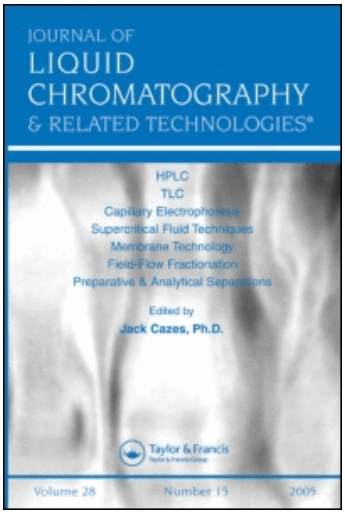

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DETERMINATION OF THE ANTI-HIV DRUG 2'-β-FLUORO-2',3'-DIDEOXYADENOSINE IN BIOLOGICAL FLUIDS BY REVERSED-PHASE HPLC

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ABSTRACT

2'-β-Fluoro-2',3'-dideoxyadenosine (F-ddA) is a synthetic dideoxynucleoside analogue that has been designed to overcome the acid stability problems of the anti-AIDS drug didanosine. F-ddA is also a clinical candidate and will be tested in AIDS patients upon completion of its preclinical evaluation. We have developed a straightforward reversed-phase HPLC method to measure both F-ddA and its deaminated metabolite, 2'-β-fluoro-2',3'-dideoxyinosine, in plasma and urine. This method employs an adenosine deaminase inhibitor to prevent sample degradation, an internal standard for quantitation, and C_{18} solid-phase extraction to isolate and concentrate the fluorinated dideoxynucleosides. Gradient HPLC analysis on a reversed-phase phenyl column with UV detection at 260 nm gives a limit of quantitation of 50 ng/ml (0.2 μM) for both analytes. This assay has been applied to preclinical studies in rats and monkeys to determine drug stability, disposition, metabolism and plasma kinetics.

INTRODUCTION

2'-β-Fluoro-2',3'-dideoxyadenosine (F-ddA, Figure 1) is a synthetic analogue of dideoxyadenosine (ddA) in which the 2'-β-hydrogen of the dideoxyribose sugar has been replaced by a fluorine atom (1). This modification retains the anti-HIV activity of ddA, confers acid stability, and reduces the rate of degradation by adenosine deaminase (ADA) on the fluorinated derivative (1-4). Both F-ddA and its deaminated catabolite, 2'-β-fluoro-2',3'-dideoxyinosine (F-ddI, Figure 1) possess *in vitro* activity and potency against HIV that is similar to that of 2',3'-dideoxyinosine (ddI, didanosine, Videx), a drug approved by the FDA to treat AIDS (1,2,5). These purine dideoxynucleosides all exhibit almost complete protection against the cytopathic effects of HIV in infected ATH8 cells over the concentration range 5-100 μM (6).

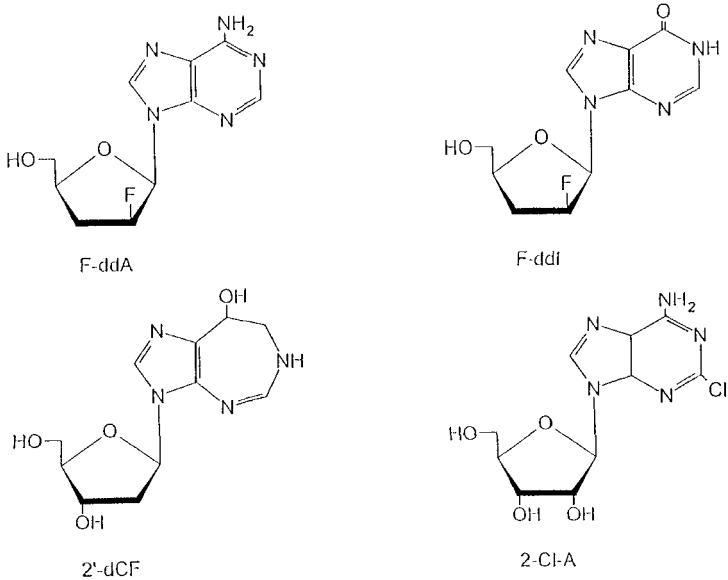


FIGURE 1. Structures of various nucleosides.

The hydrolytic instability of ddI toward acidic conditions ($t_{1/2}$ = 30 sec at pH 1) (2) complicates oral administration, which is preferred because chronic dosing is required (7,8). F-ddA offers possible advantages in this regard because of its better oral bioavailability (9), which results from its acid stability, and its potentially greater central nervous system (CNS) penetration by virtue of its higher lipophilicity (log P = -0.183 versus log P = -1.242 for ddI)(10). This latter property is important because the CNS can be a sanctuary for HIV as well as a site of significant physiologic damage as shown by the wide occurrence of AIDS-related dementia (11,12). For these reasons, the NCI has undertaken the preclinical studies necessary to bring F-ddA to clinical trial.

Chromatographic methods for the bioanalysis of anti-HIV agents, and antiviral agents in general, have recently been reviewed (13). Most HPLC methods for the measurement of purine dideoxynucleosides in biological samples employ solid-phase extraction to isolate and concentrate the compounds of interest (14-19). Reversed-phase HPLC is then combined with UV detection to achieve a limit of quantitation in the 25-200 ng/ml range. (14-21). Fluorescence detection following precolumn fluorogenic derivatization has also been used in conjunction with solid-phase extraction and reversed-phase HPLC for the analysis of ddA and ddI (22). Although this latter procedure is potentially more sensitive than UV-based analyses, it is also more complicated.

This report describes a reversed-phase HPLC method to measure both F-ddA and its primary catabolite, F-ddI, in plasma and urine at submicromolar concentrations. Solid-phase extraction is used to remove protein, isolate the fluorinated dideoxynucleosides from the biological matrix in high yield and concentrate the analytes. The fluorinated dideoxynucleosides are then separated by gradient elution on a phenyl reversed-phase column and measured by their UV absorption. We have employed this method to determine plasma and urine drug and catabolite levels during preclinical studies in rats and monkeys.

MATERIALS

Reagents and Chemicals

F-ddA (NSC-613792) and F-ddI (NSC-616290) were supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program (DTP), NCI (Bethesda, MD). The adenosine deaminase inhibitor, 2'-deoxycoformycin (2'-dCF, NSC-218321) (23) was obtained from the Drug Synthesis and Chemistry Branch, DTP, NCI (Bethesda, MD). 2-Chloroadenosine (2-Cl-A) was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade methanol, acetonitrile and water, as well as certified 1.00 N sodium hydroxide solution, were bought from Fisher Scientific (Fair Lawn, NJ). Spectrophotometric grade DMSO and sodium azide were purchased from Aldrich Chemical Co., (Milwaukee, WI), while monobasic potassium phosphate was from Mallinkrodt (St. Louis, MO). All chemicals and reagents were used without further purification, although the purity of F-ddA, F-ddI and 2-Cl-A was confirmed by HPLC analysis before use. Phosphate buffer was vacuum filtered through a 0.45 μm nylon membrane before mixing with acetonitrile. The mobile phase was then continuously degassed by sparging with helium.

Apparatus

Chromatographic analysis was performed using two separate modular HPLC systems. One configuration consisted of an LKB 2150 pump with a low pressure mixing valve and an LKB 2152 LC controller (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Samples were injected via a Gilson Model 231 autosampler (Gilson International, Middleton, WI) and UV absorbance was monitored with a Gilson Model 116 variable wavelength UV detector. The other HPLC system included a Spectra System P2000 binary pump, an AS3000 autosampler (Spectra-Physics Analytical, San Jose, CA) and a Waters model 994 programmable photodiode array detector (Waters Associates, Milford, MA). Peak areas were integrated, processed and archived using a WINner/386 chromatography data system running

on a Compaq Deskpro 386 computer (Compaq Computer Corporation, Houston, TX) interfaced to Spectra-Physics SP-4200 and SP-4400 integrators.

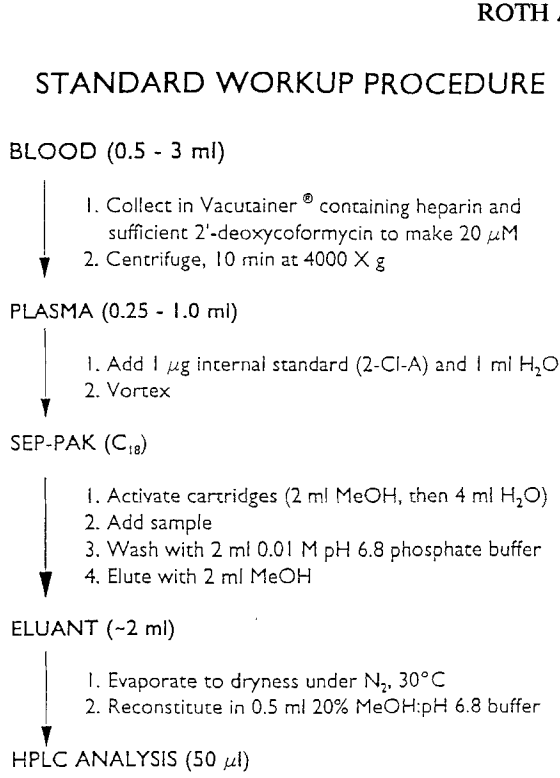
METHODS

HPLC Analysis

Separations were performed on either a 3.9 x 150 mm Waters Novapak 4 μm phenyl column or a 4.6 x 250 mm Hypersil 5 μm Phenyl-2 column (Shandon Scientific, Cheshire, UK). The analytical column was preceded by a 4.6 x 30 mm Brownlee Spheri-5 Phenyl precolumn (Applied Biosystems Inc., San Jose, CA). A 20-min linear gradient from 1% to 7% acetonitrile in 0.01 M pH 6.8 phosphate buffer was used with the Waters column, while a 22-min linear gradient from 1% to 18% acetonitrile was employed for the Hypersil column. The flow rate was 1 ml/min and UV detection was at 260 nm for all analyses.

Sample Preparation

The standard sample workup procedure is outlined in Scheme I. Blood was collected in 5 or 15 ml Vacutainer® tubes (Becton Dickinson, Rutherford, NJ) containing heparin and a sufficient amount of 2'-dCF to make the final concentration 20 μM in adenosine deaminase inhibitor. Plasma was then separated from blood cells by centrifugation at 4000 X g for 10 min. Urine was diluted 500X before processing. An aliquot of sample (0.25 to 1.0 ml), depending on availability, was then mixed with 1 μg 2-Cl-A internal standard and 1.0 ml water. The sample was loaded onto a C_{18} Sep-Pak Classic cartridge (Waters Associates, Milford, MA), preactivated with 2 ml methanol and 4 ml water. The cartridge was washed with 2 ml 0.01 M, pH 6.8 phosphate buffer and components of interest were eluted with 2 ml methanol. After the methanol eluant was evaporated to dryness under nitrogen at 30°C using an N-Evap analytical evaporator (Organomation Assoc., South Berlin, MA), the residue was reconstituted in 0.5 ml 20% methanol in 0.01



SCHEME I

M, pH 6.8 phosphate buffer. Fifty μl aliquots were injected for HPLC analysis.

Processed Sample Stability

Standards with F-ddA, F-ddI and 2-Cl-A, each at 1 μg/ml concentration, were prepared in 0.01 M, pH 6.8 phosphate buffer and in buffer mixed v/v with 10% acetonitrile, 10% methanol or 20% methanol. The samples were stored at room temperature in closed vials. Aliquots of each were taken immediately and at several times over a twelve day period for HPLC analysis. In a separate experiment, standards with 2 μg/ml each F-ddI and 2-Cl-A were prepared in phosphate buffer with or without 0.1% sodium azide. These were treated in the manner described above.

Calibration Curves, Recovery and Precision

Stock solutions of F-ddA, F-ddI and 2-Cl-A in DMSO were stored at room temperature. Standards were prepared by adding appropriate amounts of stock solutions to blank plasma, preincubated for 5 min with 2'-dCF, or to blank, diluted urine. Standard curves were generated by least-squares linear regression fit of the log of the peak area ratio of the dideoxynucleoside to the 2-Cl-A internal standard versus the log of the analyte concentration.

Analyte recovery and precision were determined at three concentrations for monkey plasma and at one concentration for rat plasma. For each experiment sufficient 2'-dCF was added to thawed plasma to make it 20 μM and the sample equilibrated for 5 min at room temperature. The appropriate amount of F-ddA and F-ddI was added to this plasma and the sample mixed. For 5.0 ml spiked human plasma, four 1.0 ml aliquots were taken at each concentration. For 2.0 ml spiked rat plasma, three 0.50 ml aliquots were used. The spiked plasma aliquots and a blank plasma (1.0 ml for human, 0.5 ml for rat) with no internal standard were all treated by the standard sample workup procedure. Dideoxynucleoside standard solutions with the appropriate amount of internal standard were prepared in triplicate in 20% methanol in 0.01 M, pH 6.8 phosphate. These absolute standards and processed plasmas were then analyzed by HPLC. Recovery was calculated by dividing the mean of the absolute peak areas of the spiked plasma samples, corrected for blank, by the mean of the absolute peak area of the appropriate dideoxynucleoside standards. The relative standard deviation of the peak area ratios of the spiked plasma samples at each concentration level was used as a measure of precision.

Plasma Protein Binding

The bound and free fractions of F-ddA and F-ddI were determined at two concentrations in both rat and monkey plasma. The two compounds were tested separately to prevent possible

binding competition. For each experiment, fresh plasma with 20 μM 2'-dCF was spiked with a dideoxynucleoside at the appropriate level, allowed to equilibrate for at least 5 min at room temperature and then ultrafiltered in three 0.50 ml aliquots by centrifuging at 1900 X **g** in Amicon Centrifree micropartition units (W.R. Grace & Co., Beverly, MA). Blank plasma ultrafiltrate, direct standards in 0.9% NaCl solution and ultrafiltered standards were also prepared. The ultrafiltered plasma samples and the direct and ultrafiltered standard solutions were then analyzed by HPLC. The absolute peak areas of the F-ddA and F-ddI were used to calculate membrane holdup and plasma protein binding at each concentration level according to the following formulas.

$$\% \text{ Membrane holdup} = 100(1-P_{ufs}/P_{ds})$$

$$\% \text{ Bound} = 100[1-(P_{puf}-P_{bluf})/P_{ufs}]$$

P_{ufs} is the mean peak area of the ultrafiltered standards and P_{ds} is the mean peak area of the direct standards. P_{puf} is the mean peak area of spiked plasma ultrafiltrate and P_{bluf} is the mean peak area of the blank plasma ultrafiltrate, if any.

Plasma Stability

F-ddA stability was determined in plasma from three different monkeys and in pooled rat plasma. F-ddI stability was measured in plasma from one monkey and in pooled rat plasma. For experiments using monkey plasma, 5.0 ml plasma from freshly drawn blood was warmed to 37°C in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, IL). For the study of F-ddA and F-ddI in pooled rat plasma, a 4.0 ml aliquot was used. The sample was spiked at 10 μg/ml with the appropriate dideoxynucleoside and kept in the shaker bath. At various times, 0.50 ml aliquots were taken and added to 1.0 ml chilled water containing 1.03 μg 2-Cl-A internal standard. For samples containing F-ddA, 2'-dCF was also added at this point to prevent further deamination. Samples were then immediately processed by the usual procedure for analysis by HPLC. For F-ddA experiments the peak area ratio of F-ddA to 2-Cl-

A versus time was fit to a monoexponential decay curve using the Inplot program (GraphPad Software, San Diego, CA). Appearance of F-ddI, as measured by peak area ratio to internal standard versus time, was fit to an exponential association curve. No weighting was used.

Stability of F-ddA in rat plasma was evaluated by a similar procedure. A 1.0 ml aliquot of fresh rat plasma was heated to 37°C in an Eppendorf Model 5320 thermostated block heater (Brinkman Instruments, Westbury, NY) and then spiked with 20 μg/ml F-ddA. A 50-μl aliquot was taken at predetermined times and added to 0.45 ml water along with internal standard and 2'-dCF. Each sample was heated to 95°C for 1 min, cooled and ultrafiltered in an Amicon Centrifree micropartition unit. Absolute peak areas of F-ddA and F-ddI versus time were then curvefit using Inplot software.

In Vivo Studies

The rat studies were conducted at Battelle Memorial Institute (Columbus, OH) as part of comparative dideoxynucleoside cardiotoxicity study (24) under Contract NO1-CM3-7834 to the Developmental Therapeutics Program, DCT, NCI. Female Sprague-Dawley rats (200-250 gm) were administered intravenous bolus doses of 2.5 to 250 mg/kg F-ddA in 0.9% NaCl solution. Blood samples (approximately 1 ml) were collected from each rat 2 min after dosing and immediately mixed with EDTA and 50 μl, 0.6 mM 2'-dCF. Plasma was separated from cells by centrifugation and then frozen until analyzed. Plasma from untreated animals was used for spiked standards to generate the appropriate calibration curves.

An adult male rhesus monkey weighing 6.7 kg was administered 20 mg/kg F-ddA as a 3-min intravenous push into the right saphenous vein as part of a protocol with the Pharmacology and Experimental Therapeutics Section (Pediatric Oncology Branch, DCT, NCI) to determine drug pharmacokinetics and metabolism in primates. Blood was drawn before dosing and at predetermined

times after treatment through a catheter placed into the left jugular vein as described in "Sampling". The resultant plasma was frozen and stored at -20°C until analysis. General procedures for both rat and monkey care, housing and treatment were in accord with published guidelines (25).

RESULTS AND DISCUSSION

The aim of this work was to develop a straightforward HPLC method suitable for use in preclinical studies to measure F-ddA and F-ddI at submicromolar concentrations in plasma and urine.

Chromatography

Reversed-phase HPLC has been widely applied for the analysis of endogenous nucleosides in biological samples (26,27). Because of their more lipophilic nature, dideoxynucleosides and their analogues are even more suited to analysis by this mode of HPLC (13). Although F-ddA and F-ddI were more strongly retained on a C_{18} column, a better separation from endogenous interferences in biological samples was achieved on a phenyl reversed-phase column. This better suitability of a phenyl column has also been noted for the analysis of ddA and ddI (8,21), which have chromatographic retention similar to their fluorinated analogues. Several phenyl packing materials were evaluated and two columns were found to give suitable results - the Waters Novapak phenyl and the Hypersil Phenyl-2. The latter is packed with an end-capped, bonded 5-μm spherical silica which maintained better peak shape and separation after multiple sample injections and prolonged use. With these columns, there were minimal interferences at the retention times of the compounds of interest in either plasma or urine from monkeys (Figure 2A) or rats (Figure 3A).

Since it was desirable to determine both F-ddA and its F-ddI metabolite for pharmacology studies, and since these two dideoxynucleosides differed substantially in their chromatographic retention (Table 1), elution with a linear acetonitrile gradient was

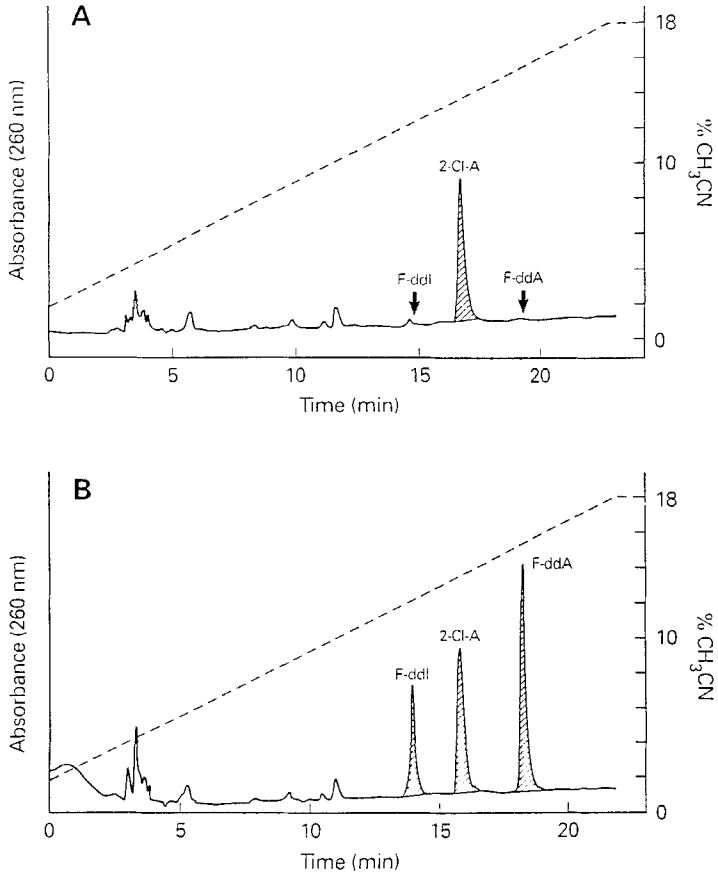


FIGURE 2. Gradient HPLC analysis of A) blank rat urine and B) rat urine spiked with F-ddA (2 μg/ml), F-ddI (2 μg/ml) and 2-Cl-A internal standard (1 μg/ml) on a 4.6 X 250 mm Hypersil Phenyl-2 column and Brownlee Spheri-5 phenyl cartridge precolumn. The dashed line shows the gradient of acetonitrile in the mobile phase. The bold arrows in chromatogram A) mark the expected elution position of the indicated analytes. See HPLC Conditions in Methods for full details.

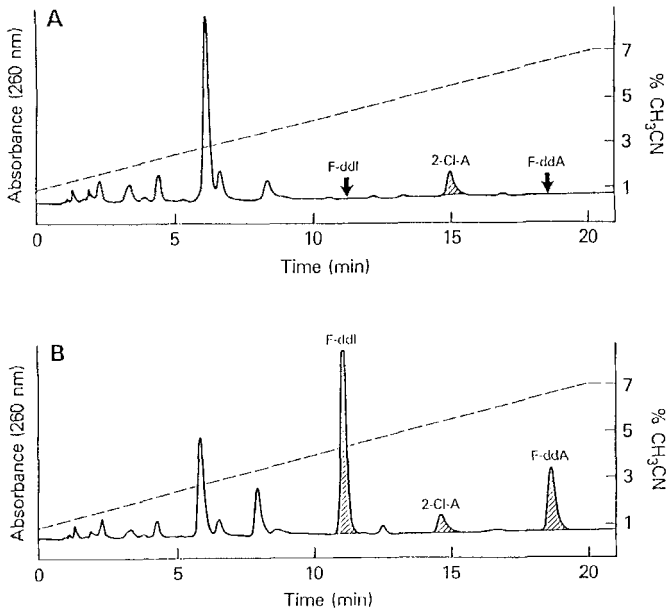


FIGURE 3. Gradient HPLC analysis on a 3.9 X 150 mm Waters Novapak phenyl column and Brownlee Spheri-5 phenyl cartridge precolumn of A) pretreatment monkey plasma and B) plasma from the same monkey 15 min after a 20 mg/kg intravenous dose of F-ddA. The measured concentration of F-ddA was 3.61 μg/ml and that of F-ddI was 17.8 μg/ml. The dashed line shows the acetonitrile gradient program.

employed to achieve an analysis cycle of 25 min. This included a 22-min gradient to final concentration, a 1-min reverse gradient to original conditions and a 2-min equilibration period (Figure 3). This latter 2-min interval, although not adequate for thermodynamic equilibrium, was sufficient to obtain reproducible retention if the column was initially conditioned by running a gradient cycle before the first injection. UV absorption at 260 nm was chosen for detection of the analytes because the greatest sensitivity was needed for F-ddA, and this wavelength is intermediate to the λ_{max} of both F-ddA and the 2-Cl-A internal standard (Table 1).

TABLE 1

Chromatographic Properties of the Analytes

Compound	RRT (Novapak)	RRT (Hypersil)	λ_{max} (nm)
F-ddA	1.25	1.10	259
F-ddI	0.78	0.88	247
2'-dCF	0.45	0.70	282
2-Cl-A	1.00	1.00	262

Internal Standard

2-Cl-A was selected as an internal standard for several reasons. Besides being commercially available, it was chemically similar to the compounds of interest in terms of UV absorption and chromatographic behavior (Table 1). Initially, stability problems were encountered with 2-Cl-A when it was left at room temperature in phosphate buffer overnight for automated HPLC analysis. Partial decomposition was noted; and, when 2-Cl-A standards in only phosphate buffer were kept at room temperature for two days, complete decomposition of 2-Cl-A and the appearance of a new, more polar peak was observed. 2-Cl-A stability was then evaluated in 10% acetonitrile in phosphate buffer as well as in 20% methanol in phosphate buffer. Standards were more stable in buffered acetonitrile, but possessed very poor chromatographic peak shape upon HPLC analysis. However, the standards in 20% methanol were stable for 12 days and the chromatography was not adversely affected. To determine if the above stability problem was due to microbial digestion, 1% sodium azide (a microbicide) was added to buffer spiked with 2-Cl-A. After 12 days at room temperature, little degradation of 2-Cl-A had occurred, confirming that the original problem was probably due to microbial contamination. Reconsti-

tuting plasma extract residues (vide infra) in 20% methanol in phosphate buffer ensures that processed samples are stable overnight at room temperature, and for at least a week, if frozen.

Sample Preparation

The sample preparation procedure in Scheme I is a modification of a method previously used for other purine dideoxynucleosides (7,28). The adenosine deaminase inhibitor, 2'-dCF, is added directly to the blood collection tubes before sampling to stop immediately any *in vitro* conversion of F-ddA to F-ddI. After plasma is separated from blood cells, 1 μg 2-Cl-A is added to a sample aliquot, giving an internal standard concentration in the middle of the expected analyte range. Then, since the dideoxynucleosides are more lipophilic than the endogenous nucleosides in plasma, a C_{18} solid-phase extraction is used to isolate and concentrate the compounds of interest. The solid-phase extraction step also serves to deproteinize the sample. The degree of sample concentration, of course, depends on the initial sample aliquot and the volume of 20% methanol in phosphate buffer used to reconstitute the residue. Typically, a volume corresponding to 10% of the original sample is used for an individual analysis.

F-ddA and F-ddI recovery using the isolation procedure of Scheme I was essentially complete for a 0.1 to 10 μg/ml range in monkey plasma (Table 2). Recovery of the 2-Cl-A internal standard, which was evaluated at the 1 μg/ml level usually used for the assay, was also comparably high. Precision, determined simultaneously with recovery, was better than 7% RSD for both F-ddA and F-ddI over all concentrations in monkey plasma. The recovery and precision in rat plasma were evaluated at 1 μg/ml for all three compounds, and the results were similar (Table 2).

Quantitation

Standard curves were evaluated for both plasma and urine from monkey and rat. These standard curves were based on the peak

TABLE 2

Analyte Recovery and Measurement Precision

Concentration	Mean Recovery (%) F-ddA	F-ddI
Monkey Plasma (n=4)		
100 ng/ml	102 ± 7	95.4 ± 14
1 μg/ml	97.2 ± 8	103 ± 12
10 μg/ml	95.4 ± 4	95.1 ± 4
Rat Plasma (n=3)		
1 μg/ml	110 ± 6	107 ± 3

Concentration	Precision (RSD, %) F-ddA	F-ddI
Monkey Plasma (n=4)		
100 ng/ml	4.0 (0.156)[a]	6.5 (0.0973)
1 μg/ml	6.2 (1.33)	6.4 (0.657)
10 μg/ml	2.8 (14.9)	2.2 (6.18)
Rat Plasma (n=3)		
1 μg/ml	10.4 (1.24)	8.8 (1.22)

[a] Mean peak area ratio

area ratios of F-ddA or F-ddI to internal standard and were linear over the range of spiked standards (0.050 to 10 μg/ml). Both the F-ddA and F-ddI standard curves had correlation coefficients better than 0.999 for all the matrices studied. For plasma standards that covered the entire concentration range, calculated concentrations were closer to actual values for low levels (< 250

ng/ml) if a log concentration *versus* log area ratio linear regression was utilized. The limit of quantitation ($S/N \geq 5$) for this method was 50 ng/ml (~0.2 μM) for both F-ddA and F-ddI.

Protein Binding

Protein binding was determined individually for each compound by centrifugal ultrafiltration in both rat and monkey plasma. This is an important therapeutic parameter because only free drug can penetrate the blood-brain barrier and reach HIV sequestered in the brain (11). Plasma protein binding of F-ddA and F-ddI was essentially zero for the monkey (Table 3). A small percent of protein binding was observed for F-ddA and F-ddI in rat plasma (Table 3). Protein binding of this extent, however, can usually be ignored when the pharmacokinetics of a compound is being determined. No ultrafiltration membrane interaction or holdup was observed for either F-ddA or F-ddI. It should also be noted that error associated with the measured plasma protein binding, which has been estimated from the sum of the RSD's of the absolute peak areas in plasma and standard solution for each compound, appears large because the percent of bound drug is so small. The parent drugs, ddA and ddI, were also observed to have no significant plasma protein binding (20,28,29).

Stability in Plasma

The stability of both F-ddA and F-ddI was investigated in fresh plasma at 37°C to determine what precautions might be required during sampling and workup. 2',3'-Dideoxyadenosine, the parent compound for F-ddA, was rapidly degraded by plasma adenosine deaminase (ADA) and had a half-life of less than 10 min in fresh rat plasma (28,30). The deamination kinetics of F-ddA was evaluated in plasma from individual monkeys as well as in pooled rat plasma. F-ddA was deaminated to form F-ddI, but with a much longer half-life of at least 4 hr in monkey plasma (Figure 4). A similar result was observed for of F-ddA in pooled rat plasma,

TABLE 3

Plasma Protein Binding

Concentration	Plasma Protein Binding (%)	
	F-ddA	F-ddI
Monkey plasma		
1 μg/ml	9.4 ± 11.1	8.2 ± 11.1
250 ng/ml	4.0 ± 10.6	0 ± 14.1
Rat plasma		
1 μg/ml	15.7 ± 2.4	15.8 ± 3.3
250 ng/ml	13.7 ± 8.1	7.3 ± 8.7

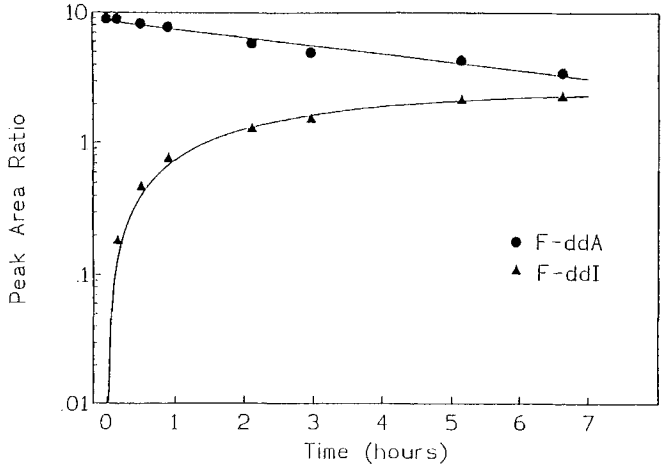


FIGURE 4. Stability of F-ddA (∘) in fresh monkey plasma at 37°C and corresponding formation of F-ddI (△). Deamination of F-ddA was curvefit to a monoexponential decay, shown by the solid line, which led to a half-life of 4.8 hr. The appearance of F-ddI, represented by the dashed line, was fit to an exponential association curve. Both fits were performed using GraphPad Inplot software with no weighting.

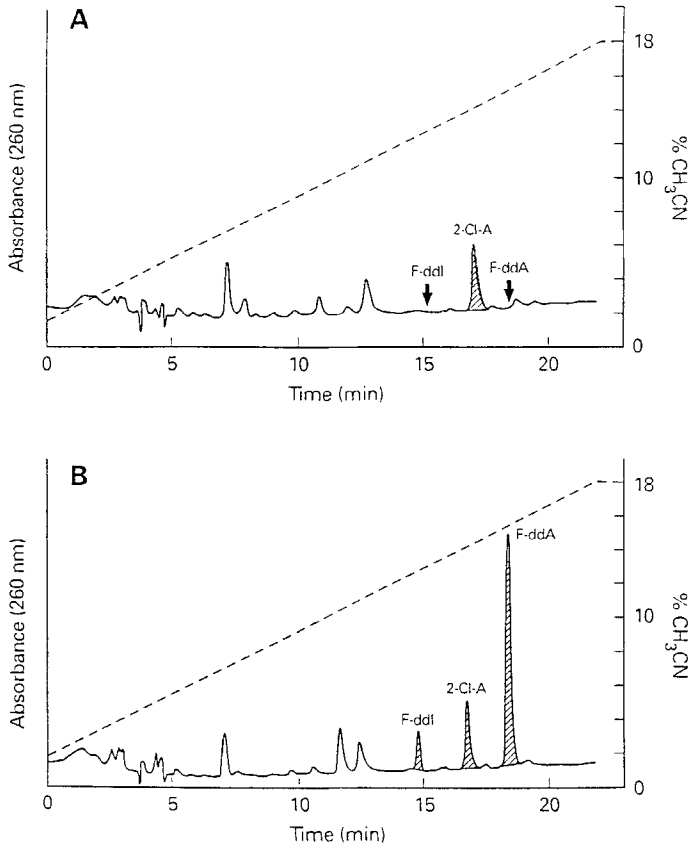


FIGURE 5. Gradient HPLC analysis on a 4.6 X 250 mm Hypersil Phenyl-2 column and Brownlee Spheri-5 phenyl cartridge precolumn system of A) pretreatment rat plasma and B) rat plasma obtained 2 min after a 12.5 mg/kg intravenous bolus dose of F-ddA. The concentration of F-ddA was 9.6 μg/ml and that of F-ddI was 3.35 μg/ml. The dashed line shows the mobile phase acetonitrile gradient.

where the half-life was 4.1 hr. Although F-ddA was a much poorer substrate for adenosine deaminase than ddA, use of 2'-dCF as an ADA inhibitor was still required during blood sampling to prevent further deamination and distortion of actual F-ddA and F-ddI blood levels. F-ddI was much more stable than F-ddA in both rat and monkey plasma, showing less than a 15% loss after 24 hr at 37°C. This was not surprising since the ddI parent showed similar stability in human plasma (28).

Application of the Method

This method has been used to document plasma concentrations of F-ddA and F-ddI following dosing at different levels and by various schedules during a comparative cardiotoxicity study of dideoxynucleosides in Sprague-Dawley rats (24). Figure 5B shows a representative chromatogram of the plasma of a rat that received F-ddA as an intravenous bolus dose. As expected, but in contrast to the rapid deamination observed with ddA, rats dosed with F-ddA had much higher levels of unchanged drug than of the F-ddI metabolite (24). This slower deamination rate of F-ddA is significant because the dideoxyinosine catabolite is more polar and presumably less able to cross the blood brain barrier. Therefore, higher levels of F-ddA relative to F-ddI may be beneficial for CNS drug penetration. The greater resistance of F-ddA to metabolic clearance from the plasma also means the drug should persist for a longer time after administration. This property may be advantageous in devising a dosing regimen. This assay has also been applied in a study of the bolus dose plasma kinetics of F-ddA in rhesus monkeys. Although deamination to F-ddI was the major route for clearance of this drug from plasma, concentrations of F-ddA corresponding to those required for *in vitro* activity persisted for an extended period (Figure 3).

Conclusion

A straightforward reversed-phase HPLC method that measures both F-ddA and F-ddI at submicromolar sensitivity in biological

samples has been developed and analytically evaluated. This method has a quantitative range of 0.2 - 10 μM (0.05 - 10 μg/ml) and a measurement precision of better than 10%, making it suitable for use in pharmacokinetic studies. Since solid-phase extraction forms the basis of the isolation procedure from the biological matrix, full assay automation using a robotic sample preparation unit is possible. This may be of value in adapting this method for the analysis of HIV-infected human samples during a future clinical trial of this anti-HIV drug.

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