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Short Communication

High-performance liquid chromatographic determination of 2′,3′-didehydro-3′-deoxythymidine, a new anti-human immunodeficiency virus agent, in human plasma and urine

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ABSTRACT

Sensitive and selective high-performance liquid chromatographic techniques have been developed for the determination of 2′-3′-didehydro-3′-deoxythymidine, d4T (BMY-27857), in human plasma and urine. The methods had linear standard curves over the concentration ranges 0.025–25.0 and 0.5–100 μg/ml for the plasma and urine matrices, respectively. Both methods used solid-phase extraction for isolating d4T and the internal standard, thymidine oxetane, from the biological matrix. In addition, the analytical column, mobile phase, instrumentation and chromatographic conditions used for both methods were identical. The ultraviolet absorbance of the column effluent was monitored at 266 nm. Results of analysis of quality control samples indicated that the intra-assay precision values, as measured by percent relative standard deviation, were within 12 and 3%, and accuracy samples deviated less than 10 and 5% from nominal values for the plasma and urine assays, respectively.

INTRODUCTION

2′,3′-Didehydro-3′-deoxythymidine, d4T (BMY-27857), has *in vitro* activity against human immunodeficiency virus (HIV) comparable to that of 3′-azido-2′,3′-dideoxythymidine (AZT or zidovudine) in a number of *in vitro* assay systems [1]. However, its toxicity to cells in culture, including bone marrow progenitor cells, is markedly less than AZT [2,3]. Dideoxynucleoside analogues have been the focus of a considerable research effort due to their inhibitory effect on HIV. It is thought that inhibition occurs by incorporation of the dideoxynucleotide at the 3′-end of unintegrated viral DNA. This terminates elongation of the DNA chain since, without a hydroxyl group at the 3′-position of the sugar moiety, addition of the next nucleotide cannot occur.

d4T is currently undergoing Phase I clinical trials in AIDS patients. Selective and sensitive high-performance liquid chromatographic (HPLC) methods were developed and validated for the quantification of d4T in plasma and urine matrices for pharmacokinetic analysis.

Heat treatment at 56°C has been reported to inactivate HIV in the laboratory setting [5]. The stability of d4T to heat inactivation was tested during assay validation. If the inactivation treatment did not degrade d4T significantly, it would be applied during sample processing to reduce the risk of viral infection. The concomitant ther-

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apeutic agents most likely to be encountered in the plasma of AIDS patients were screened in the HPLC system. Those compounds eluting near or at the retention time of one of the analytes would be noted.

This report describes assay methods for the quantification of d4T in plasma and urine and the additional work undertaken in support of ongoing clinical studies.

EXPERIMENTAL

Chemicals and reagents

d4T and the internal standard (I.S.), thymidine oxetane, were supplied by Bristol-Myers Squibb (Syracuse, NY, USA). The structures of both compounds are shown in Fig. 1. Triethylamine (TEA) was purchased from Eastman Kodak (Rochester, NY, USA). All other chemicals and solvents were of ACS analytical or HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was used throughout and was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Instrumentation and chromatographic conditions

The HPLC systems for both the plasma and urine assays consisted of the following equipment. The primary system included an M-45 solvent-delivery system pump, a WISP 710 autoinjector and a Lambda-Max 481 LC spectrophotometer, all from Waters Assoc. (Milford, MA, USA). d4T and I.S. were resolved from endogenous interference on an Apex octadecyl column (250 mm × 4.6 mm I.D., 5 μm, Jones Chromatography, Littleton, CO, USA), at a mobile phase flow-rate of 0.8 ml/min. The mobile phase consisted of 10 m*M* ammonium phosphate–acetonitrile, (9:1, v/v) with 7.2 m*M* TEA added. The pH was adjusted to 2.5 with 85% phosphoric acid. Column effluent was monitored via UV detection at 266 nm. Typical retention times for d4T were 7 and 7.5 min, and for I.S. 9 and 10.5 min in the plasma and urine matrices, respectively. Detector output was collected on an HP 3357 mini-computer system. The data management system used to acquire and process data has been described [6].

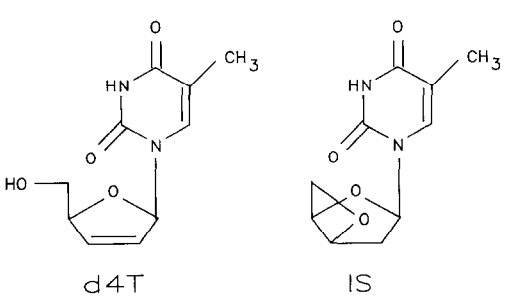

Fig. 1. Structures of d4T and internal standard (IS).

Preparation of standards and quality controls

A stock d4T spiking solution (25 μg/ml) was serially diluted to prepare a seven-point standard curve over the range 0.025–25 μg/ml. The standard curve in urine was prepared similarly over the range 0.5–100 μg/ml. Quality control samples (QCs) consisted of known concentrations of d4T in the appropriate matrix which were analyzed along with the clinical samples to assess assay accuracy and precision.

Assay procedures

Plasma assay. Each 0.5-ml plasma sample was extracted with a fresh 1-ml C_{18} Bond Elut column using a Vac Elut vacuum assembly (Analytichem International, Harbor City, CA, USA). The column was first activated by consecutive rinses with methanol and water, respectively. The plasma sample and 50 μl of I.S. were then aspirated through the column and the column rinsed with two column volumes of water. d4T and I.S. were eluted with 1.0 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen in a 37°C water-bath. The samples were reconstituted in 125 μl of mobile phase and 100 μl were injected on the HPLC column.

Urine assay. Urine samples were extracted using 3-ml phenyl solid-phase extraction columns (Bakerbond SPE, J. T. Baker, Phillipsburg, NJ, USA). Column activation and washing procedures were optimized in order to resolve d4T from endogenous interference. The columns were rinsed with one column volume (3 ml) of metha-

nol followed by two column volumes of 20 m*M* potassium phosphate (pH 8.0). A 0.5-ml aliquot of urine sample was transferred to the column followed by 50 μl of I.S. After aspiration, the columns were washed with one column volume each of 20 m*M* potassium phosphate (pH 4.1), 20 m*M* potassium phosphate (pH 8.0) and water. d4T and I.S. were eluted in two steps each using 500 μl of elution solvent. The elution solvent consisted of methanol–water, (7:3, v/v) with 1.4 m*M* TEA. The collected eluate was then diluted with 500 μl of 20 m*M* potassium phosphate (pH 7.2), briefly vortex-mixed, and 75 μl were injected on the HPLC column.

Validation procedures

The urine and plasma assay methods were evaluated and confirmed using an identical set of analytical procedures. The limit of detection and the lower limit of quantitation were determined by spiking plasma and/or urine from each of ten different individuals with d4T at 0, 16 and 26 ng/ml in plasma and 0, 0.25 and 0.5 μg/ml in urine. The lowest concentrations which were significantly different from zero (via a one-tailed paired *t*-test) would be established as the limit of detection. The accuracy and precision of each assay were assessed by analyzing blinded quality control samples in three separate analytical runs on different days. The QCs were prepared at concentrations of 0.20, 20.0 and 83.0 μg/ml in plasma and 2.8, 71.4 and 713 μg/ml in the urine matrix. The upper-most concentration of each group served as a dilution QC.

HIV inactivation methods

The stability of d4T during heat inactivation experiments (at 56°C) for 1, 3 and 5 h was examined. Three matrices were tested (2.5 μg/ml): human plasma, water and 50 m*M* potassium phosphate (pH 7.4). Mean absolute area was used to compare treatment groups to control samples.

RESULTS AND DISCUSSION

Typical chromatograms of spiked plasma and urine samples are shown in Fig. 2. No interfering peaks were present at the retention time of d4T in either matrix. Two late-eluting peaks were observed in the urine matrix during assay development (*ca.* 28 and 43 min). In order to avoid interference from these peaks in subsequent injections, three test injections were made prior to the start of an analytical run. Using the test injections as a guide the auto-injector run time was adjusted to ensure resolution of d4T and I.S. from the endogenous late-eluting peaks.

The results of the limit of detection (LD) and lower limit of quantitation (LQ) experiment are presented in Table I. The necessary LQ concentration was decided upon based on the results of non-clinical pharmacokinetic studies. These studies were indicative of the sensitivity necessary to obtain relevant pharmacokinetic data in clinical trials. Based on the precisions and accuracy results the lower limits of quantitation were set at 0.025 and 0.5 μg/ml for the plasma and urine assays, respectively. Peak response was significantly different than background at 0.016 μg/ml in plasma and 0.25 μg/ml in urine. Based on observed response the extrapolated limit of detection (at a signal-to noise ratio of 3) for d4T would be *ca.* 0.006 μg/ml in plasma and 0.150 μg/ml in urine. The intra- and inter-assay precision and accuracies of the methods are presented in Table II. The precision (R.S.D., %) and accuracy (percentage deviation) values were within 5% for all three QC concentrations in the urine matrix. Precision values were within 12.3% and accuracy deviated by not more than 10.1% in plasma.

The human plasma assay was linear over a d4T concentration range of 0.025–25 μg/ml using peak-area ratio as the quantitation method. The human urine assay was linear over a d4T concentration range of 0.5–100 μg/ml using peak-height ratio as the quantitation method. The correlation coefficients of the regression lines were ≥ 0.999 for all three validation runs in both matrices.

With one exception (Ethambutol), no interference was found at the retention times of either d4T or I.S. for therapeutic and structurally related compounds tested. Several compounds were late-eluting (*i.e.* fluconazole, dapsone, ganciclovir, sulfamethoxazole and ketoconazole), coming

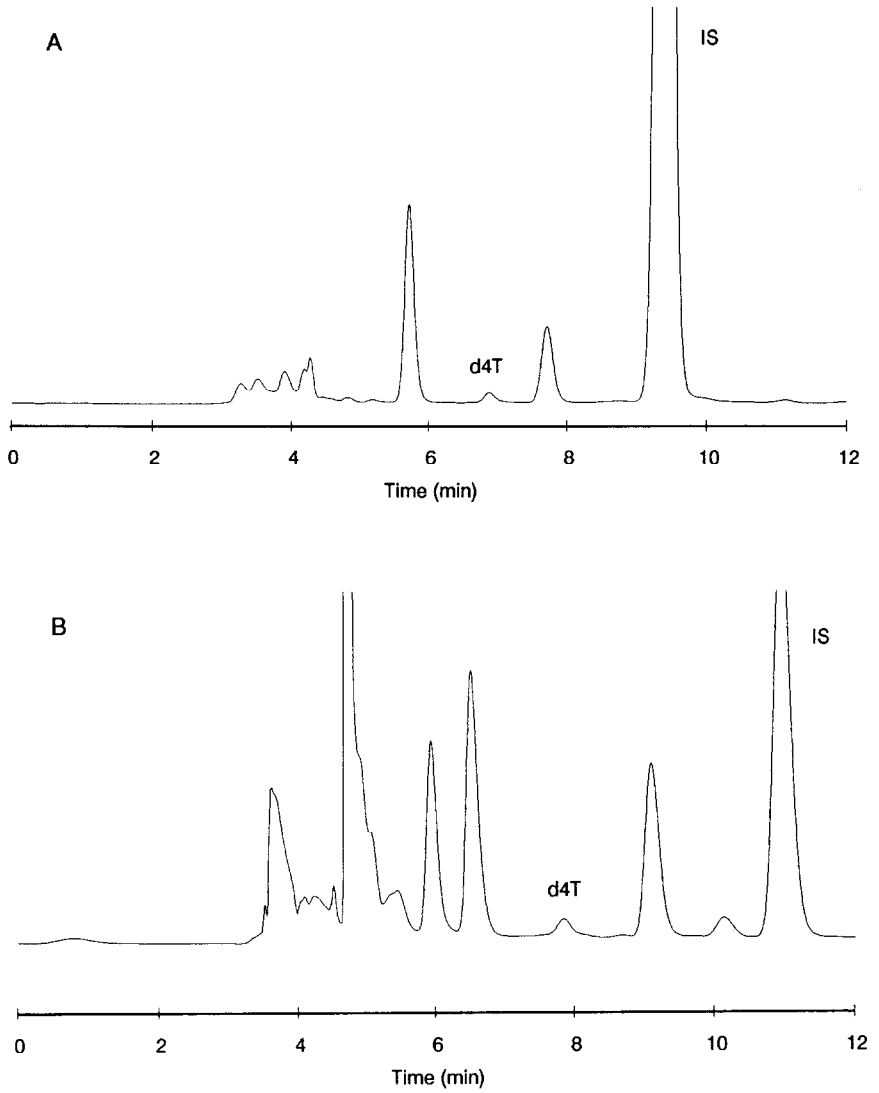

Fig. 2. Typical chromatograms for d4T and internal standard (IS) in human plasma and urine. (A) Human plasma containing 0.025 μg/ml d4T; (B) human urine containing 0.5 μg/ml d4T.

TABLE I

LOWER LIMIT OF QUANTITATION AND LIMIT OF DETECTION FOR d4T IN HUMAN PLASMA AND URINE (n = 10)

Matrix	Concentration (μg/ml)		Precision (R.S.D., %)	Accuracy (percentage deviation)	Mean signal-to-noise ratio
	Nominal	Predicted			
Plasma	0	–	–	–	–
	0.016	0.015	20.0	9.2	8.1
	0.026	0.025	23.0	4.5	13.1
Urine	0	–	–	–	–
	0.25	0.239	12.7	4.4	46.3
	0.50	0.482	6.8	3.6	104.5

TABLE II

INTER- AND INTRA-ASSAY PRECISION AND ACCURACY DATA

Matrix	Concentration (μg/ml)		Inter-assay		Intra-assay	
	Nominal	Predicted	R.S.D. (%)	Deviation (%)	R.S.D. (%)	Deviation (%)
Plasma	0.20	0.21	5.3	4.3	3.8	1.5–10.1
	19.99	20.16	3.9	0.8	2.3	0.5–5.3
	83.05	83.41	5.4	0.4	12.3	3.6–9.4
Urine	2.86	2.90	2.3	1.7	1.7	1.1–3.5
	71.39	70.81	0.9	0.8	2.3	0.7–1.7
	713.90	730.10	1.7	2.3	1.3	0.9–4.4

off the column at times greater than 30 min.

Heat treatment is an unsuitable inactivation method for use with d4T. Values for percentage deviation in plasma from time zero ranged from a 6.0 gain to a 20.0% loss for the 1-h samples. Using three different lots of plasma, irreproducible degradation patterns were observed for d4T. Significant degradation of d4T also occurred in both water and potassium phosphate buffer. The course of degradation was similar in these two matrices ranging from *ca.* 5% to 30% in 1 to 5 h.

The plasma and urine assay methods described herein are currently being used for the assay of clinical study samples. Examples of plasma concentration *versus* time and cumulative urinary excretion profiles in an adult male patient treated with 1 mg/kg intravenous and 4 mg/kg oral doses are depicted in Fig. 3. The peak d4T concentration 1 h after the end of the intravenous infusion was 1.57 μg/ml and the peak concentration following the oral dose was 7.51 μg/ml. Disappearance of this drug from plasma is very rapid with a terminal half-life of *ca.* 1 h. Within 6 h, the urinary excretion of d4T is almost complete. Over a

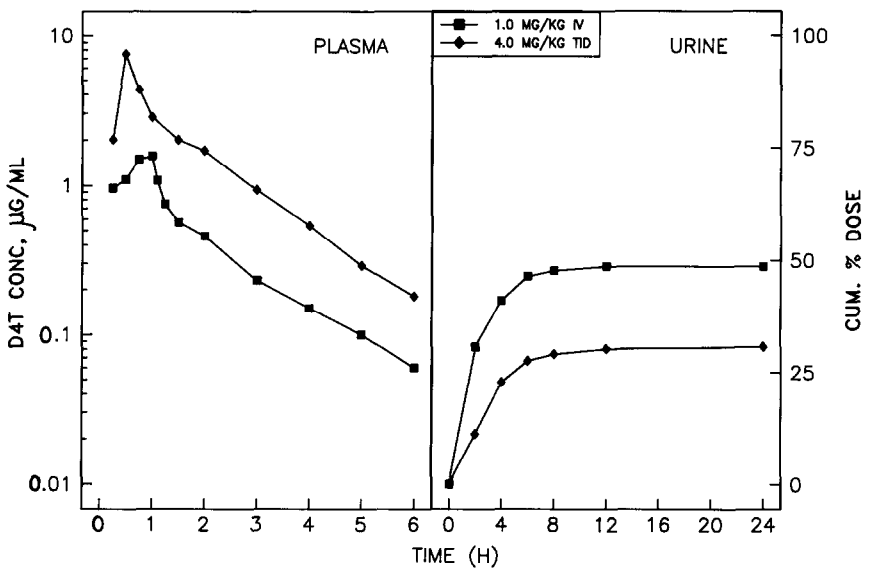

Fig. 3. Plasma concentration *versus* time and cumulative urinary excretion profiles in an adult male patient treated with 1 mg/kg intravenous and 4 mg/kg oral doses.

24-h period, 49 and 31% of the intravenous and oral doses, respectively, were excreted as the parent compound.

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