Evaluation of a fluorogenic derivatization method for the reversed-phase HPLC analysis of 2′-β-fluoro-2′,3′-dideoxyadenosine, a new anti-AIDS drug

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Abstract

High sensitivity (10^{-7} to 10^{-9} M) reversed-phase high-performance liquid chromatography (HPLC) analysis of adenine nucleosides and nucleotides, especially in a biological matrix, is difficult using only ultraviolet detection. Derivatization coupled with fluorescence detection has been investigated as a means of enhancing sensitivity for the reversed-phase HPLC analysis of 2′-β-fluoro-2′,3′-dideoxyadenosine (F-ddA), an experimental, acid-stable, anti-AIDS drug. The reaction of chloroacetaldehyde with the adenine base has been employed to form fluorescent $1,N^6$-etheno derivatives of F-ddA and 5′-deoxyadenosine, which is used as an internal standard. These derivatives give an analytically useful fluorescence emission at 416 nm after excitation at 230, 265, or 275 nm. Derivatization, fluorescence detection and reversed-phase chromatography have been optimized for the analysis of nanomolar concentrations of F-ddA in human plasma. This method has potential for the measurement of F-ddA at low concentration and in limited volume samples from in vivo biological studies. © Published by Elsevier Science B.V.

Keywords: High-performance liquid chromatography; 2′-β-Fluoro-2′,3′-dideoxyadenosine; Lodenosine; Fluorescence detection; Nucleoside; Chloroacetaldehyde derivatization

1. Introduction

2′-β-Fluoro-2′,3′-dideoxyadenosine (F-ddA, lodenosine) is a synthetic analog of 2′,3′-dideoxyadenosine (ddA) in which the 2′-β-hydrogen of the dideoxyribose sugar has been replaced by a fluorine atom (Fig. 1) [1]. This substitution greatly enhances both the acid and enzymatic stability of F-ddA relative to that of ddA and its inosine

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analog ddI (dideoxyinosine, didanosine) (Fig. 1). F-ddA is stable under conditions that approximate the acidity of the human stomach (pH 1, 37°C), while both ddA and ddI are acid-labile in this environment [1–4]. Consequently, ddI requires buffering or the use of antacids to inhibit decomposition and enhance oral bioavailability [5–7]. Metabolically, F-ddA is slowly deaminated by adenosine deaminase, in contrast to ddA which is rapidly converted to ddI by this enzyme in vivo [4]. Since adenine nucleosides are almost an order of magnitude more lipophilic than their inosine analogs [8], it is hoped that the slow deamination of F-ddA will allow enhanced drug penetration of the central nervous system (CNS) before metabolism occurs. The in vitro activity and potency of F-ddA against HIV is similar to that of ddI (didanosine) [1–3], a drug currently approved

to treat AIDS. In addition, F-ddA has been shown to have an in vivo anti-HIV activity comparable with azidothymidine, zidovudine in the intraperitoneal, hu-PBL/SCID mouse model [9]. Because of these favorable characteristics, the drug is undergoing clinical investigations at the National Cancer Institute (NCI).

Chromatographic methods for the bioanalysis of anti-HIV agents and other nucleoside analogs have been reviewed [10,11]. Most high-performance liquid chromatography (HPLC) methods for the measurement of purine dideoxynucleosides in biological samples employ solid-phase extraction for isolation and sample concentration, and rely on ultraviolet (UV) absorbance for detection. The current HPLC-UV method developed in this laboratory for measuring F-ddA in biological samples uses solid-phase extraction and has a
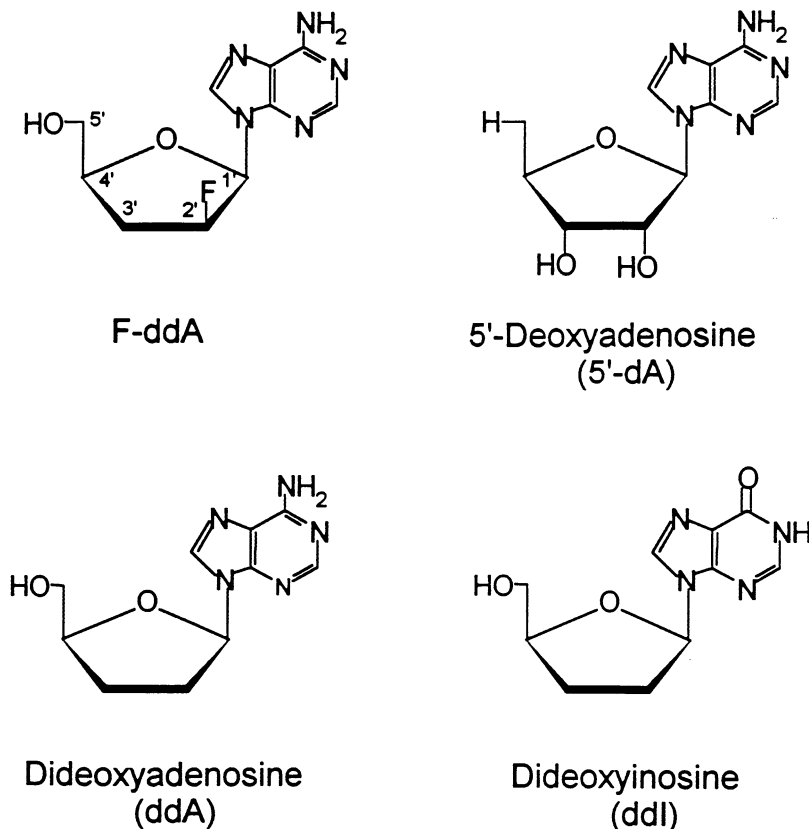

Fig. 1. Chemical structures of the purine dideoxynucleosides of interest and the internal standard (5′-dA). Positional numbering is indicated on the structure of F-ddA.
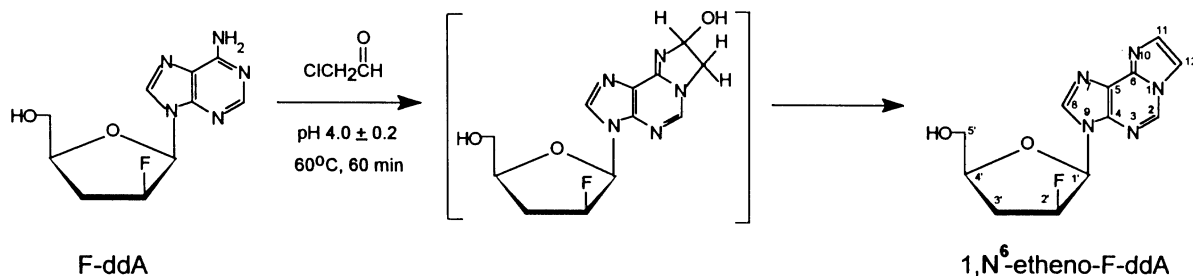
Fig. 2. Reaction of F-ddA with chloroacetaldehyde to produce 1,N^6-etheno-F-ddA. Conditions indicated are for the microscale derivatization of plasma ultrafiltrate.

limit of quantitation of 200 nM (10 pmol on-column) using a 0.5 ml processed plasma sample [12]. A method with additional sensitivity has been sought for carrying out pharmacokinetic studies and for assessing bioavailability and CNS penetration. Such a method would ideally require a smaller sample size due to the multiple plasma and cerebrospinal fluid (CSF) samples needed for pharmacokinetic studies. This is especially true for studies in pediatric patients where plasma and CSF samples will be volume limited.

Adenine nucleosides, a class of compounds of which F-ddA is a member, can be converted into highly fluorescent 1,N^6-etheno derivatives by reaction with chloroacetaldehyde (Fig. 2) [13]. Similar precolumn derivatization methods for the HPLC analysis of adenine nucleosides have been reported [14–17]. An approach involving fluorescence has the advantage of enhanced selectivity. Because fluorescence requires both excitation and emission at specific wavelengths, only compounds possessing the 1,N^6-etheno-adenine fluorophore are likely to be detected. As a result, the chromatogram should be greatly simplified compared with that from a less selective UV detection at 260 nm. In situ fluorogenic derivatization and fluorescence detection has been investigated to enhance the sensitivity of the current RP-HPLC methodology for F-ddA. This report details the studies to develop and optimize this fluorescence detection-based HPLC approach to enhance sensitivity for F-ddA and to evaluate the feasibility of its use for quantitation of this new anti-AIDS drug in biological matrices.

2. Experimental

2.1. Materials and reagents

Pharmaceutical grade F-ddA (NSC 613792) was provided by the Pharmaceutical Resources Branch, Developmental Therapeutics Program (DTP), NCI (Bethesda, MD, USA). The adenosine deaminase inhibitor 2′-deoxycoformycin (2′-dCF) (NSC 218321) was obtained from the Drug Synthesis and Chemistry Branch, DTP, NCI. 5′-Deoxyadenosine (5′-dA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), while chloroacetaldehyde, 45% (w/w) in water, was bought from Lancaster Synthesis Inc. (Windham, NH, USA). HPLC grade acetonitrile and water, as well as certified 1.00 N sodium hydroxide solution, were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Phosphate-buffered saline solution (PBS) (0.154 M NaCl, 6.67 mM (pH 7.4) phosphate) was purchased from Biofluids (Rockville, MD, USA). Silica gel flash chromatography was performed on silica gel 60, 400–630 mesh (E. Merck, Darmdstat, Germany). Scintillation grade Triton X-100 was purchased from Research Products International Corp. (Mount Prospect, IL, USA). All chemicals and reagents were used without further purification. For convenience, stock solutions of 2.03 mM F-ddA and 2.02 mM 5′-dA were prepared in dimethylsulfoxide (DMSO) (99.9%, spectrophotometric grade; Aldrich Chemical Co.). These solutions were refrigerated when not in use and could be stored for up to 3 months without detectable

decomposition. The mobile phase was continuously degassed by sparging with high-purity helium (Matheson Gas Products, East Rutherford, NJ, USA).

An analytical standard of $1,N^6$-etheno-F-ddA (Fig. 2) was synthesized by a modification of the procedure of Barrio et al. [13]. F-ddA (100 mg, 0.40 mmol) was dissolved in 2.8 M chloroacetaldehyde (14 ml, 39.2 mmol), and the pH was adjusted to approximately 4.3 with 1 N NaOH. This solution was maintained at 37°C in a metabolic shaking incubator (Precision Scientific, Chicago, IL, USA) for 24 h. The progress of this derivatization was followed by taking 5 µl aliquots of the reaction mixture at regular time intervals. After diluting these aliquots 1:4000 with H_2O, 50 µl was injected for kinetic analysis by HPLC. At the end of the 24-h period, when HPLC analysis indicated complete derivatization, the crude reaction mixture was concentrated on a rotary evaporator and purified by silica gel flash chromatography using methanol-ethyl acetate (1:3) as the eluent. After repeated dissolution in diethyl ether and evaporation to dryness under reduced pressure, white, crystalline etheno-F-ddA was obtained: ^{1}H-NMR (250 MHz, DMSO-d_6): $\delta = 9.3$ (s, 1H, H-2), 8.5 (s, 1H, H-12), 8.1 (s, 1H, H-8), 7.6 (s, 1H, H-11), 6.5 (dd, $J_{1'F} = 14.5$, 4 Hz, 1H, H-1'), 5.5 (ddd, $J_{2'F} = 54$, 5.7, 5.1 Hz, H-2'), 5.1 (1H, OH), 4.2 (m, 1H, H-4'), 3.6 (m, 2H, H-5$'_a$, H-5$'_b$), 2.2–2.8 (m, 2H, H-3$'_a$, H-3$'_b$). Positive-ion FAB/MS m/z (relative intensity): 278 (MH$^+$, 100), 160 (bH$_2^+$, 39). UV maxima: 230 nm, 265 nm, 275 nm. Fluorescence spectrum: excitation maximum, 230 nm; emission maximum, 416 nm. The absolute purity of the synthesized $1,N^6$-etheno-F-ddA was estimated as 94% by comparison of its UV spectrum with that of $1,N^6$-ethenoadenosine, since the chromophore in both cases is the same.

2.2. Apparatus

A Spectra-Physics P2000 binary pump coupled with an AS3000 autosampler (Thermo Separation Products, San Jose, CA, USA) was used for solvent delivery and sample injection. Injected samples were separated on a 30×4.6 mm Brownlee phenyl guard column (Applied Biosystems, Inc. Foster City, CA, USA) connected to a 250×4.6 mm phenyl-Hypersil®-2 analytical column (Keystone Scientific, Inc. Bellefonte, PA, USA). Sample analysis during the derivatization optimization experiments was carried out under isocratic conditions using a mobile phase of acetonitrile-phosphate buffer (pH 7.0; 0.01 M) (10:90, v/v). An isocratic mobile phase of acetonitrile-phosphate buffer (pH 7.0; 0.01 M) (15:85, v/v) was subsequently employed to analyze samples to generate standard curves and to conduct recovery studies. A Gilson Model 116 UV detector (Gilson Medical Electronics, Middleton, WI, USA) was used for measurement of UV absorption at 260 nm, and a SpectroVision FD-300 fluorescence detector (SpectroVision, Chelmsford, MA, USA) was used to monitor the on-line fluorescence intensity. Fluorescence excitation and emission spectra for $1,N^6$-etheno-F-ddA were scanned over the range indicated in Fig. 3 under stopped-flow conditions using a SpectroVision DA-10 scan controller in conjunction with the FD-300 detector. HPLC peak areas were integrated, processed and archived using a Spectra-Physics Chromjet 4400 integrator (Thermo Separation Products, San Jose, CA, USA) interfaced to a Winner On Windows chromatography data system (Thermo Separation Products) running on a Zeos 486/66 mHz personal computer (Zeos International, Minneapolis, MN, USA). An Eppendorf Model 5436 Thermomixer (Brinkmann Instruments, Westbury, NY, USA) was employed for controlled heating and shaking of samples during derivatization. Proton NMR spectra were obtained in DMSO-d_6 on a Bruker AC-250 instrument (Billerica, MA, USA). Positive ion fast atom bombardment mass spectra (FAB-MS) were acquired on a VG 7070E mass spectrometer (VG Analytical, Altrincham, UK). Glycerol was used as the sample matrix and xenon atoms were used to ionize the sample. UV spectra (190–350 nm) were obtained on a Shimadzu UV-2101 PC UV–VIS scanning spectrometer (Shimadzu, Kyoto, Japan).

2.3. Optimization of derivatization parameters

In order to determine optimum reagent concentration, 1.6 M (pH 4.3) chloroacetaldehyde was diluted with pH 7.4 phosphate-buffered saline to create a range of concentrations. A 0.50 ml aliquot of chloroacetaldehyde (0.1–1.6 M) was mixed with 2.5 µl of a 4.1 mM F-ddA DMSO stock solution in a 1.0-ml screw-cap, polypropylene Eppendorf vial to give a reaction mixture containing 20 µM F-ddA. The vial was tightly capped, vortexed to thoroughly mix, and then heated with shaking at 60°C for 60 min in an Eppendorf Thermomixer. An aliquot of each reaction mixture was taken after heating and analyzed by HPLC with fluorescence detection.

For determination of the optimum temperature and reaction time, the derivatization with chloroacetaldehyde was then carried out at 50, 60, and 70°C. Aliquots of each reaction mixture were taken at timed intervals over the period 0–3 h and subjected to HPLC analysis with fluorescence detection.

2.4. Sample preparation

Human blood samples (1–3 ml) were collected in 10-ml Vacutainer® tubes (Becton Dickinson, Rutherford, NJ, USA) containing sodium heparin and a sufficient amount of 2′-dCF, an adenosine deaminase inhibitor [18], to make its final concentration 15 µM. Plasma was separated from blood cells by centrifugation at $1000 \times g$ for 10 min and then Triton X-100 was added to yield a final concentration of 0.5%. A 0.50 ml aliquot of this plasma was then mixed with 1.8 µl of 67 M 5′-deoxyadenosine, the internal standard, to make it 240 nM. The sample was then ultrafiltered in an Amicon Ultrafree micropartition unit (Beverly, MA, USA) at $2500 \times g$ for 50 min. A 0.250 ml aliquot of the resulting plasma ultrafiltrate was transferred to an Eppendorf tube and mixed with 0.125 ml (pH 4.2) of 3.6 M chloroacetaldehyde. After sealing tightly, this mixture was heated at 60°C for 60 min with shaking. Upon cooling to room temperature, a 40 µl aliquot of the reaction mixture was injected for HPLC analysis.

The effect of using 0.5% Triton-X as an inactivating agent of plasma HIV [19,20] was evaluated after a standard sample preparation procedure was established. Fifty microliters of Triton X-100 was added to 10 ml human plasma, and 0.5 ml aliquots of this plasma were used to ascertain whether this non-ionic surfactant had any effect on derivatization, HPLC separation or fluorescence detection. This was carried out by compar-
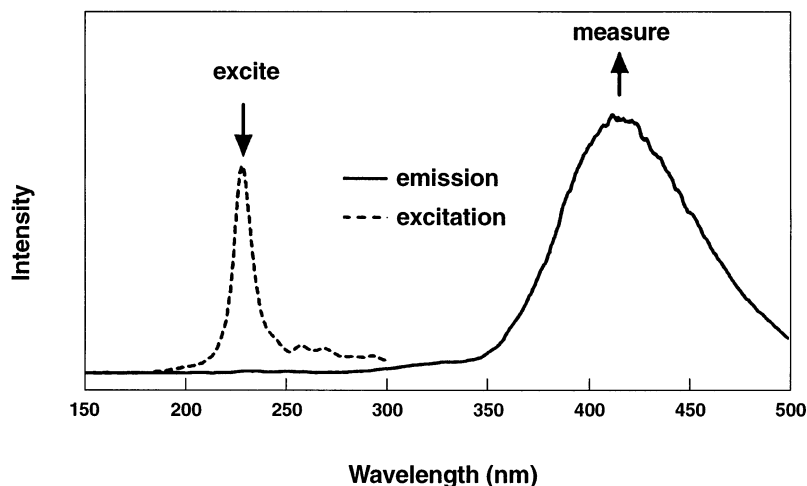

Fig. 3. Fluorescence excitation and emission spectra of 1,N^6-etheno-F-ddA. The bold arrows indicate the optimum wavelengths for excitation and for measurement of the resulting fluorescence, respectively.

ing the standard curves generated in untreated sample matrices (phosphate buffered saline and plasma) with those in 0.5% Triton X-100 treated matrices (vide infra).

2.5. Standard curves

Standards were prepared by serial dilution of the F-ddA and 5′-dA DMSO stock solutions and addition of the appropriate volumes to 0.5 ml aliquots of phosphate-buffered saline solution or blank plasma containing 2′-dCF. Spiked standards were then processed by the standard sample work-up procedure and analyzed as already described. The F-ddA standard curve was then generated by least-squares linear regression fit of the peak area ratio of F-ddA to the 5′-dA internal standard versus the spiked F-ddA concentration.

2.6. Recovery, precision and accuracy

F-ddA recovery and precision were determined at four concentrations for human plasma. For each concentration, 2.5 ml blank human plasma was spiked with the appropriate amount of F-ddA and 5′-dA internal standard. Four 0.5 ml aliquots were taken for analysis at each concentration. The spiked plasma aliquots and a blank plasma (0.5 ml) with no internal standard were all treated by the standard sample work-up procedure. An absolute standard was prepared in phosphate-buffered saline solution in triplicate for each concentration using synthesized etheno-F-ddA. These absolute standards and the processed plasma samples were analyzed by HPLC as already described.

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 etheno-F-ddA standard PBS solution. 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.

The percent difference between the measured concentration and the known concentration of F-ddA in a series of spiked samples was used as a measure of the method's accuracy. Duplicate

aliquots were taken from each spiked sample and analyzed using a standard curve covering the appropriate concentration range. Samples outside the linear range of the method were diluted before analysis. Samples were processed and analyzed on two separate days and a fresh standard curve was prepared each day.

3. Results and discussion

3.1. Derivatization of F-ddA

The first step in enhancing the method by introducing a fluorophore into the adenine base of F-ddA was the miniscale synthesis of $1,N^6$-etheno-F-ddA (Fig. 2) to provide an analytical standard for structural characterization and for comparison during optimization of the micro- and nanoscale derivatization of biological samples. Reaction of chloroacetaldehyde with F-ddA proceeded smoothly under mild conditions to produce a single major product, although silica gel flash chromatography was required before crystalline material could be obtained. ^{1}H-NMR and UV analysis of this synthetic $1,N^6$-etheno-F-ddA gave spectra consistent with the expected structure and similar to those reported for $1,N^6$-ethenoadenosine [13]. The positive ion FAB mass spectrum also showed the expected 24 Da increase in mass for the ions indicating both molecular weight ($MH^+ = 278$) and nucleic acid base ($bH_2^+ = 160$) relative to those observed for underivatized F-ddA [21]. As expected, $1,N^6$-etheno-F-ddA exhibited fluorescence excitation and emission spectra similar to those of $1,N^6$-ethenoadenosine. Since these two derivatives possess the same chromophore and fluorophore, the purity of $1,N^6$-etheno-F-ddA was estimated by HPLC with both UV and fluorescence detection using $1,N^6$-ethenoadenosine as a reference standard.

3.2. Fluorescence of $1,N^6$-etheno-F-ddA

A 1×10^{-7} M solution of synthetic $1,N^6$-etheno-F-ddA was used to obtain fluorescence spectra under stopped-flow conditions (Fig. 3).

Table 1
1,N^6-Etheno-F-ddA[a] fluorescence intensity as a function of pH

Buffer	Apparent pH	Emission intensity[b] (counts)
0.001 M HCl	3	27.7
0.1 M Acetate	4	15.8
0.1 M Acetate	5	65.7
0.01 M PO$_4$	6	66.4
0.01 M PO$_4$	7	93.9

[a] Fluorescence response was determined under stopped flow conditions using 1×10^{-7} M 1,N^6-etheno-F-ddA.
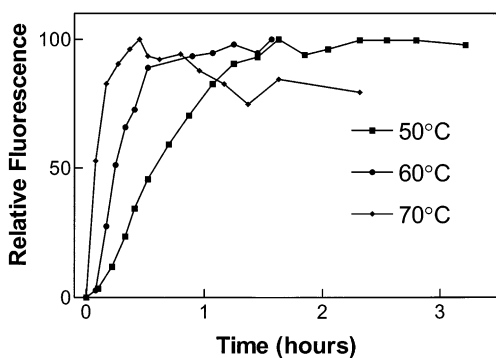[b] Emission intensity was measured at 416 nm after excitation at 230 nm.

Fig. 4. Optimization of derivatization temperature and reaction time using 20 μM F-ddA. See Section 2 for procedural details.

Characteristic excitation maxima were observed at 230, 265 and 275 nm, while an emission maximum of 416 nm was measured at all excitation settings. Since the emission intensity was approximately sevenfold higher after excitation at 230 nm than after excitation at the other maxima, this wavelength was chosen for excitation with our detector and the emission intensity was monitored at 416 nm for all subsequent experiments. Once the optimum excitation and emission wavelengths had been chosen, the effect of pH on fluorescence intensity was also evaluated. Table 1 indicates that, over the pH range compatible with chromatography on silica-based columns, the greatest fluorescence emission occurs at neutral pH.

3.3. Optimization of derivatization conditions

The kinetics of derivatization of the adenine base by chloroacetaldehyde is typical of that of an A → B → C reaction [22]. The overall rate of this reaction depends on both formation and dehydration of a non-fluorescent intermediate, which is depicted for F-ddA in Fig. 2. Since only 1,N^6-etheno-F-ddA exhibits fluorescence, reaction conditions were evaluated with the goal of maximizing formation of this product in a rapid and reproducible manner.

Because the time required with the mild conditions used for the milliscale synthesis of an analytical standard of 1,N^6-etheno-F-ddA would not be optimum for analytical derivatization, the reaction rate and yield was evaluated at 50, 60 and 70°C. As can be seen in Fig. 4, reaction at 70°C rapidly produced 1,N^6-etheno-F-ddA, but prolonged heating beyond 25 min resulted in product loss. In contrast, reaction at 50°C required in excess of 2 h before 1,N^6-etheno-F-ddA yield maximized. At 60°C, an intermediate situation existed where 1,N^6-etheno-F-ddA formation maximized within 60 min with minimal subsequent decomposition. Thus 60°C and 60 min were chosen as the best microscale derivatization conditions.

The effect of chloroacetaldehyde concentration on 1,N^6-etheno-F-ddA formation was investigated using 20 μM F-ddA (10 nmol), the maximum concentration that would be expected in a biological sample. When F-ddA was derivatized at 60°C for 60 min, the extent of fluorescent product increased as chloroacetaldehyde concentration increased from 0.1 to 1.2 M. However, further increase in reagent concentration did not provide increased formation of 1,N^6-etheno-F-ddA, as can be seen in Fig. 5. Thus, 1.2 M chloroacetaldehyde was chosen as the optimal reagent concentration and used in all subsequent experiments and analyses. This reagent concentration represents a 6×10^4 molar excess.

3.4. Chromatography

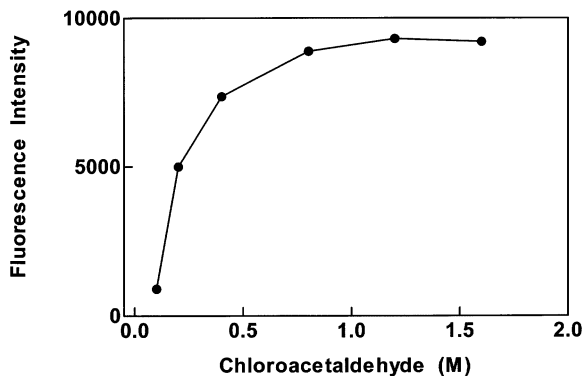The methodology had been developed previously for the reversed-phase HPLC analysis of

Fig. 5. Optimization of chloroacetaldehyde concentration for derivatization reaction using 20 μM F-ddA. See Section 2 for procedural details.

underivatized F-ddA using elution with an acetonitrile gradient in pH 6.8 phosphate buffer on a Hypersil Phenyl-2 column [23]. This HPLC system was adapted for the separation and analysis of $1,N^6$-etheno-FddA since the mobile phase pH was already close to the optimum for maximum fluorescence, and adjustment of the acetonitrile content of the mobile phase could control the retention of the more lipophilic etheno derivatives. Since simpler chromatograms were also expected because of the specificity of fluorescence detection, isocratic elution was initially investigated and found to be suitable. Once a suitable internal standard was selected (see later) and chromatography was optimized, it was possible to use a mobile phase of acetonitrile-phosphate buffer (pH 7.0; 0.01 M) (15:85, v/v) to separate all components of a derivatized biological sample in less than 15 min.

3.5. Internal standard

An internal standard was desirable for this assay to make it more quantitatively rugged in view of the sample handling steps that were required before HPLC analysis. A properly chosen internal standard would minimize any error resulting from incomplete or variable derivatization as well as compensate for any inconsistencies in sample handling. The major requirement for a

suitable internal standard was that it either be fluorescent under the conditions used to detect $1,N^6$-etheno-F-ddA or it be amenable to derivatization with chloroacetaldehyde to form a fluorescent derivative with these properties. Additionally, such a compound also needed to be chromatographically similar, yet not identical, to $1,N^6$-etheno-F-ddA. A third consideration was that it be compatible with and not endogenous to the sample matrix, which in this case would be plasma. A fourth condition was that the derivatization kinetics of the internal standard be as similar as possible to those of F-ddA, so that the method would be robust to slight changes in reaction time and temperature.

These requirements meant that the most likely candidate would be an adenosine analog with an unmodified adenine base. Among the several internal standard candidates evaluated, 5′-deoxyadenosine (5′-dA, Fig. 1) best met the stated criteria. This nucleoside underwent smooth reaction with chloroacetaldehyde with minimal formation of side products when the optimal F-ddA derivatization conditions of 60°C and 60 min were used. Furthermore, the rate and extent of formation of the $1,N^6$-etheno derivative of 5′-dA was almost the same as that of F-ddA, with the formation of the fluorescent derivatives of both compounds reaching and maintaining a plateau after 60 min (Fig. 6). The chromatography was easily adjusted so that the etheno derivative of 5′-dA was well-separated from both $1,N^6$-etheno-F-ddA
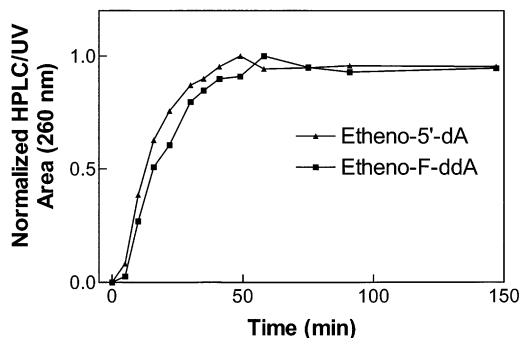

Fig. 6. Comparison of the rate of formation of the $1,N^6$-etheno derivative for F-ddA and the 5′-deoxyadenosine internal standard using optimized reaction conditions.
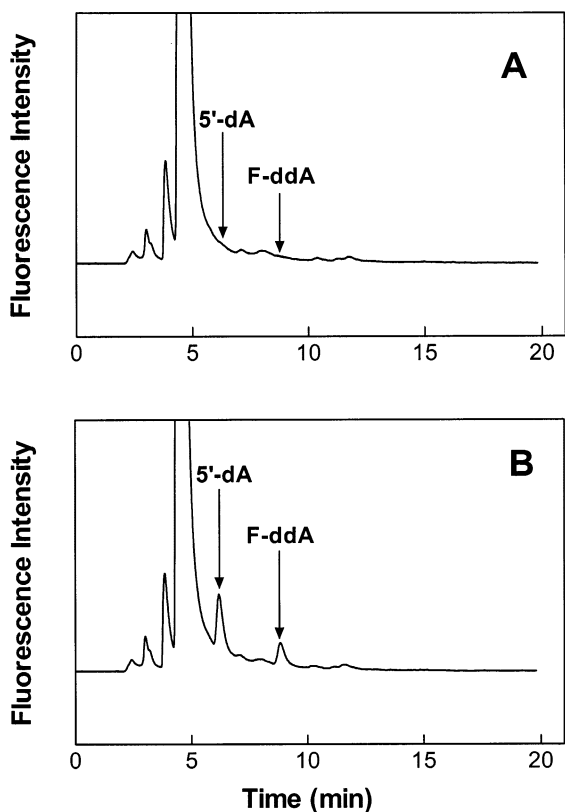
Fig. 7. Chromatograms obtained using fluorescence detection after direct derivatization of plasma ultrafiltrate. (A) Blank human plasma and (B) human plasma spiked at 100 nM F-ddA and 240 nM 5'-dA.

and $1,N^6$-ethenoadenosine, which was always present because of adenosine endogenous to the plasma matrix (Fig. 7b).

3.6. Sample preparation

The sample preparation procedure was chosen to minimize sample handling and to be as simple as possible given the chemical and biochemical characteristics of the analytes and the biological matrix. Because F-ddA is a poor substrate for adenosine deaminase [2,4], the inhibitor 2'-dCF was added directly to the blood collection tubes before sampling to stop immediately any in vitro conversion of F-ddA to F-ddI. The blood sample was then handled as quickly and carefully as

possible to minimize the hemolysis of red blood cells. Hemolysis tended to generate excess amounts of plasma adenosine from degradation of intracellular ATP by nucleosidases. Since adenosine is readily derivatized by chloroacetaldehyde [13], high plasma concentrations resulted in a large fluorescent peak that tailed into that of 5'-dA and complicated peak integration (Fig. 7A and Fig. 8A). After aliquots of plasma were taken for analysis, 5'-dA was added to each sample to give an internal standard concentration of 240 nM. Ideally, the amount of internal standard
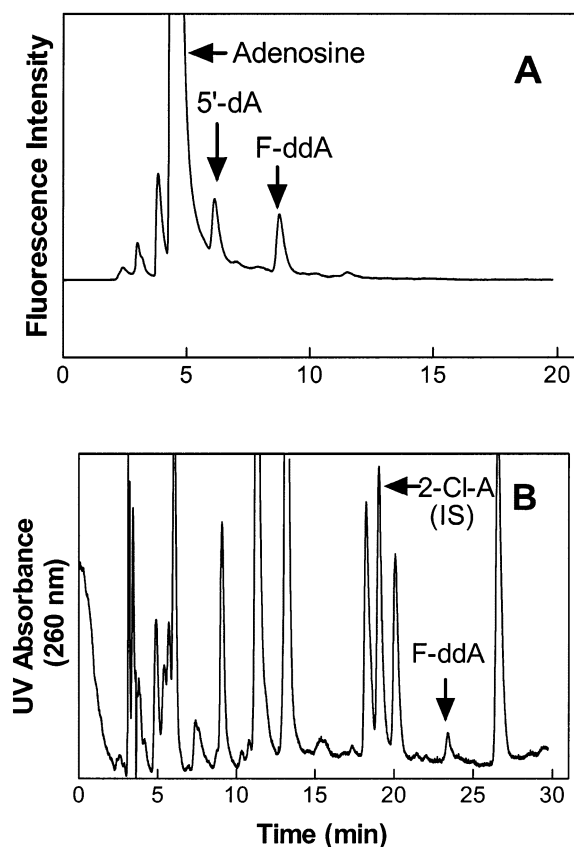

Fig. 8. Comparison of HPLC methods for the analysis of F-ddA using human plasma spiked with 200 nM F-ddA. (A) Chromatogram following chloroacetaldehyde derivatization and fluorescence detection at 416 nm after excitation at 230 nm. The 5'-dA internal standard concentration was 240 nM. (B) Chromatogram following solid-phase extraction and UV detection at 260 nm. The 2-chloroadenosine (2-Cl-A) internal standard concentration was 1 µg/ml (3.95 µM).

Table 2
F-ddA standard curve characteristics for various sample matrices

Parameter	PBS[a] solution	Human plasma[b]	Triton X-100 treated plasma[b]
Slope	0.00520	0.00577	0.00568
S.E.[c]slope	0.000229	0.000166	0.000180
Intercept	−0.131	−0.124	−0.094
S.E. intercept	0.0836	0.0591	0.0550
Range (nM)[d]	37.5–3750	37.5–3750	37.5–3750
Number of standards	6	6	6
Correlation coefficient (r^2)	0.998	0.999	0.999

[a] Phosphate-buffered saline.
[b] Plasma was obtained from the same individual.
[c] Standard error.
[d] Concentration range over which samples were spiked.

Table 3
Analytical recovery and measurement precision for F-ddA-spiked human plasma

Concentration (nM)	Recovery[a] (%)	Precision[b] (R.S.D.,%)	Mean area ratio
50	79	4.6	0.224
240	90	2.9	1.267
496	89	1.5	2.713
5000	92	5.5	26.58

[a] Mean of four independent work-up procedures and analyses.
[b] Determined from peak area ratio of F-ddA to internal standard (5′-dA). The internal standard concentration was 240 nM in all cases.

should be adjusted so that the analyte peak area ratio will be in the range 0.1–10. This was achieved for standard curves when F-ddA was present in the anticipated clinical range of 37 nM–3.75 μM. (Occasional higher level samples in the 4–20 μM range were reprocessed and diluted before analysis.) Ultrafiltration, using a commercial micropartition unit with a 30 kDa cut-off, was then used to deproteinize the plasma. This simple, efficient method can be employed because F-ddA has minimal plasma protein binding (8–11%) and no membrane hold-up [12]. An aliquot of the resulting ultrafiltrate was then derivatized directly by reaction with 1.2 M chloroacetaldehyde. Aliquots of these reaction mixtures could then be injected directly onto the described HPLC system for repeated analysis without problem.

Plasma samples from AIDS patients present an additional challenge, since they are contaminated with HIV. Although P-2 biological safety proce-dures are followed when handling patient samples [24], inactivation of HIV to provide an additional measure of protection is desirable. Among the procedures that have been employed, addition of a non-ionic surfactant such as Triton X-100 [19,20] appeared to be the most benign in terms of effect on the analyte and the sample matrix. Addition of Triton X-100 at a concentration of 0.5% or higher has been shown to effectively inactivate HIV in human plasma [12]. The question that existed was whether this amount of surfactant in the plasma sample would have any effect on the derivatiza-tion, chromatography and subsequent quantita-tion. To answer this question, spiked standards were prepared in Triton X-100 treated human plasma and used to generate a standard curve. No difference in retention or resolution was seen in the chromatograms of these samples and the resul-tant standard curve was similar to those generated from untreated plasma or buffer (Table 2).

3.7. Recovery and precision

A study to determine the overall recovery of F-ddA from a plasma matrix using the standard sample work-up procedure was conducted at four concentrations in the range 50 nM–5 μM. Absolute recoveries for the three highest levels of F-ddA, determined by comparison with equivalent concentrations of solutions of standard 1,N^6-etheno-F-ddA were very consistent and averaged 90% (Table 3). This indicated that both ultrafiltration and direct derivatization of the aqueous ultrafiltrate were quite efficient. The less than quantitative recovery observed for this concentration range of F-ddA could easily be explained by loss of protein-bound F-ddA during ultrafiltration. A somewhat lower recovery of 79% was observed for 50 nM F-ddA (Table 3). Although sample losses were slightly more than would be expected from plasma protein binding, this phenomenon probably also accounts for the majority of the unrecovered material at this level. It is even possible that F-ddA plasma protein binding is concentration dependent at very low, subtherapeutic concentrations, and thus the observed results reflect this. It should be noted that since the F-ddA standard curve is based on spiked plasma standards that are processed in the same manner as unknown samples, the effect of protein binding should cancel out and be transparent to quantitation. Measurement precision for F-ddA, which was also determined at the same time as recovery, was found to be 5.5% or better for all concentrations (Table 3). It may be noteworthy that the

lowest precision occurs where the analyte to internal standard area ratio is outside the optimum range of 0.1–10.

Recovery of the internal standard was measured indirectly, since no pure standard of the etheno derivative of 5′-dA was available. However, the fluorescence of the derivatized compound is the same as that of etheno-F-ddA at the same molar concentration. A comparison of the mean peak area of the derivatized internal standard in processed plasma, corrected for concentration, with that of standard etheno-F-ddA yields a recovery of 71%.

3.8. Assay characteristics

Linear calibration curves of comparable slope and intercept could be generated for F-ddA in phosphate-buffered saline solution and in both untreated and Triton X-100 treated human plasma (Table 2). The 37 nM–3.75 μM range of the standard curves in Table 2 was chosen to cover the range of F-ddA concentration that would most likely be encountered in clinical trials of F-ddA in AIDS patients. Occasional higher level samples could be diluted and reanalyzed. The limit of quantitation (LOQ) using this method was determined by generating a low-level calibration curve ($y = 0.00508x - 0.0196$; $r^2 = 0.999$) for the range 10–500 nM. The 10 nM standard, whose HPLC peak had a signal-to-noise ratio of 8, was considered to represent the LOQ. Thus, derivatization and fluorescence detection resulted in a 20-fold increase in measurement

Table 4
Accuracy of fluorogenic derivatization method for the measurement of F-ddA in spiked human plasma

Nominal concentration (nM)	Dilution factor	Measured concentration (nM)	Deviation from nominal (%)
67	Undiluted	58	−13.4
		66	−1.5
150	Undiluted	145	−3.3
		160	6.7
2020	Undiluted	2040	1.0
		2040	1.0
2050	5	1890	−7.8
		1840	−10.2
20 500	20	19 800	−3.4
		20 800	1.5

sensitivity over the 200 nM LOQ obtainable with our previously reported HPLC-UV method [12]. The increase in specificity and shorter analysis time is shown in Fig. 8, in which the analysis of spiked human plasma by the two methods is compared.

The accuracy of this fluorescence-based HPLC assay was evaluated using Triton X-100 treated human plasma that had been spiked with F-ddA at concentrations that were unknown to the analyst. Five different F-ddA spiked plasma concentrations were used: (1) two low concentrations in the range 50–200 nM that were below the LOQ of the HPLC-UV method already discussed, (2) two intermediate concentrations in the range 0.5–5 μM that represented target therapeutic concentrations for patients, and (3) a high concentration in the range 10–50 μM that represented the range of greatest in vitro activity. Blank human plasma containing 0.5% Triton X-100 was used to construct appropriate calibration curves and independent, duplicate analyses of each spiked concentration level were carried out. Overall, the average deviation of measured values from spiked F-ddA concentrations was 5.0% ($n = 10$) with a range of 1.0–13.4% (Table 4). Of particular note were the duplicate analyses of the low level samples containing 67 and 150 nM F-ddA, respectively, that gave an average deviation of measured values from actual concentrations of 6.2% ($n = 4$), which was only slightly greater than the overall mean deviation.

4. Conclusion

An isocratic, reversed-phase HPLC method incorporating fluorogenic derivatization and fluorescence detection has been developed, optimized and evaluated for the measurement of F-ddA in human plasma at both low and therapeutically relevant concentrations. The specificity of fluorescence detection allows minimal sample preparation and generates a simpler chromatogram in reduced analysis time as compared with HPLC-UV. Fluorogenic derivatization coupled with fluorescence detection also results in a 20-fold enhancement in measurement sensitivity.

This allows measurement of low levels of drug in biological samples and a reduction in the sample volume required for determination of F-ddA in higher concentration samples. This latter attribute may have substantial utility in the analysis of samples that are volume-limited such as cerebrospinal fluid or plasma from pediatric AIDS patients. Unlike the HPLC-UV method, however, this analysis approach only allows measurement of parent drug and not the anti-HIV-active catabolite 2′-β-fluoro-2′,3′-dideoxyinosine. The hypoxanthine base does not react with chloroacetaldehyde under the conditions employed to produce a fluorescent derivative.

A similar approach employing chloroacetaldehyde for fluorogenic derivatization should be useful for developing high-sensitivity, high-specificity HPLC methods for other adenosine analogs such as the antiviral agents Vidarabine (arabinosyladenosine) and Neplanocin A (cyclopentenyl adenosine). In addition, derivatization with chloroacetaldehyde shows promise as a method for the sensitive detection of intracellular adenine nucleotides [25–27]. An approach employing this strategy has been developed in our laboratory for the measurement of intracellular F-ddATP, the triphosphate and active metabolite of F-ddA [28].

Acknowledgements

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