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Determination of 2′-β-fluoro-2′,3′-dideoxyadenosine, an experimental anti-AIDS drug, in human plasma by high-performance liquid chromatography

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Abstract

2′-β-Fluoro-2′,3′-dideoxyadenosine (F-ddA, lodenosine) is an experimental anti-AIDS drug currently being evaluated in a Phase I clinical trial. A simple and specific HPLC method with UV detection, suitable for use in clinical studies, has been developed to determine both F-ddA and its deaminated catabolite, 2′-β-fluoro-2′,3′-dideoxyinosine (F-ddI) in human plasma. After inactivation of plasma HIV by 0.5% Triton X-100, the compounds of interest are isolated and concentrated using solid-phase extraction. Processed samples are separated by use of a pH 4.8 buffered methanol gradient on a reversed-phase phenyl column. The method has a linear range of 0.05–5 μg/ml (0.2–20 μ*M*) and intra-assay precision is better than 8%. Analyte recovery is quantitative and plasma protein binding is minimal. In addition, drug and metabolite levels measured in Triton-treated human plasma remain stable for at least 5 months when samples are stored frozen without further treatment. Compound concentrations determined after samples are processed and then frozen for up to 1 month before analysis are also unchanged. Published by Elsevier Science B.V.

Keywords: 2′-β-Fluoro-2′,3′-dideoxyadenosine

1. Introduction

2′-β-Fluoro-2′,3′-dideoxyadenosine (F-ddA, lodenosine Fig. 1) is an experimental anti-AIDS drug that has been rationally designed to have improved chemical stability relative to its parent compound, dideoxyadenosine (ddA) or the currently approved anti-AIDS drug, dideoxyinosine (ddI, didanosine, Videx) [1]. This synthetic dideoxynucleoside has a fluorine atom substituted for the 2′-β-hydrogen of the dideoxyribose sugar in ddA. This structural modification increases the hydrolytic and metabolic stability of the analog relative to its parent compound. In fact, F-ddA is completely stable in acid at pH 1 for more than 24 h and is degraded by adenosine deaminase (ADA) at a much slower rate than ddA [2,3]. It is hoped that these properties will lead to enhanced oral bioavailability, an important consideration for AIDS drugs because chronic dosing is likely to be required. In preclinical testing, F-ddA has shown better oral bioavailability than ddI in dogs [4]. However, the addition of fluorine has not

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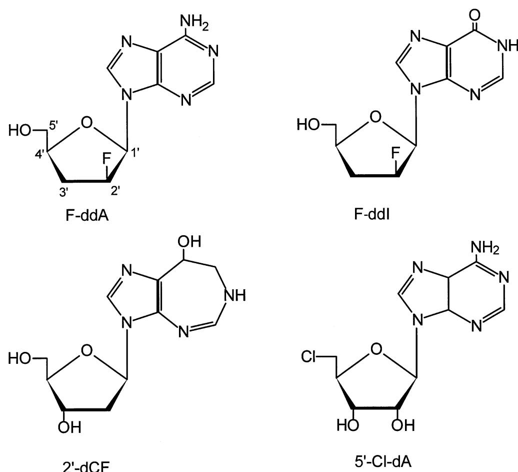

Fig. 1. Chemical structures of relevant nucleoside analogues; F-ddA, 2′-β-fluoro-2′,3′-dideoxyadenosine (positional numbering is indicated for the dideoxyribose moiety); F-ddI, 2′-β-fluoro-2′,3′-dideoxyinosine, the major catabolite of F-ddA; 2′-dCF, deoxycoformycin, an adenosine deaminase inhibitor; and 5′-Cl-dA, 5′-chloro-5′-deoxyadenosine, the internal standard.

affected the compound's in vitro activity or potency against HIV. In fact, F-ddA protects ATH8 cells against HIV over the range 5–100 μ*M* which is similar to didanosine, one of the five reverse transcriptase inhibitors (AZT, ddC, ddI, d4T and 3TC) approved by the Food and Drug Administration for the treatment of AIDS [5,6]. F-ddA is currently being evaluated in both adult and pediatric Phase I clinical trials at the National Cancer Institute (NCI) [7].

Chromatographic methods for analysis of antiviral agents, including anti-AIDS drugs, were reviewed a few years ago [8]. More recently, other methods for the analysis of nucleoside analogs, especially didanosine, have appeared [9–17]. We reported an HPLC method for the analysis of F-ddA and its major metabolite, 2-β-fluoro-2′,3′-dideoxyinosine (F-ddI), in biological fluids used for analysis of samples from preclinical studies of rodents and primates [14,18]. That methodology employed solid-phase extraction (SPE) followed by gradient HPLC analysis on a phenyl reversed-phase column to achieve a limit of quantitation of 50 ng/ml (0.2 μ*M*) for both compounds. However, that method was inadequate for analysis of clinical samples from AIDS patients since the sample work-up was not compatible with the necessary HIV viral decontamination procedure. In addition, the endogenous interferences in human plasma were different from those in animal plasma.

In this report we describe a modified method appropriate for the analysis of both F-ddA and F-ddI in plasma from patients who have AIDS or are HIV positive. Although based on the original analytical strategy, major modifications were necessary in the procedure to permit analysis of human samples. For safer sample handling, we chose a nonionic detergent, Triton X-100, to inactivate the HIV without adversely affecting the compounds of interest. The use of this detergent necessitated a modification of the SPE procedure. In addition, the chromatography was adjusted to optimize the separation in human plasma and a new internal standard was chosen and validated. Sample storage conditions were also evaluated since the large volume of samples from a clinical study means samples often have to be stored before analysis. This modified method is being used to analyze samples from the Phase I F-ddA clinical trials conducted by the NCI.

2. Experimental

2.1. Chemicals and reagents

F-ddA (NSC 613792), F-ddI (NSC 616290), dideoxycytidine (ddC, zalcitabine), dideoxyadenosine (ddA) and dideoxyinosine (ddI, didanosine) were supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment, Diagnosis and Centers (DCTDC), NCI (Bethesda, MD, USA). The adenosine deaminase inhibitor, 2′-deoxycoformycin (2′-dCF, NSC 218321) [19] was obtained from the Drug Synthesis and Chemistry Branch, DTP, DCTDC, NCI. The experimental anti-AIDS drug, 1592U89 (abacavir, (−)-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-eyclopentene-1-methanol) and the nonnucleoside reverse transcriptase inhibitor nevirapine were provided by the NCI clinic. The internal standards, 5′-chloro-5′-deoxyadenosine (5′-Cl-dA) and 2-chloroadenosine (2-Cl-A), as well as

several drugs used for retention time determination on this system, were purchased from Sigma (St. Louis, MO, USA). Scintillation grade Triton X-100 was manufactured by Research Products International Corporation (Mount Prospect, IL, USA). Betadine® solution, 10% povidone–iodine complex (equivalent to 1% available iodine), was produced by Purdue Frederick Company (Norwalk, CT, USA) and the Wescodyne® solution, 1.6% titratable iodine, by West Chemical Products (Tenafly, NJ, USA). Chlorox® bleach (5.25% sodium hypochlorite) was manufactured by the Chlorox (Oakland, CA, USA). HPLC grade methanol and water, as well as certified 1 *M* sodium hydroxide solution, were bought from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium formate used for buffer preparation was purchased from Fluka (Buchs, Switzerland). Spectrophotometric grade DMSO, used for preparation of concentrated stock solutions of analytes and internal standards, was purchased from Aldrich (Milwaukee, WI, USA) and monobasic potassium phosphate used for buffer preparation was obtained from Mallinkrodt (St. Louis, MO, USA). Phosphate buffered saline (PBS, 0.9% NaCl, pH 7.0) was procured from Biofluids (Rockville, MD, USA). Human plasma, collected with sodium heparin, was obtained from normal volunteers.

2.2. Chromatographic analysis

The HPLC instrumentation was identical to that previously described [14] except for the study on retention time comparisons in which a TSP UV 2000 detector (Thermo Separation Products, San Jose, CA, USA) was used. Aliquots (50 μl) of processed sample were analyzed on a 250×4.6 mm Hypersil phenyl-2 analytical column (Shandon Scientific, Cheshire, UK) preceded by a 30×4.6 mm Brownlee Spheri-5 Phenyl precolumn (Applied Biosystems, San Jose, CA, USA). A 30-min linear gradient from 2 to 36% methanol in 0.01 *M* (pH 6.8) phosphate buffer, followed by a 1-min return to initial conditions and a 2-min equilibrium period were employed. The buffer was later changed to 0.01 *M* ammonium formate (pH 4.8) as described in Section 3.1. The flow-rate was 1 ml/min and detection was by UV absorbance at 260 nm.

2.3. HIV inactivation and sample stability

Studies were initially conducted to determine the effects of heat and various chemical inactivation reagents for HIV on the stability of F-ddA and F-ddI. For the heat inactivation experiment, fresh human plasma containing 2′-dCF (20 μ*M*) was heated to 56°C and spiked with F-ddA or F-ddI at 5 μg/ml. Aliquots were taken from each immediately and over a 5-h period and analyzed by HPLC. Various agents reported as effective for inactivation of the retrovirus were also investigated to determine if they influenced F-ddA or F-ddI stability or chromatography. One-half per cent sodium hypochlorite (10% Chlorox bleach), 2% Wescodyne, 2% Betadine and 0.5% Triton X-100 were added separately to F-ddA and F-ddI solutions in 0.01 *M* phosphate buffer (pH 6.8), allowed to equilibrate for 30 min at room temperature and analyzed by HPLC. If the nucleosides were stable to the agent in buffer, then plasma samples spiked with F-ddA, F-ddI and 5′-Cl-dA were prepared with the addition of that reagent and allowed to stand at least 15 min at room temperature before further processing.

The activity of Wescodyne, Betadine and Triton X-100 to inactivate HIV-1_{LAW} was determined as follows. The virus (100 μl of 10^9 virus particles/ml preparation) was inoculated in fresh human plasma (900 μl) to which 20 μl Wescodyne or Betadine or 100 μl Triton X-100 (5% v/v in PBS) were added. Each plasma sample was incubated at room temperature for 2 h and was then subjected to centrifugation at 90 000 *g* for 45 min at 4°C in a Beckman (Fullerton, CA, USA) type 35 rotor. The pelleted virus was resuspended in the appropriate amount of RPMI-1640 to make a final concentration 20 times greater than the original. Each sample was diluted between 1:50 and 1:75 in 1% uranyl acetate and was subjected to transmission electron microscopy as previously described [20]. A portion of each resuspended virus preparation was also subjected to testing for infectivity. MOLT-4 cells were exposed to the virus preparation at 10, 10^2, 10^3 and 10^4 dilutions ($n=8$) and the HIV-1 induced cytopathic effect was used as an endpoint for assessing infectivity. Fresh human plasma, inoculated with HIV-1 but not exposed to decontaminants, was similarly treated and served as a control.

2.4. Sample preparation

Triton X-100 (2.5 μl) was mixed with an 0.5-ml aliquot of plasma to make a final concentration of 0.5% v/v. This treated plasma was mixed with 1 ml distilled water and 1 μg 5′-Cl-dA internal standard. The sample was then loaded onto a Sep-Pak® C_{18} cartridge (Waters Corporation, Milford, MA, USA) activated by washing with 4 ml methanol followed by 2 ml water. The cartridge was washed with 2 ml 0.01 *M* phosphate buffer (pH 6.8) and the compounds of interest were eluted with 2 ml methanol–water (70:30, v/v). The eluent was evaporated under nitrogen at 40°C using a TurboVap LV evaporator (Zymark Corporation, Hopkinton, MA, USA) and the residue was reconstituted in 0.5 ml 20% methanol in 0.01 *M* phosphate buffer (pH 6.8).

2.5. Recovery, intra-assay precision and limit of quantitation

To determine intra-assay precision and recovery, fresh human plasma containing 20 μ*M* 2′-dCF was spiked at 1 μg/ml each F-ddA and F-ddI. Triton X-100 was added to make the concentration 0.5% v/v and allowed to equilibrate for 1 h at room temperature. Triplicate 0.5-ml aliquots of the spiked plasma and a blank plasma sample with Triton X-100 were then processed by the procedure described above. A standard solution of F-ddA and F-ddI in the methanol–phosphate buffer (20:80, v/v) used to reconstitute plasma samples was also prepared. Triplicate aliquots of this direct standard were then taken and internal standard added to each. At a later time, quadruplicate aliquots of human plasma spiked at 50 ng/ml each F-ddA and F-ddI were processed and analyzed by HPLC. Recovery was calculated (at 1 μg/ml only) from the mean of the absolute HPLC peak areas of the spiked plasma samples, corrected for blank, divided by the corresponding mean of the absolute peak areas in the direct standards. Intra-assay precision was subsequently determined by the relative standard deviation (R.S.D.) of the peak area ratio in the spiked plasma samples. Limit of quantitation was evaluated by calculating the concentration of the nominal 50 ng/ml precision samples using a log–log standard curve (GraphPad PRISM version 2, San Diego, CA, USA) from 20 ng/ml to 5 μg/ml prepared by a different person.

2.6. Plasma protein binding

Plasma protein binding for F-ddA and F-ddI was determined in human plasma at 250 ng/ml and 1 μg/ml. Fresh plasma containing 20 μ*M* 2′-dCF was spiked with F-ddA or F-ddI and allowed to equilibrate at least 5 min at room temperature. Three 0.5-ml aliquots were then taken and ultrafiltered by centrifuging at 1900 *g* for 75 min at room temperature in Amicon Centrifree micropartition units with a 30 000 molecular mass cut-off (W.R. Grace, Beverly, MA, USA). Blank plasma ultrafiltrate, direct standards in phosphate buffered saline (pH 7.4) and ultrafiltered direct standards were also prepared. All samples of buffer and ultrafiltrate were analyzed directly by HPLC and absolute peak areas of the compounds of interest were used to calculate membrane hold-up and plasma protein binding according to the following formulas:

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

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

where 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.

2.7. Plasma stability

F-ddA stability was determined in fresh plasma from four different human volunteers and F-ddI stability was measured in pooled human plasma. Each sample of plasma (4 ml) was heated to 37°C in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, IL, USA) and then spiked to 5 μg/ml with F-ddA or F-ddI. Aliquots of 0.5 ml were taken at various times over a 24-h period and added to a chilled mix of 1.0 ml water, 1 μg 2-Cl-A and 1.3 μg 2′-dCF. Samples were immediately processed by the SPE procedure described above, with the exception that the compounds of interest were eluted

with 2 ml of 100% methanol instead of 70% methanol. The peak area ratio of F-ddA to 2-Cl-A versus time was curvefit to a monoexponential decay using the GraphPad INPLOT program.

2.8. Assay validation

Accuracy of this method was determined by analysis of human plasma spiked at three different unknown concentrations of F-ddA and F-ddI. Fresh plasma collected with sodium heparin and 2′-dCF was used as the matrix. Each 10 ml spiked sample was deactivated with 50 μl Triton X-100 and duplicate 0.5-ml aliquots were taken from it. Blank plasma was used to create a standard curve in the range of 50–5000 ng/ml F-ddA and F-ddI. Unknowns and standards were processed and analyzed as described above. Concentrations were calculated using a least-squares regression line, weighted by the inverse of the concentration. The mean of the four values calculated for each level, from duplicate injections of the two aliquots taken from each unknown sample, was then compared with the actual spiked concentration. Accuracy was measured by percent deviation from the nominal value.

The spiked plasma samples from the assay validation study were also used to determine sample stability of completely processed and Triton-treated plasma. Aliquots of Triton-treated spiked plasma were thawed and processed after frozen storage for one week and again after 1 month. Also, processed samples that had been frozen were reanalyzed at these times. A newly prepared standard curve in thawed blank plasma was used to calculate concentrations.

Day-to-day sample variability and analyte stability in frozen plasma were also evaluated. For this determination, three 10-ml aliquots of human plasma were prepared at 0, 90 or 900 ng/ml F-ddA and F-ddI. After addition of Triton X-100 (0.5% v/v) and 2′-dCF (20 μ*M*), the plasma was vortexed and 0.5-ml aliquots were taken and immediately frozen. Samples were thawed one set (consisting of a blank, 90 ng/ml and 900 ng/ml aliquot) at a time over a 5-month period and processed with patient samples by the standard procedure. The mean concentrations and R.S.D. values were determined.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of blank and spiked human plasma, using the initial mobile phase buffer at pH 6.8, are shown in Fig. 2. The same reversed-phase phenyl column was utilized for the analysis of F-ddA and F-ddI in human plasma as was previously used for analysis in the biological fluids of rat and monkey [14]. However, it was necessary to change the gradient program and internal standard due to the presence of different endogenous peaks in the human samples. A 30-min linear program from 2–36% methanol led to reasonable separation. It can be seen that there are no interfering peaks under these conditions at the retention times of the compounds of interest. As before, a 2-min between run

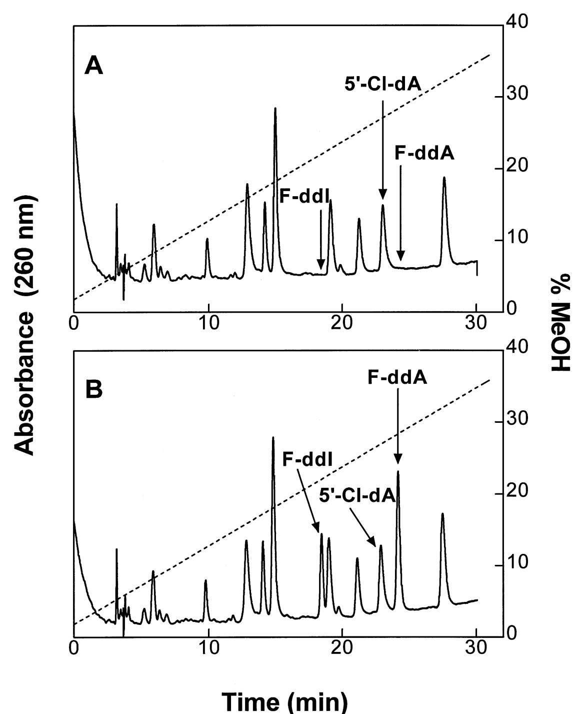

Fig. 2. HPLC chromatograms obtained using a 250×4.6 mm Hypersil phenyl-2 column and Brownlee Spheri-5 phenyl cartridge precolumn with phosphate buffer (pH 6.8) of (A) blank human plasma and (B) human plasma spiked at 2 μg/ml each F-ddA and F-ddI. Each sample was processed according to the procedure outlined in Section 2.4. The dotted line shows the methanol gradient program.

equilibration time was necessary to obtain reproducible retention times, leading to a 35-min analysis cycle using an autosampler.

The new internal standard, 5′-chloro-5′-deoxyadenosine (5′-Cl-dA) was selected because it is structurally similar to F-ddA yet with a chromatographic retention time that is intermediate between that of F-ddA and F-ddI. In addition, 5′-Cl-dA is commercially available.

The chromatographic characteristics of various other drugs that might be prescribed for HIV positive patients were also investigated (Table 1). It was determined after the first patient samples were received that Bactrim (sulfamethoxazole and trimethoprim), a prophylactic antibiotic often prescribed to individuals with AIDS, had a metabolite that interfered with determination of F-ddI ($t_R = 17.7$ vs. 18.1 min) using the original HPLC conditions (phosphate buffer, pH 6.8). Changing to a pH 4.8 formate buffer in the mobile phase eliminated the problem (Figs. 3 and 4). This adjustment also led to better separation between F-ddA and AZT. Since anti-AIDS drugs are often given in combination [21], this is an added advantage. A list of the compounds evaluated and their retention times using the two different mobile phases is shown in Table 1. Several experimental or recently approved anti-HIV drugs were not directly evaluated on this system since published chromatographic methods indicated they would not cause interference problems in this assay. For example, indinavir lacks significant UV absorption above 220 nm [22] while ritonavir is greater than 96% plasma protein bound and would be removed with the proteins in the SPE step [23]. Saquinavir, nelfinavir and delavirdine are much more lipophilic than F-ddA and so much longer retention times are expected [24–26].

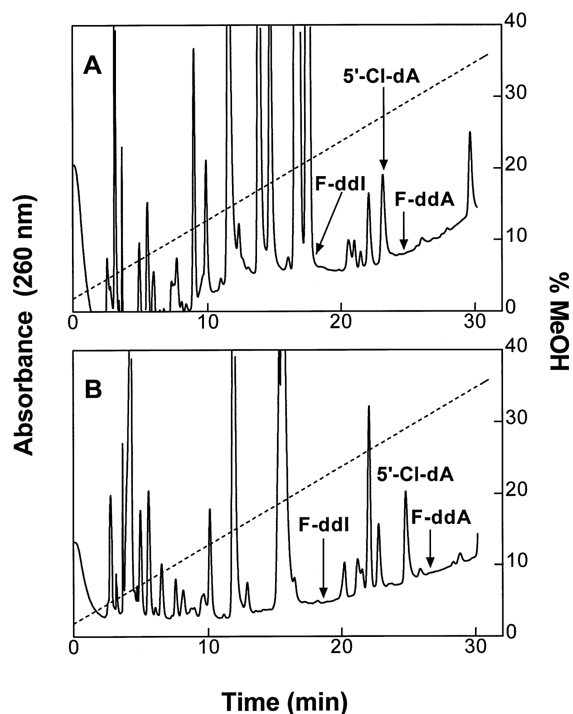

Fig. 3. HPLC chromatograms showing predose plasma from F-ddA Phase I clinical trial of patient taking Bactrim™ antibiotic obtained using a 250×4.6 mm Hypersil phenyl-2 column and Brownlee Spheri-5 phenyl cartridge precolumn using (A) original mobile phase with phosphate buffer (pH 6.8) and (B) mobile phase containing formate buffer (pH 4.8). The sample was processed according to the procedure outlined in Section 2.4. The dotted line shows the methanol gradient program.

3.2. HIV inactivation

Since biological samples from patients with AIDS will be analyzed during the clinical trial, an important consideration is minimizing the risk of HIV infection to the analyst and other laboratory personnel. Several procedures to inactivate the virus, of varying efficiency and complexity, have been reported. Standard methods for decontamination of aqueous samples include treatment with heat, bleach, detergents, activated iodine complexes and phenolics [27,28].

The first method considered was heat inactivation because it has the advantage of simplicity with no sample dilution or manipulation. Heating at 57°C for 3 h was used for the decontamination of plasma and urine samples from patients receiving ddI in a Phase I clinical trial [29]. Since others indicated that various heating times, from 30 min to 5 h, were necessary for complete loss of HIV infectivity [30,31], we tested the stability of 5 μg/ml F-ddA (with 20 μ*M* 2′-dCF, an adenosine deaminase inhibitor [19]) and F-ddI in separate human plasma aliquots heated at 56°C for the full 5 h. F-ddI concentration, as measured by HPLC peak area, did not change for the entire period. However, a decay of about 15% was observed for F-ddA over this time.

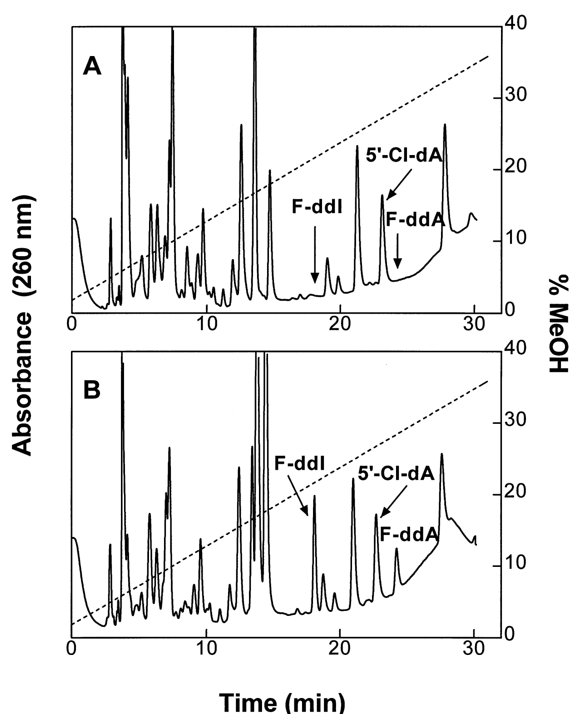

Fig. 4. HPLC chromatograms using a 250×4.6 mm Hypersil phenyl-2 column and Brownlee Spheri-5 phenyl cartridge pre-column with formate buffer (pH 4.8) in the mobile phase from a patient treated with 1.6 mg/kg F-ddA as part of a Phase I clinical trial. (A) Predose plasma and (B) plasma obtained 85 min after the beginning of a 100-min intravenous infusion. Each sample was processed according to the procedure outlined in Section 2.4. The dotted line shows the methanol gradient program.

Therefore, this inactivation procedure was not considered further.

Various chemical decontamination agents were then investigated. Addition of bleach (0.5% v/v sodium hypochlorite) to standard buffer solutions caused complete decomposition of F-ddA within 10 min. However, F-ddA and F-ddI were stable in buffer or plasma after the addition of either Wescodyne, Betadine or Triton X-100 at room temperature for at least 15 min. Previous research indicated 0.5% Betadine was an effective inactivation agent in 20% fetal calf serum [32]. Treatment with 0.5% Triton X-100 was reported to inactivate HIV in cell culture media [33] and was used to decontaminate plasma samples from a Phase I clinical trial of ddI in AIDS patients [34]. In addition, 0.5% Nonidet-P40, another similar detergent, completely inactivated HIV after a 1-min exposure at room temperature in 50% human plasma [31]. Therefore, Betadine, Wescodyne and Triton X-100 were further tested for their ability to inactivate HIV-1 in fresh human plasma.

Inactivation experiments in HIV-inoculated human plasma indicated that the infectivity of the virus was completely abrogated when the virus was treated with 0.5% Triton X-100 for 2 h and resuspended in RPMI-1640. No retrovirus particles displaying characteristics normally found in HIV-1 were identified in this Triton X-treated sample when examined by transmission electron microscopy (Fig. 5C). In contrast, neither of the iodine complexes (Betadine and Wescodyne) had any significant effect on the morphology of the virus (Fig. 5B) when compared to control (Fig. 5A). Indeed, 2% Betadine-treated HIV-1 failed to completely inactivate the virus. When MOLT-4 cells were exposed to 10-fold serially diluted Betadine-treated HIV-1 preparations and the HIV-1-induced cytopathic effect (CPE) was assessed, CPE was detected in 8/8 and 1/8 tests by day 14 of culture whereas the control HIV-1 produced CPE in 8/8 and 4/8 at 10-fold and 100-fold dilutions, respectively. In contrast, both the 2% Wescodyne-treated HIV-1 and the Triton X-treated HIV failed to produce infection of MOLT-4 cells at all dilutions tested. These data suggested that although electron microscopy did not reveal a significant morphological change of the viral structure, the infectivity of HIV-1 was also destroyed by the 2% Wescodyne exposure. However, since the 0.5% Triton X-100 was proven effective by both morphology and infectivity tests, it was chosen as the inactivation agent.

3.3. Sample preparation

In our previous method, used for analysis of F-ddA and F-ddI in biological fluids from healthy rats and monkeys, the compounds of interest were eluted from the SPE cartridge with 2 ml methanol. However, the Triton X-100, added to inactivate the plasma HIV, was also eluted out of the cartridge using this procedure. When the processed samples were injected, the Triton X-100 caused rapid HPLC column degradation which was not alleviated by additional washing. Changing the final SPE cartridge elution to 2 ml of methanol–water (70:30, v/v) led to sufficient solvent strength to elute the compounds

Table 1
Chromatographic characteristics of anti-HIV drugs and potential interferences

Compound	Retention time (min)		λ_{max} (pH 4.8)[a] (nm)
	pH 6.8	pH 4.8	
Isonicotinic acid	3.5	4.4	264
Gancyclovir	8.7	8.8	252, 271(sh)
AZT-glucuronide	–	9.1	266
Acyclovir	11.1	11.1	252, 271(sh)
ddC (zalcitabine)	13.3	15.8	273
3TC (lamivudine)	15.4	17.0	272
Sulfamethoxazole	16.9	32.6	267[c]
Interfering peak[b]	17.7	35.0	264
F-ddI	18.1	18.6	248
d4T (stavudine)	18.7	19.1	265
ddI (didanosine)	19.3	19.6	249
2-Cl-A	20.0	20.2	264
5′-Cl-dA	23.0	25.0	267
AZT (zidovudine)	24.4	23.8	265
F-ddA	24.7	26.4	258
ddA	25.9	27.3	260
Trimethoprim	34.8	32.5	269, 235
Nevirapine	–	43.6	236, 282
Abacavir (1592U89)	–	44.2	290, 256

[a] Determined with Shimadzu 2101 UV–vis scanning spectrophotometer using 10 m*M* ammonium formate buffer (pH 4.8).
[b] Peak collected from patient plasma that interfered with F-ddI analysis. Patient was on Bactrim.
[c] Determined on-the-fly using Perkin-Elmer LC-235 diode array detector with 0.1 *M* ammonium formate (pH 4).

of interest yet still retain most of the detergent [34]. This adjustment eliminated the chromatographic problems, although it lengthened the evaporation time by about 30 min.

3.4. Assay characteristics

Standard curves were calculated based on the peak area ratio of F-ddA or F-ddI to internal standard and were linear over the range of 50 ng/ml to 5 μg/ml (0.2–20 μ*M*). For a range covering the full two orders of magnitude, calculated concentrations for low standards (50–500 ng/ml) were more accurate if a weighting factor of $1/x$ or a log–log standard curve was used. Typically, a blank and 4–7 spiked plasmas led to standard curves with a coefficient of determination (r) better than 0.998. The linearity was also confirmed by use of the residual runs test. (A residual is the distance of a point from the regression line. A run consists of a series of consecutive residuals which are either all positive or all negative. If the number of runs deviates too far from the expected value, this indicates the data systematically deviate from the line.) The recovery of F-ddA, F-ddI and 5′-Cl-dA was complete as can be seen in Table 2. Precision was better than 8% for each compound at 1 μg/ml. The limit of quantitation for F-ddA and F-ddI in human plasma was 50 ng/ml (0.20 μ*M*). Replicate spiked samples at this level had an R.S.D. of 12% for F-ddA and 17% for F-ddI and the deviation from the nominal concentration was +8.0% for F-ddA and +5.3% for F-ddI. The limit of detection, as defined at $S/N=5$ for spiked plasma standards, was 10 ng/ml (0.04 μ*M*) for F-ddA and 15 ng/ml (0.06 μ*M*) for F-ddI.

3.5. Plasma protein binding and stability

Plasma protein binding was determined for F-ddA and F-ddI at two different concentrations in fresh human plasma by centrifugal ultrafiltration. The two compounds were tested separately to prevent possible binding competition. Drug binding to plasma protein is of particular importance for these anti-HIV agents because only free drug can penetrate the blood–brain barrier to reach the central nervous

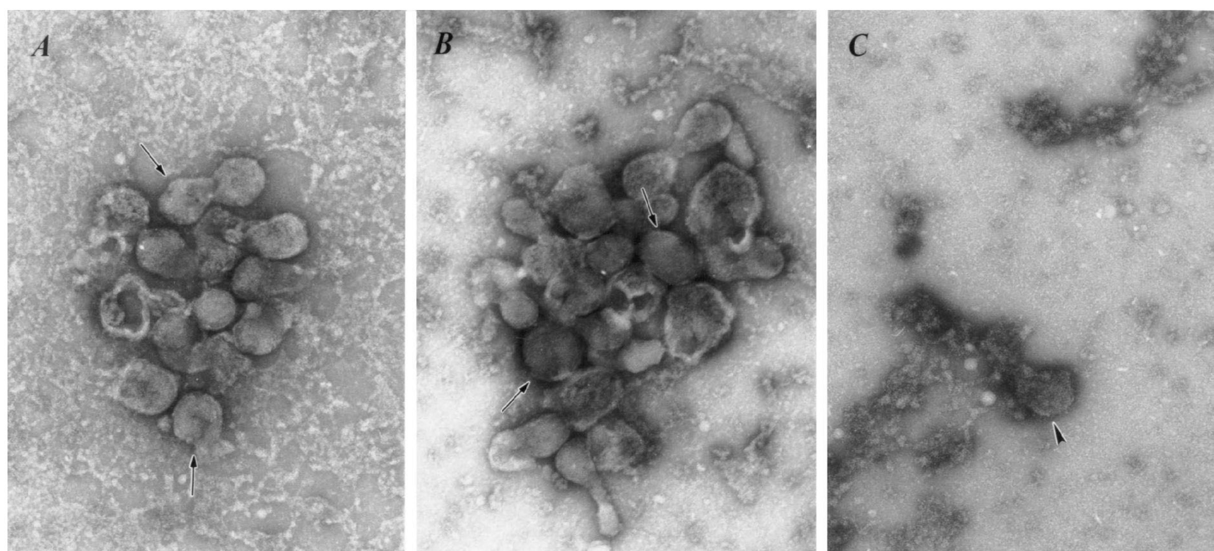

Fig. 5. Morphology of HIV-1 in human plasma treated with Betadine and Triton X-100. HIV-1 samples were untreated or treated with 2% Betadine or 0.5% Triton X-100 and subjected to ultracentrifugation, resuspension, negative staining and transmission electron microscopy analysis. (A) Retrovirus particles were found in small aggregates throughout the untreated HIV-1 preparation. The particles which best represent how normal HIV-1 virions appear are marked with arrows. (B) In the HIV-1 preparation treated with 2% Betadine, as in the untreated preparation, virus particles exhibiting a prominent exterior envelope were seen, as marked with arrows. (C) In the HIV-1 preparation treated with 0.5% Triton X-100, there were no particles which appeared to display the characteristics normally found in HIV-1 negative stain preparations. Some spherical particles were found in certain areas (marked with arrowheads); however, such particles did not display prominent envelopes and all appeared to be lysed.

system, which is known to be a sanctuary for HIV [35]. There was no significant protein binding for F-ddA or F-ddI at either 0.25 or 1.0 μg/ml. In addition, no membrane hold-up was observed for either compound using the Amicon Centrifree units which contain membranes with a molecular mass cut-off of 30 000.

Table 2
Assay characteristics

	F-ddA	F-ddI
Recovery (%)[a,b]		
1 μg/ml (3.9 μ*M*)	109±8.0	111±7.9
Intra-assay precision (% R.S.D.)		
50 ng/ml[c] (0.20 μ*M*)	11.7	16.8
1 μg/ml[a] (3.9 μ*M*)	3.3	4.3
Plasma protein binding (%)[a]		
250 ng/ml (1.0 μ*M*	10.9±10.2	16.2±14.3
1 μg/ml (3.9 μ*M*)	8.2±5.0	8.0±11.2

[a] Determinations were performed in triplicate for this study.
[b] Recovery of 5′-Cl-dA was 101±6.5%.
[c] Determinations were performed in quadruplicate for this study.

The stability of both F-ddA and F-ddI at 37°C in fresh human plasma from four normal volunteers was also investigated. The F-ddI concentration, as measured by its ratio to internal standard, remained constant at 1.4±2.7% over the 24-h period it was evaluated. When F-ddA was incubated in plasma without the addition of an adenosine deaminase inhibitor, a very slow decay of the compound was observed with the concomitant slow formation of F-ddI. The halflife for F-ddA was greater than 24 h in each of the samples tested. Both compounds were more stable in human plasma than in that of monkey or rat [14].

3.6. Assay validation, sample stability and day-to-day variability

A summary of the assay validation study is shown in Table 3. Predicted concentrations for unknown spiked samples were within 7% of the nominal values for both F-ddA and F-ddI. The only exception was the lowest level F-ddI sample (0.15 μ*M* or 38

Table 3
Assay validation

	Concentration (μM)		Deviation from nominal (%)
	Nominal	Measured	
A. F-ddA			
Fresh plasma			
Low conc.	0.15	0.14	−6.7
Medium conc.	2.05	2.02	−1.5
High conc.	20.5	21.85	+6.6
Frozen 1 week			
Low conc.	0.15	0.15	+0.0
Medium conc.	2.05	1.93	−5.9
Medium proc.[b]	2.05	2.00	−2.4
High conc.	20.5	20.3	−1.0
Frozen 4 weeks			
Low conc.	0.15	0.14	−6.7
Medium conc.	2.05	2.00	−2.4
Medium proc.[b]	2.05	1.93	−5.9
High conc.	20.5	19.78	−3.5
B. F-ddI			
Fresh plasma			
Low	0.15	0.23	+53
Medium	2.50	2.63	+5.2
High	9.92	10.48	+5.6
Frozen 1 week			
Low	0.15	0.21	+40
Medium	2.50	2.42	−3.2
Medium proc.[b]	2.50	2.32	−7.2
High	9.92	9.59	−3.3
Frozen 4 weeks			
Low	0.15	0.19	+27
Medium	2.50	2.33	−6.8
Medium proc.[b]	2.50	2.41	−3.6
High	9.92	10.26	+3.4

[a] For each test, duplicate aliquots were processed and each was injected twice.
[b] Samples spiked at medium level and processed for HPLC analysis before freezing.

ng/ml) in which the actual spiked concentration was just below the limit of quantitation. It should be noted that the current method involves monitoring UV absorption at 260 nm, the F-ddA λ_{max}. To maximize sensitivity for F-ddI at the cost of simplicity and calculation time, dual wavelength monitoring at 260 nm and 248 nm, the F-ddI λ_{max}, could be used.

Plasma samples treated only with Triton X-100 were stored frozen and reprocessed after 1 week and again after 1 month. In addition, selected processed samples were also stored frozen for up to 1 month before reanalysis by HPLC. New standard curves were calculated using freshly prepared spiked plasma standards. The predicted concentrations of either set of frozen samples showed no significant difference over this period. Thus, processed human plasma samples from patients treated with F-ddA may be stored frozen at least a month without any deterioration. Unprocessed plasmas may be stored even longer, up to 5 months, based on the day-to-day variability and stability study. In this experiment, thawed plasma samples that had been previously spiked with F-ddA and F-ddI showed no significant

Table 4
Day-to-day variability

Sample[a]	Nominal conc. (μM)	Measured conc. ($\mu M\pm$R.S.D. %)	Deviation from nominal (%)
Low			
F-ddA	0.357	0.328±19.3	−8.0
F-ddI	0.360	0.331±17.9	−8.2
High			
F-ddA	3.57	3.74±13.2	+4.9
F-ddI	3.60	3.80±19.4	+5.6

[a] $n=8$ over 5-month period.

change in measured concentrations when analyzed by HPLC over this period. A summary of these results is presented in Table 4.

4. Conclusions

A robust, semi-automated method has been developed that is suitable for the analysis of F-ddA and F-ddI in clinical samples. It is based on an earlier method that was utilized for preclinical animal studies, but several modifications have been made. Because Triton X-100 is used to inactivate HIV, the SPE step has been changed to prevent HPLC column contamination and degradation. In addition, a different gradient program and new internal standard are employed to improve the chromatographic separation of the components in human plasma. This method has a quantitative range of 0.05–5 μg/ml (0.2–20 μM). Processed samples may be stored for up to a month and unprocessed plasma with added Triton X-100 and ADA inhibitor may be kept frozen for as long as 5 months with no significant loss of drug or metabolite.

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