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Technical Note

Analytical Method for the Quantification of 2′,3′-Didehydro-3′-Deoxythymidine, a New Anti-Human Immunodeficiency Virus (HIV) Agent, by High-Performance Liquid Chromatography (HPLC) and Ultraviolet (UV) Detection in Rat and Monkey Plasma

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KEY WORDS: 2′,3′-didehydro-3′-deoxythymidine determination; high-performance liquid chromatography; rat plasma; monkey plasma.

INTRODUCTION

2′,3′-Didehydro-3′-deoxythymidine (d4T or BMY-27857; Fig. 1) was synthesized in 1966 by Horowitz and coworkers at the Detroit Institute of Cancer Research (1). The potent anti-human immunodeficiency virus (anti-HIV) activity of d4T has been demonstrated by a number of investigators using a variety of assays (2–7). Biochemical studies indicate that, in common with other related nucleoside analogues, this compound acts at the level of reverse transcriptase of HIV and other retroviruses (8–13); following phosphorylation by cellular kinases, d4T-triphosphate is produced which preferentially inhibits viral reverse transcriptase activity, with relatively little inhibition of host cell DNA polymerases. Although the *in vitro* activity of d4T against HIV is almost comparable to that of 3′-azido-3′-deoxythymidine (AZT or zidovudine) (4,6), its toxicity to cells in culture, especially to human bone marrow progenitor cells, is markedly less than that of AZT (7).

Currently, d4T is undergoing preclinical evaluation. In anticipation of the analysis of plasma samples from toxicologic and pharmacokinetic nonclinical studies, a selective and sensitive high-performance liquid chromatographic (HPLC) method was developed for the quantification of d4T in rat and monkey plasma, respectively. In order to expedite the assay validation process, a simultaneous validation in rat and monkey plasma was carried out.

MATERIALS AND METHODS

Materials

D4T reference standard, Lot No. 26630-21A, 98.0% purity, and thymidine oxetane (internal standard, Fig. 1), Lot No. 29867-075, 98.2% purity, were supplied by the Reference Standards Department and Department of Chemical Processing and Development, respectively, Pharmaceutical Research and Development Division, Bristol-Myers Co., Syracuse, N.Y. The water was of Milli-Q quality (resistivity, >10 MΩ · cm) produced by the Milli-Q water purification system (Millipore Corp., Bedford, Mass.). HPLC-grade monobasic potassium phosphate and Optima-grade methanol were purchased from Fisher Scientific Co., Fair Lawn, N.J. Control Sprague-Dawley rat plasma with EDTA, Lot No. S-3515, and control cynomolgus monkey plasma with EDTA, Lot No. 880222, were purchased from Cocalico Biologicals, Inc., Reamstown, Pa. Rat plasma was obtained from an in-house rat colony.

Instrumentation and Accessories

The HPLC instrumentation consisted of a M-45 solvent delivery system (Waters Associates, Inc., Milford, Mass.), a WISP 710B autoinjector (Waters), and a UV detector, Lambda-Max Model 481 LC spectrophotometer (Waters). The detector wavelength was set at 254 nm. The chromato-

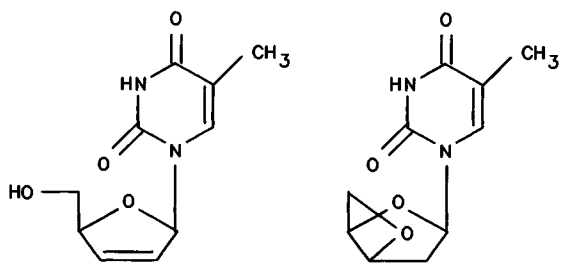

Fig. 1. Chemical structures of 2′,3′-didehydro-3′- deoxythymidine, d4T (1a), and thymidine oxetane, internal standard (1b).

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graphic separations were performed at ambient temperature on a 5-μm Apex octadecyl (250 × 4.6-mm i.d.) column (Jones Chromatography, Littleton, Colo.) using a mobile phase of 0.05 *M* potassium phosphate–methanol, 80:20 (v/v), at a flow rate of 1.0 ml/min. The HPLC column was preceded by a guard column (Part No. C-135B, Upchurch Scientific, Inc., Oak Harbor, Wash.) packed with Pellcular ODS, 37–53 μm (Whatman, Inc., Clifton, N.J.). The chart recorder (Model SE120, BBC Goerz Metrawwtt, Broomfield, Colo.) operated at a chart speed of 12 cm/hr and a 10-mV full-scale deflection. Data acquisition was done on the HP 3357 Laboratory Automation System (Hewlett Pack-

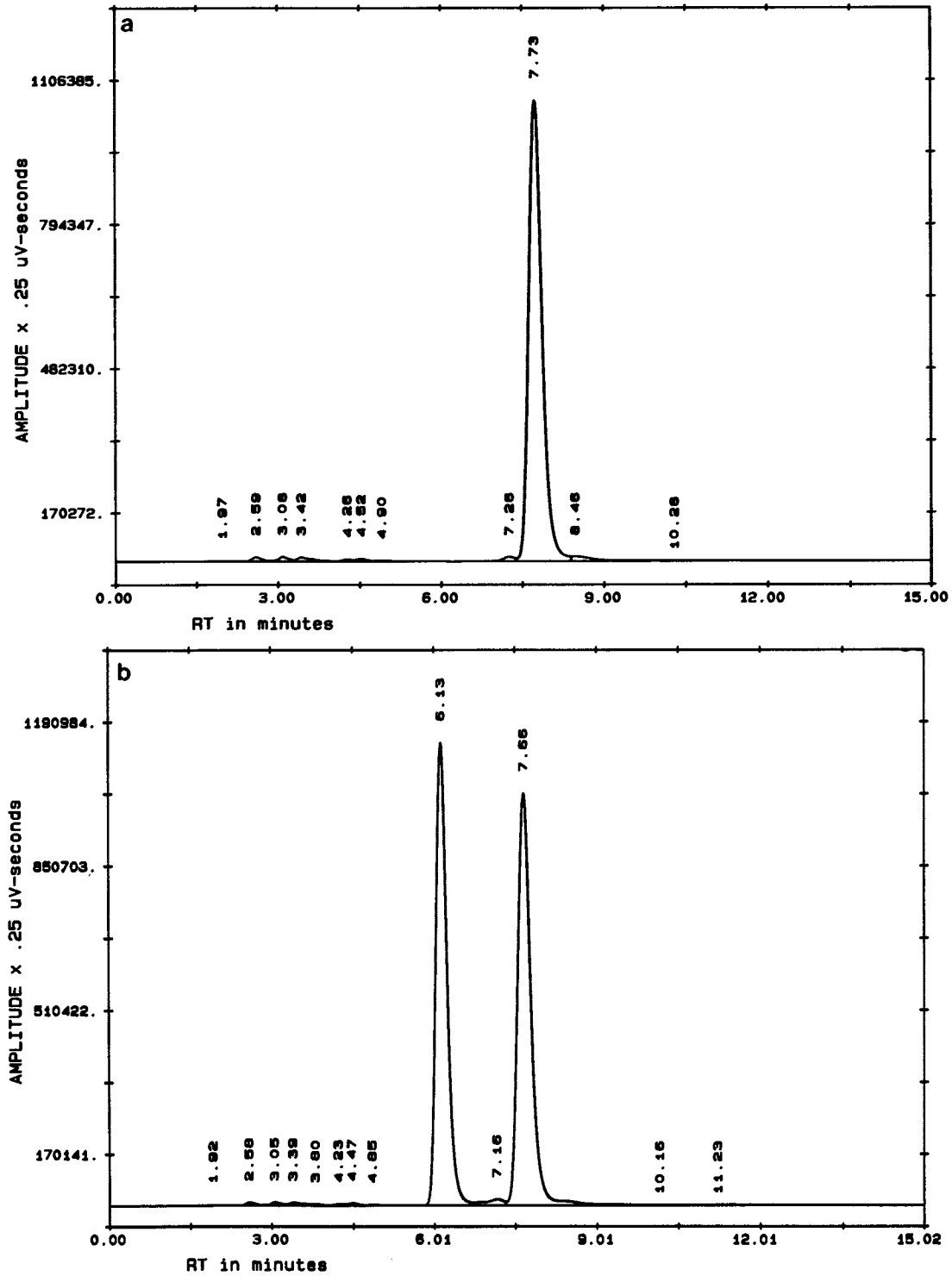

Fig. 2. Typical chromatograms for d4T and internal standard. (a) Blank rat plasma containing 50 μg/ml of internal standard; (b) rat plasma containing 50 μg/ml of d4T and internal standard. The approximate retention times of d4T and internal standard are 6 and 8 min, respectively.

Table I. Limit of Detection and Lower Limit of Quantitation Data

Concentration (μg/ml)		Precision	Accuracy	Mean peak	Mean signal/ noise
Nominal	Predicted	(% RSD)[a]	(% dev)[a]	area[b]	ratio
0	—	—	—	164	—
0.050	0.049	18	3	3144*	19
0.101	0.096	9	5	7187	44

[a] % RSD, percentage relative standard deviation; % dev, percentage deviation.
[b] $N = 10$.
* Significantly different from the mean peak area for the blank samples (0 μg/ml) by paired t test, $P < 0.001$.

ard, Palo Alto, Calif.). Peak purity and specificity of the assay method were evaluated using the Model 1040M diode-array detector and chemstation (Hewlett Packard). All extractions were performed on a Vac-Elut extraction system using 1.0-ml Bond Elut C_{18} columns (Analytichem International, Inc., Harbor City, Calif.). Samples were vortex-mixed on a touch-mixer (Fisher). The extracted samples were evaporated in an N-EVAP analytical evaporator at 35°C (Organomation Associates, Inc., South Berlin, Mass.).

Standard, Quality-Control, and Internal Standard Solutions

Stock solutions of d4T (1000 μg/ml) and internal standard (1000 μg/ml) were prepared in Milli-Q water. A working internal standard solution (125 μg/ml) was prepared from the stock solution. Plasma standards were prepared by spiking control rat or monkey plasma, to yield a concentration range of 0.1–100 μg/ml, with a plasma spiking solution (100 μg/ml). Quality-control samples (QCs), which were stored at −20°C, were prepared in sufficient quantity at the following concentration levels: rat plasma, 1.05, 50.2, 83.6, and 418.0 μg/ml; and monkey plasma, 6.70, 47.0, 84.6, and 470,0 μg/ml. The 50.2- and 47.0-μg/ml QCs were analyzed as freeze-thaw QCs and the 418.0- and 470.0-μg/ml QCs were analyzed after a 1:5 dilution.

Assay Method

Frozen samples were thawed at room temperature, mixed, and sampled. To a 10 × 75-mm disposable culture tube were added 0.25 ml of plasma sample and 0.1 ml of internal standard (125 μg/ml), and the samples were mixed by vortexing. To a 1.0-ml Bond Elut C_{18} column, attached to a Vac-Elut extraction system under vacuum, was added 2 column volumes of HPLC-grade methanol followed by 2 column volumes of water. The vacuum was relieved and the contents of the culture tube were transferred to the column. The system was evacuated and the column was washed with 2 column volumes of water. The vacuum was relieved and a clean 10 × 75-mm culture tube was positioned under the column. The system was evacuated and d4T and internal standard were eluted with 1.0 ml of methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen at 35°C. The residue was reconstituted in 200 μl of the mobile phase, then transferred to a WISP limited-volume insert, and 50 μl was chromatographed. The standards and QC samples were randomly dispersed on the WISP tray. In each analytical run, the standards and QCs were assayed in replicates of two and five, respectively.

Table II. Summary of d4T Plasma Standard Curves

Plasma species	Slope	Intercept	Correlation coefficient
Rat	0.01897	−0.00047	1.000
Monkey	0.01935	−0.00058	1.000
Rat	0.01905	−0.00090	1.000
Monkey	0.01906	−0.00039	1.000

Validation Procedure

The limit of detection and the lower limit of quantitation were determined by spiking plasma from 10 different rats at 0, 0.05, and 0.1 μg/ml of d4T and analyzing them versus a 7-point standard curve. The measured peak areas were recorded for each paired sample and subjected to a one-tailed paired t test. On 4 separate days, four standard curve validation experiments were carried out—two each using control rat or monkey plasma. To assess the accuracy and precision of the assay method, in each run, all the QC samples (both rat and monkey) were analyzed. The stability of d4T at −20°C and its freeze–thaw stability were determined by analyzing the QCs. The stability of the compounds in the reconstituted extracts was demonstrated by processing samples and making replicate injections at various time points. The recovery of d4T was determined by comparing the slope of a standard curve (peak area versus concentration) extracted from rat or monkey plasma to the slope of a nonextracted standard curve in mobile phase. The extraction recovery of the internal standard was determined by comparing the mean peak area from the above standard curves. The specificity of the assay method was evaluated by analyzing plasma samples obtained from dose-ranging studies using the diode-array detector.

Data Processing and Documentation

The detector output was recorded on the strip-chart recorder and collected by the HP 3357 Laboratory Automation System by a previously described method (14). At the end of each analytical run, the standards data (peak area ratio versus concentration) were fit to a linear regression equation weighting each standard by the reciprocal of its concentration and tested for outliers by the method of Prescott (15). The concentration of d4T in the unknown samples was determined by inverse prediction from the regression line.

RESULTS AND DISCUSSION

Chromatograms of blank and spiked rat plasma are shown in Fig. 2. No interfering peaks were present at the retention time (approximately 6 min) of d4T. Similar chromatograms were obtained from monkey plasma (not shown). Analysis of rat and monkey plasma samples from dose-ranging studies indicated the absence of potentially interfering metabolites (not shown); spectral scans using the diode-

Table III. Inter- and Intraassay Precision and Accuracy Data

Plasma species	Concentration (μg/ml)		Interassay[a,c]		Intraassay[b,c]	
	Nominal	Predicted	% RSD	% dev	% RSD	% dev
Rat	1.05	1.08	4.4	2.8	2.5	2.4–8.5[d]
	83.60	85.07	1.6	1.8	4.1	0.2–4.9
	418.00	414.21	1.2	0.9	1.8	0.1–2.3
Monkey	6.70	6.76	1.0	0.8	2.0	0.0–2.9
	84.60	87.89	1.2	3.8	1.6	2.3–5.8
	470.00	462.63	1.6	1.6	1.8	0.0–3.6

[a] $N = 20$.
[b] $N = 5$.
[c] % RSD, percentage relative standard deviation; % dev, percentage deviation.
[d] Range from four validation standard curve experiments.

array detector indicated perfect peak match and purity with reference spectra of d4T and internal standard (not shown).

The results of the limit of detection and lower limit of quantitation experiment are presented in Table I. The peak area of d4T from the 0.05-μg/ml samples was significantly greater than that of the blank samples (0 μg/ml) by paired *t* test. Consequently, the lower limit of detection of the assay method was ≤0.05 μg/ml. At this concentration, the mean signal-to-noise ratio was 19. Based on the precision and accuracy results (Table I), the 0.1-μg/ml concentration was selected as the lower limit of quantitation.

A 7-point standard curve, 0.1, 0.5, 1, 25, 50, 75, and 100 μg/ml, was constructed for the validation experiments. The standard curve parameters are presented in Table II. The assay was linear over the concentration range of 0.1–100 μg/ml with highly reproducible slopes and intercepts. In all cases, the correlation coefficient of the regression lines was 1.0. These results indicate that there is no difference between the standard curves prepared in rat and those in monkey plasma.

The intra- and interassay precisions and accuracies of the assay method are presented in Table III. The precision values (%RSD) were less than 5% and accuracy deviated by not more than 8.5%.

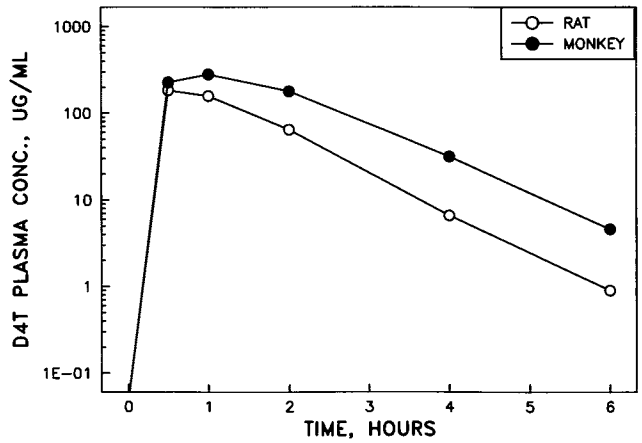

Fig. 3. Plasma concentration–time profiles of d4T in a rat and monkey after oral administration of a single dose of 200 and 300 mg/kg, respectively.

D4T was stable at −20°C for at least 21 days and through three freeze–thaw cycles. Both d4T and internal standard were stable in the injection solvent (mobile phase) for at least 70 hr at ambient temperature. The recoveries of d4T and internal standard were similar from rat and monkey plasma and were 86 and 82%, respectively.

The assay method has been successfully used to analyze nonclinical study samples. An example of d4T plasma concentration–time profile in a rat and monkey is shown in Fig. 3.

It is of interest to note that the chromatograms of plasma samples from dosed animals contained one major peak (of d4T) compared to blank rat or monkey plasma. In addition, preliminary data from ^{14}C-d4T studies in the rat and monkey indicated that the urines are devoid of any glucuronide or other metabolites (data on file, Bristol-Myers Co). This is in contrast to the metabolism of a structurally related analogue, AZT. Like man, 60–80% of a given dose is metabolized to a glucuronide (GAZT) and excreted in the urine in the monkey (16). In the rat, GAZT is a minor metabolite, as urinary radioactivity, 78% of the dose, consists predominantly of AZT (17). Consequently, GAZT is detected in the plasma of these two species.

In conclusion, the assay method described in this report is suitable for the assay of d4T in rat or monkey plasma. The validated standard curve range is 0.1–100 μg/ml. The assay procedure yields a matrix that is free of endogenous and metabolite interfering peaks at the retention times of d4T and internal standard. D4T spiked rat and monkey QC samples gave similar results for accuracy, precision, recovery, specificity, and stability. Therefore, the simultaneous validation of rat and monkey plasma methods was successful without compromising the results for either species.

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