was 3 79 μg/ml after intravenous administration and 2.59 μg/ml after ddI was given orally. The apparent elimination half-life was approximately 1.5 h, regardless of the route of administration. The absolute bioavailability of ddI was 47%. The urinary recovery of ddI in this patient was 32% after intravenous administration and 29% after ddI was given orally.

The sensitivity of the ddI plasma assay is comparable to that obtained in the HPLC assay for azidothymidine [11], a nucleoside analogue that is currently the only approved therapeutic agent for AIDS. The HPLC retention times of a number of other compounds, either structurally related to ddI or used in the treatment of AIDS patients, have been determined using both the plasma and urine chromatographic conditions (Table III) None of the compounds listed interfered

TABLE III

COMPOUNDS THAT DO NOT INTERFERE CHROMATOGRAPHICALLY WITH THE QUANTITATION OF ddI IN HUMAN PLASMA OR URINE

| | |
|---|---|
| Acetominophen | Hypoxanthine |
| Acyclovir | Ibuprofen |
| Amikacin | Inosine |
| Amphotericin B | Ketoconazole |
| Aspirin | Nystatin |
| AZT | Ribavarin |
| Clindamycin | Rifabutin |
| Dapson | Rifampicine |
| 2'-Deoxyinosine | Sulfadiazine |
| Dextran Sodium Sulfate | Sulfamethoxazole |
| Fluconazole | Thymidine |
| 5-Fluorocytosine | Thymine |
| Ganciclovir | Trimethoprim |
| | Xanthine |

with the quantitation of ddI or the internal standards. The assays have proven to be rugged and provide consistent data even when more than one analytical facility is processing samples. Interferences in urine samples analyzed using plasma methodology necessitated the development of different extraction and chromatographic conditions for the quantitation of ddI in urine. TEA was used to minimize peak tailing in the plasma HPLC system but was not necessary in the mobile phase used for urine samples.

These methods are particularly useful since they incorporate a step to inactivate HIV. Inactivation of the AIDS virus through heat treatment is an efficient decontamination step that can be used as soon as samples are received, thereby minimizing exposure of laboratory personnel to infectious samples. Heat inactivation is advantageous in that additional sample manipulation is not required, such as occurs when a virucidal agent is added, and samples are not unnecessarily diluted.

In conclusion, the validated methods for the quantitation of ddI in human plasma and urine are sensitive, accurate and precise Samples are stable when heated at 56°C to inactivate the AIDS virus, or upon storage at −20°C for at least one year.

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