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Analysis of various nucleosides in plasma using solid phase extraction and high-performance liquid chromatography with UV detection

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Abstract

The National Cancer Institute (NCI) has screened many nucleosides for antiviral activity to the HIV-1 virus. Drugs demonstrating antiviral activity are tested in animal models to evaluate their toxicity and pharmacokinetic characteristics. These drugs are subsequently evaluated for efficacy in human clinical trials. Sensitive analytical methodology is needed to quantify nucleosides in plasma and other biological matrices in support of these studies. Battelle has modified and validated a reversed phase high-performance liquid chromatography (HPLC) method for several of these nucleosides that could be easily adapted for similar compounds. Methods have been validated for 6-chloro-2′,3′-dideoxyguanosine (6CldG), 6-chloro-2′,3′-dideoxyinosine (6CldI) and their primary metabolites 2′,3′-dideoxyguanosine (ddG) and 2′,3′-dideoxyinosine (ddI) in both rat and dog plasma containing EDTA. The method has also been validated for 2′-fluoro-2′,3′-dideoxyara-adenosine (βFldA) and its primary metabolite 2′-β-fluoro-dideoxyinosine (βFdI) in rat plasma containing heparin. Calibration plasma standards were prepared over ranges of 0.1–10 μg ml^{-1} for βFldA and βFdI, 0.1–50 μg ml^{-1} for 6CldG and ddG, and 0.25–50 μg ml^{-1} for 6CldI and ddI in plasma containing 4 μg ml^{-1} pentostatin. The addition of pentostatin to the plasma samples inhibits in-vitro deamination of the drug after collection. Quality control (QC) standards were prepared containing the appropriate anticoagulant and 4 μg ml^{-1} pentostatin at concentrations within each of the bracketed calibration ranges in plasma. These methods have been successfully applied to plasma samples generated during various animal studies.

Keywords: Nucleoside; HPLC; Plasma; Analysis; Solid phase extraction; Antiviral

1. Introduction

The National Cancer Institute (NCI) has screened many nucleosides for antiviral activity to

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Table 1
Internal standard concentrations and volumes

Analyte	IS	IS concentration (μg ml^{-1})	IS volume (μl)
βlddA and βFlddI	2ClA	50	20
6CldG and ddG	6CldI	10	100
6CldI and ddI	6CldG	10	100

Table 2
Analysis parameters

Analyte/IS	Injection volume (μl)	Detection wavelength (nm)	Retention time (min)
βFlddA	10	260	10.5
βFlddI	10	260	6.5
2ClA	10	260	8.3
6CldG	50	240	14.1
ddG	50	240	6.3
6CldI	50	240	17.4
ddI	50	240	7

the HIV-1 virus. Dideoxyanalogues of adenosine, guanosine, cytosine, and thymine have demonstrated the ability to suppress the infectivity, replication, and cytopathic effect of the human immunodeficieiency virus [1–4]. Drugs demonstrating antiviral activity are tested in animal models to profile their toxicity and pharmacokinetic (PK) characteristics. Those drug formulations having adequate safety margins with characterized pharmacokinetics are then evaluated in human clinical trials for efficacy, safety, toxicity, and to establish optimal dosing schedules. Sensitive analytical methods for determination of these nucleosides and their major metabolites in biological fluids are essential for support of these studies. High-performance liquid chromatography (HPLC) has been successfully used to quantitate these nucleosides in biological fluids [5–10]. Battelle has modified and validated an existing analytical method for several of these nucleosides and their primary metabolites. The method has been successfully validated for analysis of samples of dog and rat plasma. These methods can be easily adapted to tissues as well; however, the deamination of the nucleosides without addition of pentostatin observed in plasma suggests that analysis of tissue samples may not be practical.

2. Experimental

2.1. Materials

6-Chloro-2′,3′-dideoxyguanosine (6CldG), 6-chloro-2′,3′-dideoxyinosine (6CldI), 2′,3′-dideoxyguanosine (ddG), 2′,3′-dideoxyinosine (ddI), 2′-fluoro-2′,3′-dideoxyara-adenosine (βFlddA), metabolite 2′-β-fluorodideoxyinosine (βFddI), and 2-chloroadenosine (2ClA) were supplied by the developmental therapeutics program (DCTDC, NCI, MD, USA). Solid phase extraction (SPE) C18 cartridges were purchased from Baxter (Columbia, MD, USA). Purified water (resistivity of ≥ 18 MΩ was generated using a Milli-Q system (Millipore, Milford, MA, USA). All other reagents were reagent grade.

2.2. Methods

2.2.1. Standard preparation

Two stock solvent standards were prepared by dissolving a weighed amount of each analyte in methanol. Working standards were prepared by dilution of the stock standards with 0.01 M phosphate buffer (pH 6.8):methanol (80:20 v/v). Calibration standards were prepared by diluting the working standards 1:10 with plasma containing

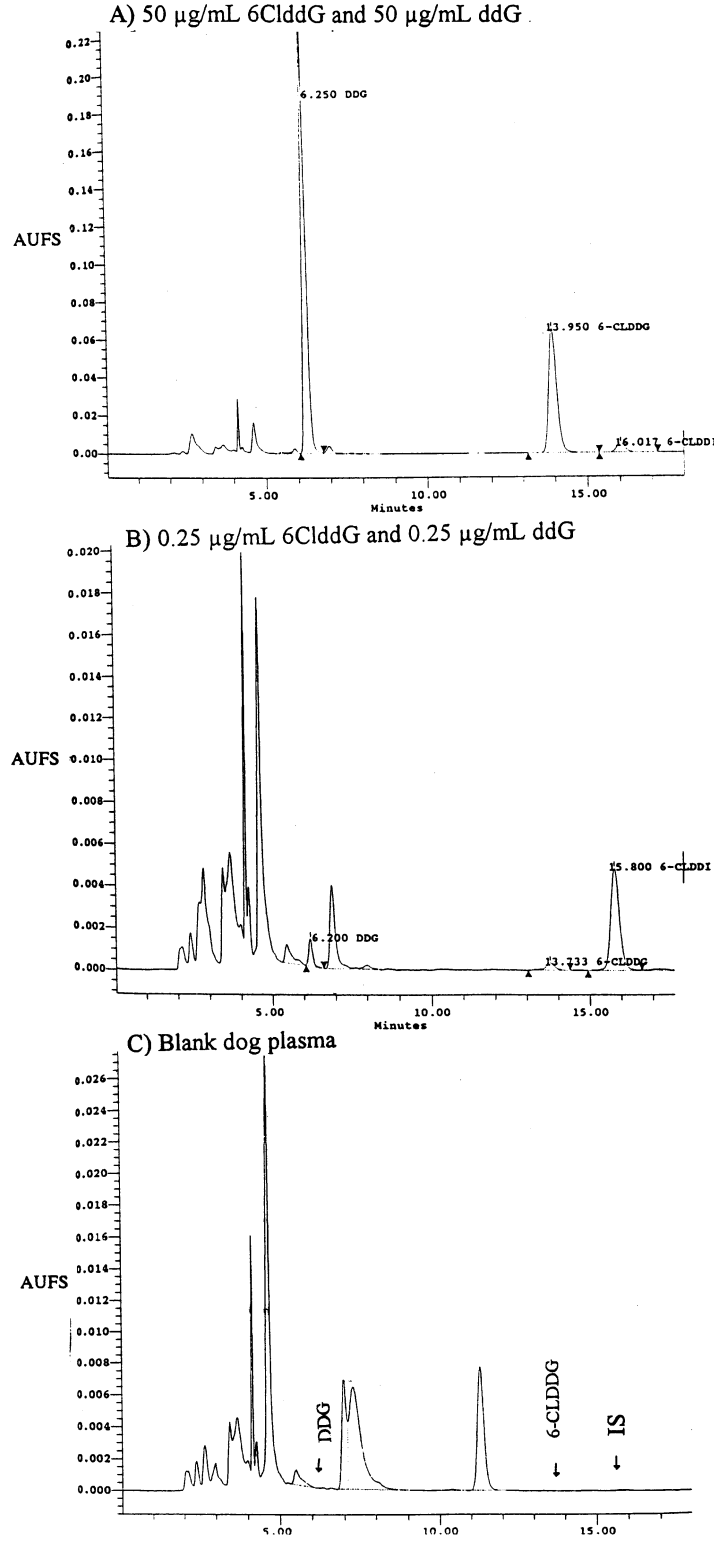

Fig. 1. Example chromatograms of the 6CldG and ddG calibration standards in dog plasma, and blank dog plasma: (A) high (50 µg ml^{-1}); and (B) low (0.25 µg ml^{-1}).

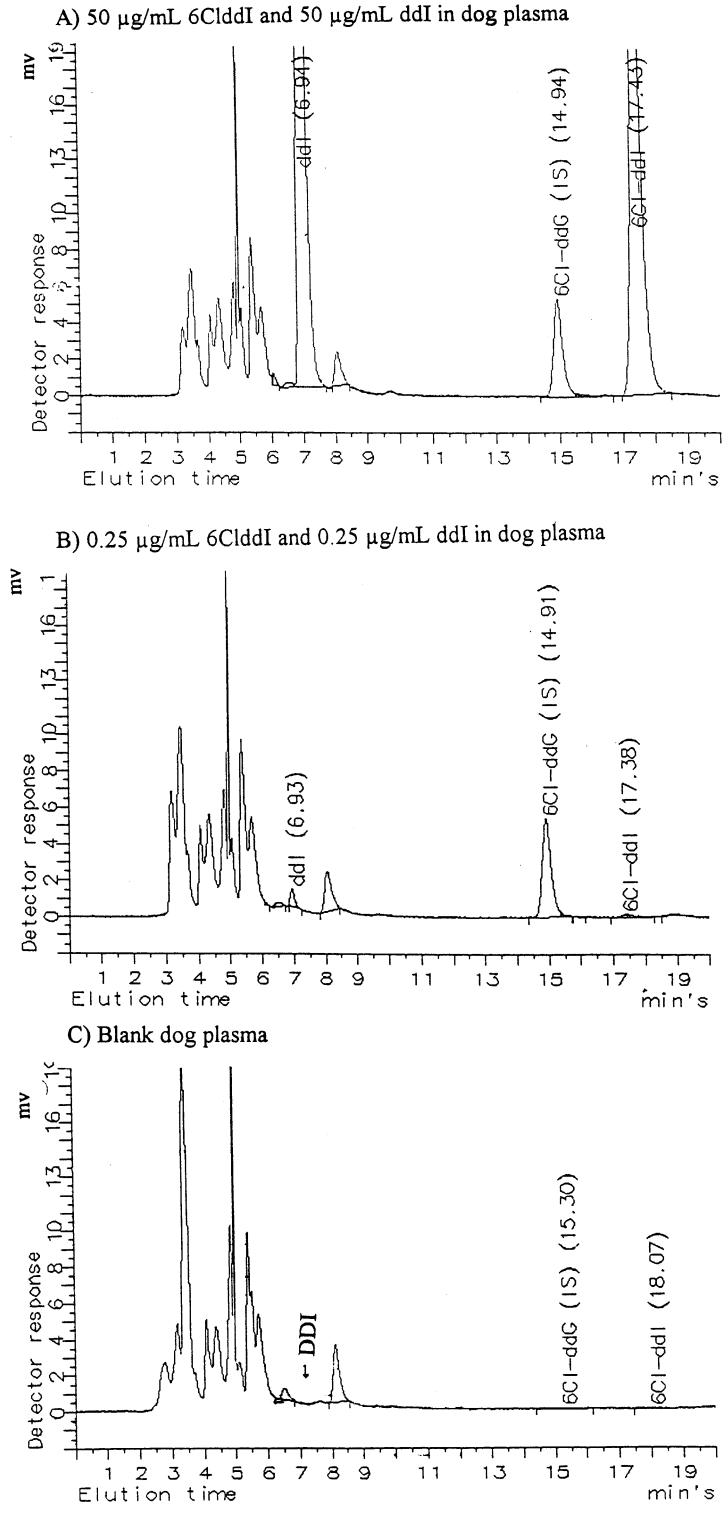

Fig. 2. Example chromatograms of the 6CldI and ddI calibration standards in dog plasma, and blank dog plasma: (A) high (50 µg ml^{-1}); and (B) low (0.25 µg ml^{-1}).

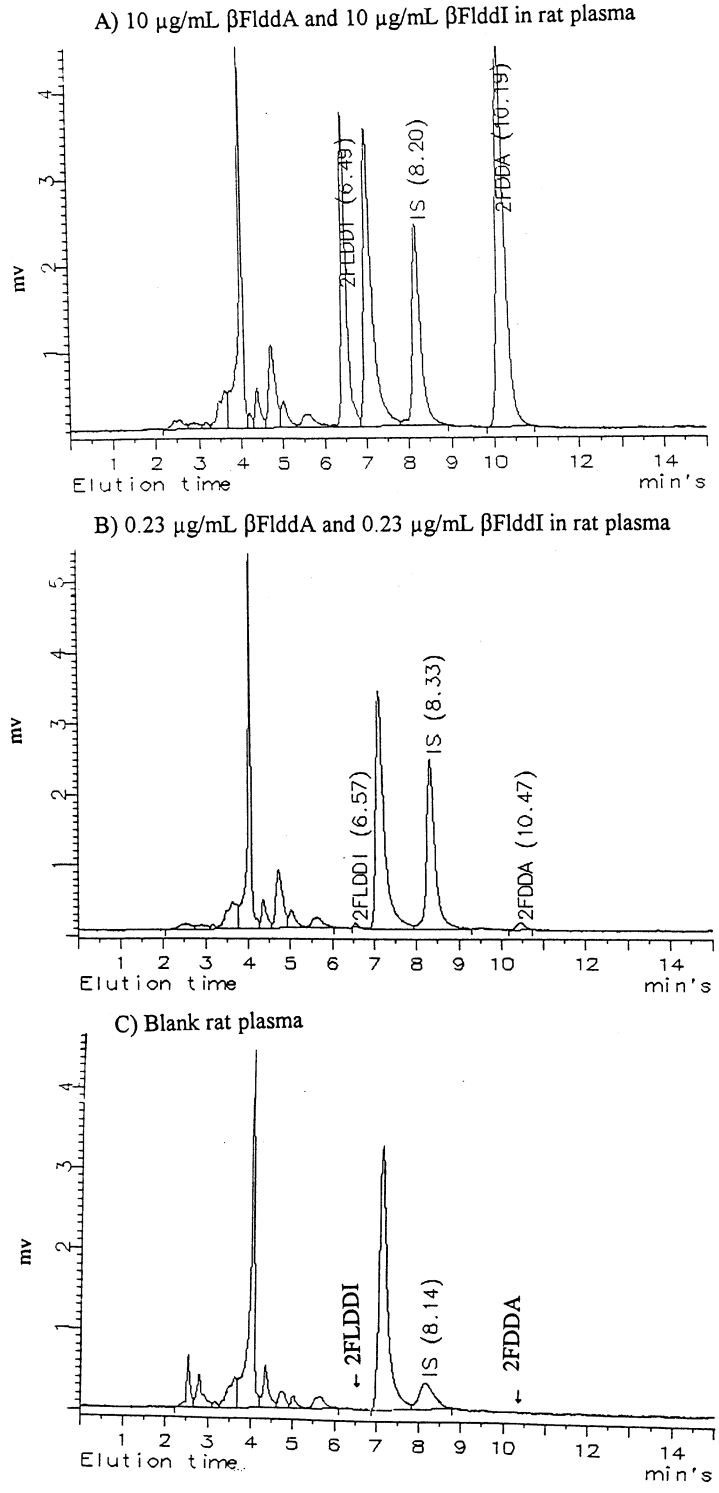

Fig. 3. Example chromatograms of the βFlddA and βFlddI calibration standards in rat plasma, and blank rat plasma: (A) high (10 µg ml^{-1}); and (B) low (0.23 µg ml^{-1}).

Table 3
Calibration standard ranges

Analyte	Calibration standard range (μg ml^{-1})
βFlddA	0.10–10
βFlddI	0.20–10
6CldG	0.10–50
ddG	0.10–51
6CldI	0.25–50
ddI	0.25–50

the appropriate anticoagulant and pentostatin. The pentostatin was added to all blank plasma as a stabilizer for the analytes and was added to all test animal samples at the time of their collection.

2.2.2. Extraction

Plasma samples (200 μl) were combined with internal standard as shown on Table 1. One ml of purified water was added. The diluted samples were transferred to SPE cartridges, which had been preconditioned with 2 ml methanol followed by 4 ml purified water. The cartridges were washed with 2 ml 0.01 M potassium–phosphate buffer (pH 6.8). The analytes were eluted with 2 ml methanol. The extract was evaporated to dryness under nitrogen at ≈30°C for ≈3–4 h using a Techne Dri-Block DB3, reconstituted in 0.5 ml 0.01 M phosphate buffer (pH 6.8) and filtered into a microvial insert and analyzed by HPLC.

2.2.3. HPLC conditions

Several different manufacturer's instrumental systems were used during the validation of these three methods. The standard instrumental system included an HPLC pump, auto injector, and UV detector. The data were acquired using either Waters millennium software version 2.0 or Beckman peak pro version 2.0. The analytical column was a Metachem hypersil phenyl, 250 × 4.6 mm ID, with a Metachem hypersil 5μ phenyl guard column. The analytes were eluted with an isocratic mobile phase of 90:10 (0.01M potassium–phosphate buffer (pH 5.5):ACN) (v/v) at a flow rate of 1 ml min^{-1}. The injection volumes, detection wavelengths, and retention times are reported in Table 2.

2.2.4. Method validation design

Calibration standards were prepared over the appropriate range in quadruplicate at each concentration. Calibration standard ranges are given in Table 3. Quality control (QC) standards were prepared at three concentrations within the calibration range. Concentrations close to the low, the middle, and high end of the calibration range were selected. These QC standards were prepared in bulk on the first day of validation and stored at −20°C until used. A full validation design included 3 sets of analyses with four calibration standards at each concentration and six QC standards at each concentration. One set also included analysis of blank plasma specificity samples from six individual sources, QC standards which were exposed to at least two freeze thaw cycles prior to analysis, and solvent standards at the same theoretical concentrations as the calibration standard following processing for determination of recovery. For the validation of the 6CldG, ddG, 6CldI, and ddI methods, at least two different analysts and two different analytical columns were used to demonstrate the ruggedness of the method. Extract stability was determined by analyzing dried QC extracts which were stored frozen

Table 4
Linearity of analyte responses

Analyte	Slope	% RSD of the slope	y-intercept	r^2
6CldI	0.117 ± 0.002	1.9	0.009	0.999 ± 0.001
ddI	0.742 ± 0.019	2.6	0.004	0.998 ± 0.001
βFlddA	0.195 ± 0.007	2.6	−0.012	0.999 ± 0.000
βFlddI	0.103 ± 0.003	3.0	−0.003	0.999 ± 0.001
6CldG	0.267 ± 0.023	8.6	−0.001	0.999 ± 0.001
ddG	0.464 ± 0.032	6.9	0.002	0.999 ± 0.001

Table 5
Dog ddG QC precision and accuracy

Nominal concentration (μg ml^{-1})	0.80			4.02			15.03		
Daily	1	2	3	1	2	3	1	2	3
N	6	6	6	6	6	6	6	6	6
Mean	0.77	0.75	0.71	3.88	3.68	4.03	14.99	14.06	15.17
s	0.02	0.02	0.02	0.07	0.08	0.10	0.15	0.94	0.51
RSD	2.5	2.8	2.2	1.9	2.1	2.5	1.0	6.7	3.4
% RE	−3.8	−7.0	−11.7	−3.6	−8.4	0.3	−2.0	−8.1	−0.9
Day to day									
N	18			18			18		
Mean	0.74			3.86			14.74		
s	0.03			0.17			0.77		
RSD	4.3			4.3			5.2		
% RE	−7.5			−3.9			−3.7		

Table 6
Dog 6CldG QC precision and accuracy

Nominal concentration (μg ml^{-1})	0.80			4.02			15.30		
Daily	1	2	3	1	2	3	1	2	3
N	6	6	6	6	6	6	6	6	6
Mean	0.79	0.83	0.84	3.94	3.84	4.12	14.66	13.58	14.90
s	0.02	0.2	0.03	0.06	0.12	0.12	0.12	0.78	0.49
RSD	2.1	2.0	3.0	1.5	3.1	3.0	0.8	5.7	3.3
% RE	−2.2	2.9	4.6	−1.9	−4.4	2.5	−2.9	−10.1	−1.3
Day to day									
N	18			18			18		
Mean	0.82			3.97			14.38		
s	0.03			0.15			0.77		
RSD	3.7			3.9			5.4		
% RE	1.76			−1.3			−4.8		

for 1 week after preparation, and one set of QC extracts in solution after storage for 2 days at room temperature. Long term stability of 6CldG, ddG, 6CldI, and ddI in rat and dog plasma was determined by analysis of QC standards after storage at −20°C for 30 days.

A cross validation included the same elements listed for validation, but only one day's data were collected. The methods for 6CldI and ddI, and 6CldG and ddG were validated for dog plasma, and cross validated in rat plasma, while the method for βFddI and βFddA was validated only in rat plasma. Because of the large volume of data and space limitations, only the validation results will be presented.

3. Results and discussion

The same basic method was applied to rat and dog plasma for validation and cross validation. The data presented here are representative of all validation data generated by the procedures described in Section 2.2.4. Results from the cross validations were similar.

3.1. Linearity

The concentration vs. chromatographic response ratio demonstrated linearity for all analytes measured. The average coefficients of determination, slopes, and average *y*-intercepts

Table 7
Dog 6CldI QC precision and accuracy

Nominal concentration (μg ml^{-1})	0.08			4.02			15.03		
Daily	1	2	3	1	2	3	1	2	3
N	6	6	6	6	6	6	6	6	6
Mean	0.81	0.74	0.83	4.13	4.07	4.23	15.15	13.94	14.60
s	0.04	0.05	0.06	0.07	0.08	0.08	0.25	0.28	0.17
RSD	4.4	6.3	7.3	1.7	2.1	2.0	1.7	2.0	1.2
% RE	1.1	−7.0	4.4	3.2	1.8	5.8	1.0	−7.0	−2.7
Day to day									
N	18			18			18		
Mean	0.80			4.14			14.56		
s	0.06			0.10			0.55		
RSD	7.6			2.5			3.8		
% RE	−0.5			3.6			−2.9		

for 6CldG, ddG, 6CldI, ddI, βFldA, and βFldI are presented in Table 4. The data demonstrated that the methods provided linear responses ($r \leq 0.99$) and the y-intercepts were not significantly different from zero. The slopes for each analyte were consistent from run to run with the largest relative standard deviation (RSD) being only 8.6% ($n = 3$).

3.2. Precision and accuracy

The acceptance criteria applied to these methods were average RSD of $\leq 15\%$ and average relative errors (RE) of $> 15\%$ for calibration standards (20% for the lowest calibration standard) and QC standards. Summaries of the precision and accuracy of the QC samples are presented in Tables 5–10. The acceptable calibration ranges for all compounds tested are presented in Table 1.

The acceptable limit of quantitation for each analyte was determined based on precision and accuracy at the low end of the respective calibration curve. The calibration curves all met the specified acceptance criteria for precision and accuracy over the ranges presented in Table 11. Two of the three individual validation runs for 6CldG produced acceptable results down to concentrations of 0.10 μg ml^{-1} in spite of having day to day RSD and RE values outside of the acceptance criteria. During actual sample analysis, the lower concentration standards were analyzed and the data was reported for all values bracketed by standards, and containing QC standards falling within the acceptance criteria. The limit of quantitation was therefore measured on an individual basis for each run.

The QC standards passed all acceptance criteria with the exception of the low (0.22 μg ml^{-1}) βFdI which had an average day to day %RE of −27.7. This was consistent with the poor performance of the low calibration standard (0.20 μg ml^{-1}). The limit of quantitation of this compound was adjusted to1 μg ml^{-1} based on these data.

3.3. Specificity

Analysis of at least six blank plasma samples per validation study showed no significant interferant in either dog or rat plasma which eluted with a retention near any of the six analytes. Representative chromatograms of the high and low standards and plasma blanks are presented in Figs. 1–3. There was a small peak corresponding to the elution time for 2ClA (an internal standard). Although this peak was observed consistently in the six specificity samples, it was not observed in the toxicokinetic samples. The specificity samples were obtained from an out-

Table 8
Dog ddI QC precision and accuracy

Nominal concentration (μg ml^{-1})	0.80			3.98			15.05		
Daily	1	2	3	1	2	3	1	2	3
N	6	6	6	6	6	6	6	6	6
Mean	0.79	0.78	0.80	4.04	4.27	4.16	14.97	15.00	14.63
s	0.03	0.02	0.02	0.06	0.09	0.09	0.28	0.32	0.12
RSD	3.3	2.7	2.2	1.6	2.2	2.1	1.9	2.1	0.8
% RE	−0.3	−2.4	0.6	1.4	7.2	4.4	−0.5	−0.3	−2.8
Day to day									
N	18			18			18		
Mean	0.79			4.15			14.87		
s	0.02			0.12			0.30		
RSD	2.9			30			2.0		
% RE	−0.7			4.3			−1.2		

side supplier and may have contained some contaminant that the study animals had not been exposed to. In addition, the variability of the internal standard peak in the spiked samples was small ($\leq 10\%$), suggesting that this interferant had little, if any, affect on the final quantitation. Future studies could easily be conducted using a larger concentration of the same IS to minimize the impact of any potential interferent present.

3.4. Recovery

The average recovery for all six compounds was greater than 70%. The average recoveries were 101.7, 83.4, and 94.9% for βFlddA, βFlddI, and the internal standard (2ClA), respectively. The average recovery for the lowest two βFlddI standards were below 80%; however, this was most likely due to method variability of the βFlddI assay in this range. Average recoveries for 6CldG, CldI, ddI, and ddG were 98.8, 98.7, 90.9, and 95.7%, respectively. For the 6CldG and 6CldI, increasing recovery was observed at decreased concentrations suggesting that there may be some plasma component interfering at low levels. This was further confirmed by the presence of these small peaks at the zero time points for many animals during the corresponding animal studies. The standards with high recoveries (above 115%) were not included in quantifiable range of the method.

3.5. Stability

Use of pentostatin to prevent in vitro deamination is critical to obtain consistent results and assure analyte stability. During method development, calibration standards, and quality controls prepared without pentostatin were unstable at room temperature over the period of time required for extraction. This instability was not quantitated, but spiked plasma standards at high concentration without pentostatin produced barely measurable peaks. All subsequent stability evaluations were performed with plasma containing 4 μg ml^{-1} pentostatin.

Freeze-thaw stability, stability of extracts stored at room temperature and dried and stored at −20°C, and stability for 30 days in plasma stored at −20°C were demonstrated for 6CldG, 6CldI, ddI, and ddG at all concentrations evaluated. The stability of βFlddI after two freeze-thaw cycles in plasma and after storage as both solution and dried extracts was acceptable only at the higher and mid concentrations. The results for βFlddI were consistent with the results for the standard curves and QC standards prepared daily, suggesting that poor performance of the assay at the concentration of the low standard was the problem rather than decreased stability at low concentrations in plasma.

The results for the βFlddA were less straightforward. The average REs and RSDs for the low

Table 9
Rat βFlddA QC precision and accuracy

Nominal concentration (μg ml^{-1})	0.22			2.24			7.47		
Daily	1	2	3	1	2	3	1	2	3
N	6	6	6	6	6	6	6	6	6
Mean	0.21	0.21	0.19	2.11	2.25	2.24	7.25	7.73	7.73
s	0.01	0.01	0.01	0.13	0.07	0.03	0.28	0.16	0.13
RSD	7.0	5.1	4.9	5.9	3.0	1.5	3.9	2.0	1.7
% RE	−5.9	−8.0	−14.2	−5.8	0.6	0.1	−3.0	3.5	3.5
Day to day									
N	17			18			18		
Mean	0.20			2.20			7.57		
s	0.01			0.10			0.30		
RSD	6.7			4.7			4.0		
% RE	−9.1			−1.7			1.4		

QCs were −16.5 and 3.5, −19.3 and 4.7, and −9.1 and 15.3% for freeze thaw stability, storage stability at room temperature, and dried extract stability at −20°C, respectively. These data were all outside of acceptance criteria either because of unacceptable REs or RSDs. The majority of the problem was probably be due to a combination of greater variability at the low end of the calibration range and the fact that the initial values for the low QC standards were slightly low relative to the nominal concentrations. Since all standards and quality control standards gave acceptable results over this range during validation poor assay performance at this concentration is unlikely. Comparison of these stability values to day 0 time points for each stability study rather than nominal values would have shown acceptable stability for βFlddA under all conditions. In the worst possible case, these data suggest that only one analysis can be done on a low concentration sample containing βFlddA. The possibility of poor freeze-thaw stability of βFlddA was not crucial for these studies since the method was applied to low volume rat samples, in which only sufficient sample was available for a single analysis.

3.6. Conclusions

The method presented has been successfully validated for analysis of several nucleosides in plasma and was applied to plasma samples from animal studies. Optimization of detection wavelengths, injection volume, and mobile phase may be necessary to obtain optimum sensitivity and resolution for other nucleoside analytes. Quantitation limits of ≈0.2 μg ml^{-1} can be obtained for most compounds. Because of the short half-life of some of these nucleosides in plasma, careful characterization of the precision and accuracy at the low end of the calibration curve is necessary in order to obtain quality data for reliable pharmacokinetic interpretation. The overall method is rugged in that it has been validated on at least four different HPLC instrumental systems with several different lots of the same column and at least five different analysts. The acceptable calibration ranges were as follows: βFlddA 0.2–10 μg ml^{-1}; βFddI 1–10 μg ml^{-1}; and 0.25–50 μg ml^{-1} for the remaining dideoxynucleosides.

Addition of pentostatin to all samples at the time of collection is critical to obtaining valid results. Method development data indicated that substantial loss of all parent analytes was observed during the sample extraction procedure in the absence of pentostatin. Analysis of structurally similar compounds may require addition of pentostatin for the same reason.

Preliminary results with spiked tissue homogenates have indicated that this method may be easily modified for analysis of nucleosides in tissues; however the instability of these nu-

Table 10
Rat βFlddI QC precision and accuracy

Nominal concentration (μg ml^{-1})	0.22			2.23			7.44		
Daily	1	2	3	1	2	3	1	2	3
N	6	6	6	6	6	6	6	6	6
Mean	0.17	0.17	0.14	2.04	2.15	2.19	7.17	7.34	7.54
s	0.02	0.02	0.03	0.17	0.12	0.05	0.22	0.30	0.26
RSD	14.3	10.9	23.2	8.4	5.4	2.3	3.0	4.1	3.4
% RE	−23.9	−23.7	−37.2	−8.6	−3.7	−1.7	−3.6	−1.4	1.4
Day to day									
N	17			18			18		
Mean	0.16			2.13			7.35		
s	0.03			0.13			0.29		
RSD	17.0			6.3			3.9		
% RE	−27.7			−4.7			−1.2		

Table 11
Acceptable calibration standard ranges based on validation data

Analyte	Nominal calibration standard range (μg ml^{-1})
βFlddA	0.10–50
βFlddI	1–10
6CldG	0.50–50
ddG	0.10–51
6CldI	0.25–50
ddI	0.25–50

cleosides would pose a substantial problem in measuring these compounds. Since there is no easy way to add pentostatin early, in the process, substantial loss of the nucleosides could occur between the time that the tissues are harvested and the time that pentostatin could be added.

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