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High-performance liquid chromatographic determination of (−)-β-D-2-aminopurine dioxolane and (−)-β-D-2-amino-6-chloropurine dioxolane, and their metabolite (−)-β-D-dioxolane guanine in monkey serum, urine and cerebrospinal fluid

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Abstract

(−)-β-D-2-Aminopurine dioxolane (APD), (−)-β-D-2-amino-6-chloropurine dioxolane (ACPD) and dioxolane guanine (DXG) are nucleoside analogues possessing potent activity against human immunodeficiency virus (HIV) and hepatitis B virus (HBV) in vitro. APD and ACPD are metabolized in vivo to yield DXG. Reversed-phase HPLC analytical methodologies were developed for the simultaneous determination of APD and DXG, and for ACPD and DXG in monkey serum, urine and cerebrospinal fluid (CSF). 2-Fluoro-2′,3′-dideoxyinosine (FDDI) served as the internal standard. The extraction recoveries of the nucleoside analogues from serum samples were similar, averaging approximately 90%. The limit of quantitation of the analytical method for serum samples was 0.1 μg/ml for DXG, and 0.25 μg/ml for APD and ACPD. The intra- and inter-day relative standard deviations for each compound at low, medium and high nucleoside concentrations were less than 9.0%. The accuracy of the assay methods was greater than 90% for prodrugs and parent compound. Similar results were observed with urine and CSF samples. Thus, these methods provide sensitive, accurate and reproducible determination of the prodrugs and parent nucleoside in biological samples.

Keywords: 2-Aminopurine dioxolane; 2-Amino-6-chloropurine dioxolane; Dioxolane guanine

1. Introduction

(−)-β-D-Dioxolane guanine (DXG) is a nucleoside analogue possessing potent activity against human immunodeficiency virus (HIV) and hepatitis B virus (HBV) in vitro [1,2]. The limited aqueous solubility of DXG renders the compound difficult to administer in vivo. (−)-β-D-2,6-Diaminopurine dioxolane (DAPD), a more water soluble nucleoside analogue also exhibiting potent antiviral activity [1,2] was previously studied in woodchucks [3] and monkeys [4] and found to have a favorable pharmacokinetic

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profile. These studies also demonstrated that DAPD is susceptible to deamination by adenosine deaminase yielding DXG. Two related compounds, (−)-β-D-2-aminopurine dioxolane (APD) and (−)-β-D-2-amino-6-chloropurine dioxolane (ACPD) which exhibit potent antiviral activity in vitro have recently been synthesized [1]. These derivatives are more water soluble than DAPD and have the potential to be metabolized in vivo to form DXG. Further, the chemical synthesis of these compounds in large scale is more readily accomplished than for DXG. The chemical structures of APD, ACPD and DXG are illustrated in Fig. 1.

Pharmacological development of these novel nucleoside analogues as potential antiviral agents necessitates preclinical pharmacokinetic and metabolism studies in relevant animal models, including monkeys. Of particular interest are the rate of formation of DXG from APD and ACPD, and the extent to which the compounds cross the blood brain barrier. Thus, as a prerequisite to preclinical pharmacokinetic investigations, sensitive and reproducible analytical methods were developed for the simultaneous determination of APD and DXG and for ACPD and DXG in monkey serum, urine and cerebrospinal fluid (CSF).

2. Experimental

2.1. Chemicals and reagents

APD, ACPD, DXG and 2-fluoro-2′,3′-dideoxyinosine (FDDI) were synthesized as previously described [1,5]. The chemical purity of the compounds, as determined by HPLC analysis, was greater than 99%. Acetonitrile, HPLC grade and all other chemicals, analytical grade were purchased from J.T. Baker (Philipsburg, NJ, USA).

2.2. Preparation of standards

Standard solutions of APD, ACPD and DXG were prepared in 3% acetonitrile in 30 m*M* potassium phosphate buffer, pH 5.0. Calibration plots for the nucleoside analogues in monkey serum were prepared by adding standard solutions to blank monkey serum yielding nucleoside concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 50 μg/ml for DXG and 0.25, 0.5, 1, 2.5, 5, 10 and 50 μg/ml for APD and ACPD. Standard curves for the analysis of urine samples were prepared in blank urine over the concentration range 1 to 50 μg/ml (1, 2.5, 5, 10, 25 and 50 μg/ml) for each compound, and those for

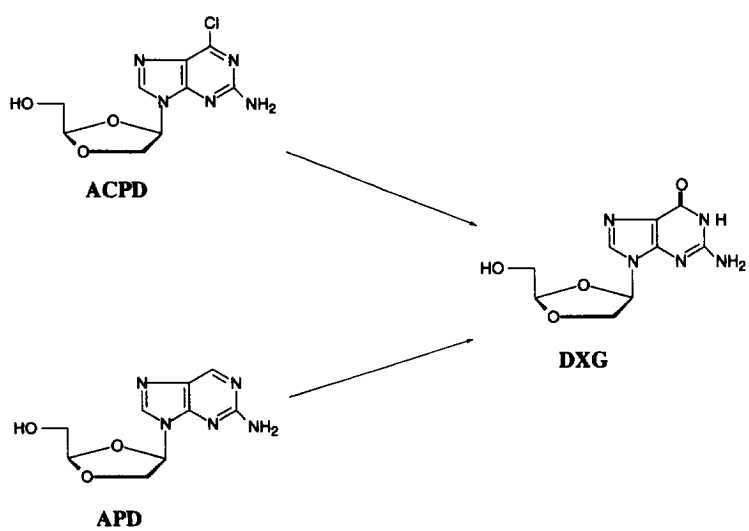

Fig. 1. Chemical structures of APD, ACPD and DXG.

CSF samples were prepared in water at concentrations of 0.5, 1, 2.5, 5, 10 and 50 μg/ml.

2.3. Extraction procedure

Serum samples (100 μl), 25 μl of FDDI (20 μg/ml) as internal standard and 25 μl of 2 *M* perchloric acid as a protein precipitant were added to propylene microcentrifuge tubes. The contents of the tubes were thoroughly mixed, and the tubes were centrifuged at 2000 *g* for 5 min. The supernatant was transferred to a clean tube and 80 μl saturated borax buffer solution (pH 6.7) for neutralizing the perchloric acid was added. The contents of tubes were thoroughly mixed, and the tubes were centrifuged at 2000 *g* for 5 min. 50 μl of the resulting supernatant was injected onto the HPLC column.

Urine samples were diluted 10- to 100-fold with distilled, deionized water. 20 μl of FDDI (20 μg/ml) as internal standard was added to 200 μl of the diluted urine sample, and 50 μl was injected onto the HPLC column. For quantitation of the compounds in CSF, 20 μl of FDDI (20 μg/ml) was added to 200 μl CSF sample and 50 μl was injected onto the HPLC column.

2.4. Chromatography

The HPLC system used for analysis of monkey serum samples consisted of Shimadzu LC-10A solvent delivery system, a Shimadzu SCL-10A system controller, a Shimadzu SIL-10A auto injector, a Shimadzu SPD-10A UV–VIS detector and a Shimadzu CR501 chromatopac integrator. The HPLC system used for urine and CSF analysis consisted of a Varian 2510 HPLC pump, a Varian 9090 autosampler, a Varian 2550 UV detector and a Varian 4290 integrator. Separation of APD and DXG in serum was performed using a Waters Bondapak C_{18} precolumn and an Alltech hypersil ODS column (4.6×250 mm; particle size, 5 μm). Separation of ACPD and DXG in serum, and separation of all the nucleoside analogues in urine and CSF was performed using a Waters Bondapak C_{18} precolumn and an Alltech hypersil ODS column (4.6×150 mm; particle size, 5 μm).

For quantitation of APD and DXG in monkey serum the mobile phase was comprised of 2% acetonitrile in 30 m*M* KH_2PO_4, pH 5.0 at a flow-rate of 1.5 ml/min. For the determination of ACPD and DXG concentrations in monkey serum, a mobile phase of 2% acetonitrile in 30 m*M* KH_2PO_4, pH 4.0 at a flow-rate of 2 ml/min was used. The UV wavelength for the detection of APD and ACPD was 300 nm and that for DXG and FDDI was 260 nm. A detector range setting of 0.005 absorbance units, full scale was used. For the quantitation of all compounds in urine and CSF, a mobile phase of 2% acetonitrile in 30 m*M* K_2HPO_4, pH 4.0, at a flow-rate of 1.5 ml/min was used. All compounds were detected at UV wavelength of 240 nm with a detector range setting of 0.005 absorbance units, full scale.

2.5. Quantitation

Concentrations of APD, ACPD and DXG in unknown samples were determined form the slopes of standard curves of the peak-area ratio (nucleoside/internal standard) versus standard nucleoside concentration. Standard curve slopes were generated by weighted ($1/x^2$) least-squares regression analysis. Use of this weighting factor yielded a normal distribution of weighted residuals over the entire range of nucleoside concentrations.

2.6. Assay specifications

The extraction recovery of APD, ACPD, DXG and FDDI was determined by comparing the peak area for six extracted monkey serum samples with that of six unextracted samples of the same amount of nucleoside prepared in mobile phase. Four concentration levels, 0.1 μg/ml (DXG) or 0.25 μg/ml (prodrugs), 0.5 μg/ml, 5 μg/ml and 50 μg/ml of the nucleoside analogues were investigated. The percentage extraction recovery was calculated from $100 \times \text{peak area}_{\text{extracted}} / \text{peak area}_{\text{unextracted}}$.

The intra-day accuracy and precision of the assay methodologies in serum were determined by assaying six samples per concentration level (0.1 μg/ml (DXG) or 0.25 μg/ml (prodrugs), 0.5 μg/ml, 5 μg/ml and 50 μg/ml). For the inter-day assay accuracy and precision, samples were analyzed on three separate days. Precision is reported as relative

standard deviations (R.S.D.) and accuracy was calculated by comparing measured nucleoside concentrations to the known values. Similar studies were performed in urine and CSF.

3. Results and discussion

Chromatograms of (A) blank monkey serum, (B) monkey serum with APD, DXG, and internal standard added, and (C) a monkey serum sample collected following oral administration of APD are illustrated in Fig. 2. The retention times of DXG, APD and FDDI were 11.6, 19.2 and 27.4 min, respectively. All peaks exhibited baseline separation, and no interfering peaks from endogenous substances were detected near the retention times of the APD, DXG and FDDI. Chromatograms of (A) blank monkey serum, (B) monkey serum spiked with ACPD, DXG and FDDI, and (C) a serum sample collected after oral administration of ACPD are depicted in Fig. 3. There were no interfering peaks from endogenous compounds. The retention times of DXG, FDDI and ACPD were 6.3, 15.7 and 30.5 min, respectively. To achieve complete separation of prodrugs from endogenous compounds, slightly different mobile phase conditions were required for the APD and ACPD analytical methods. The mobile phase for APD (pH 4.0) was less basic than that for ACPD (pH 5.0). In addition, a longer column and slower mobile phase flow-rate for APD (1.5 ml/min) was used compared to that for ACPD (2.0 ml/min). Thus, the retention times for DXG and FDDI were longer for the APD assay methodology than those for the ACPD method.

The analytical methodologies were linear over the concentration range of 0.25 to 50 μg/ml for the prodrugs APD [$y=0.15\ (0.0013)x-0.0047\ (0.0098)$, $r=0.998$] and ACPD [$y=0.13\ (0.0034)x-0.00059\ (0.00085)$, $r=0.998$]. For both methods, the range of linearity for DXG was 0.1 to 50 μg/ml [$y=0.30(0.0011)x+0.0032\ (0.0015)$, $r=0.999$]. The limit of quantitation for the methods was 0.25 μg/ml for the prodrugs and 0.1 μg/ml for DXG. The assay specifications including extraction recovery, assay precision and accuracy are presented in Table 1. The

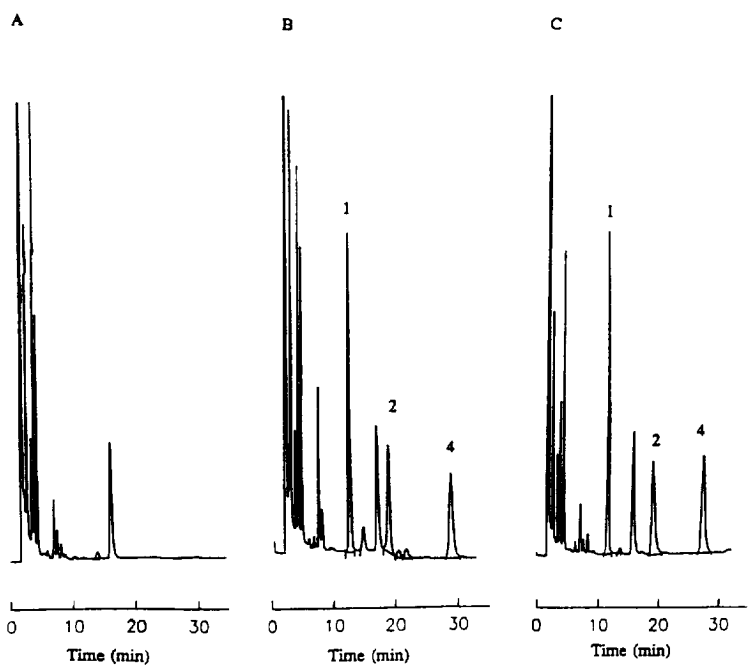

Fig. 2. Chromatograms of (A) blank monkey serum, (B) monkey serum with (1) 5.0 μg/ml DXG, (2) 5.0 μg/ml APD and (4) internal standard added, and (C) a monkey serum sample collected 1.5 h (5.8 μg/ml DXG and 0.5 μg/ml APD) after oral administration of 33.3 mg/kg APD.

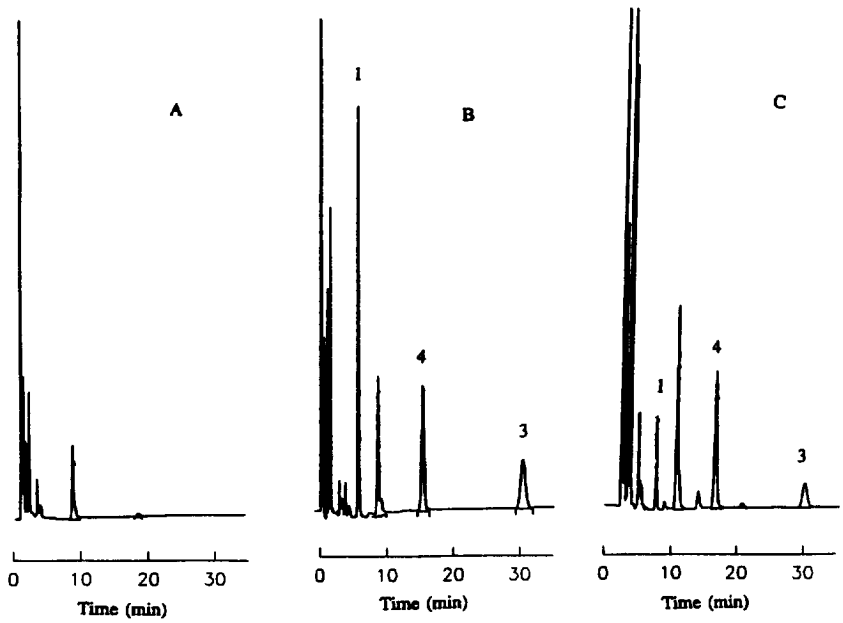

Fig. 3. Chromatograms of (A) blank monkey serum, (B) monkey serum spiked with (1) 5.0 μg/ml DXG, (3) 5.0 μg/ml ACPD and (4) FDDI, and (C) a serum sample collected 0.25 h (1.1 μg/ml DXG and 2.7 μg/ml ACPD) after oral administration of 33.3 mg/kg ACPD.

Table 1
Assay specifications for the determination of APD and DXG and for ACPD and DXG in monkey serum

Compound	Concentration (μg/ml)	Recovery[a] (%)	Precision		Accuracy	
			Intra-day	Inter-day	Intra-day	Inter-day
APD and DXG						
APD	0.25	91.0±4.7	9.0	8.3	97.5	98.7
	0.5	93.3±4.5	8.5	4.0	97.8	98.6
	5.0	94.3±5.0	6.7	5.0	99.4	99.7
	50.0	91.3±4.6	2.8	3.8	99.3	98.3
DXG	0.1	92.3±3.0	3.2	6.2	92.0	95.2
	0.5	90.5±3.2	3.0	5.1	91.7	94.7
	5.0	86.9±2.4	2.1	3.5	99.1	98.8
	50.0	87.6±4.3	2.4	4.1	90.1	91.6
ACPD and DXG						
ACPD	0.25	87.0±3.8	3.8	4.5	92.8	93.5
	0.5	86.0±2.6	3.3	2.6	90.9	92.9
	5.0	86.9±3.8	1.7	3.8	98.6	99.4
	50.0	86.5±4.1	2.2	4.1	95.4	97.4
DXG	0.1	95.0±2.6	7.5	2.8	92.5	94.2
	0.5	99.4±2.3	7.7	2.6	92.0	93.7
	5.0	86.7±3.0	7.5	3.0	97.6	99.3
	50.0	91.1±3.7	1.5	7.6	90.0	94.0

[a] Mean±S.D..

extraction recoveries of the nucleoside analogues, including internal standard FDDI (92.2±2.1%), were similar averaging approximately 90%. The intra- and inter-day precision and accuracy of the assay methodologies was satisfactory. The intra- and inter-day relative standard deviations for each compound at low, medium and high nucleoside concentrations were less than 9.0%. The accuracy of both assay methods was greater than 90% for prodrugs and parent compound (Table 1).

In contrast to the methods for serum samples, a single analytical method was suitable for the determination of APD, ACPD and DXG in urine and CSF samples. Chromatograms of blank monkey urine and urine samples collected after oral administration of APD and ACPD are shown in Fig. 4. There were no interfering peaks from endogenous compounds (Fig. 4A). The retention times of DXG, APD and FDDI were 8.0, 12.7 and 19.6 min, respectively (Fig. 4B). The retention times of DXG, FDDI and ACPD were 8.0, 19.6 and 37.8 min, respectively (Fig. 4C). The assay methodology for the compounds in urine samples were linear over the concentration range of 1.0 to 50 μg/ml for the prodrugs APD [$y=0.19(0.0021)x-0.011(0.016)$, $r=0.999$] and ACPD [$y=0.18(0.0019)x-0.040$ (0.033), $r=0.998$] and 1.0 to 50 μg/ml for DXG [$y=0.43(0.0071)x+0.0015(0.013)$, $r=0.999$]. The limit of quantitation for the analytical method was 1.0 μg/ml for the prodrugs and 1.0 μg/ml for DXG. The assay specifications for the quantitation of prodrugs and parent compound are shown in Table 2. The intra- and inter-day precision and accuracy of the assay methodology was satisfactory. The intra-

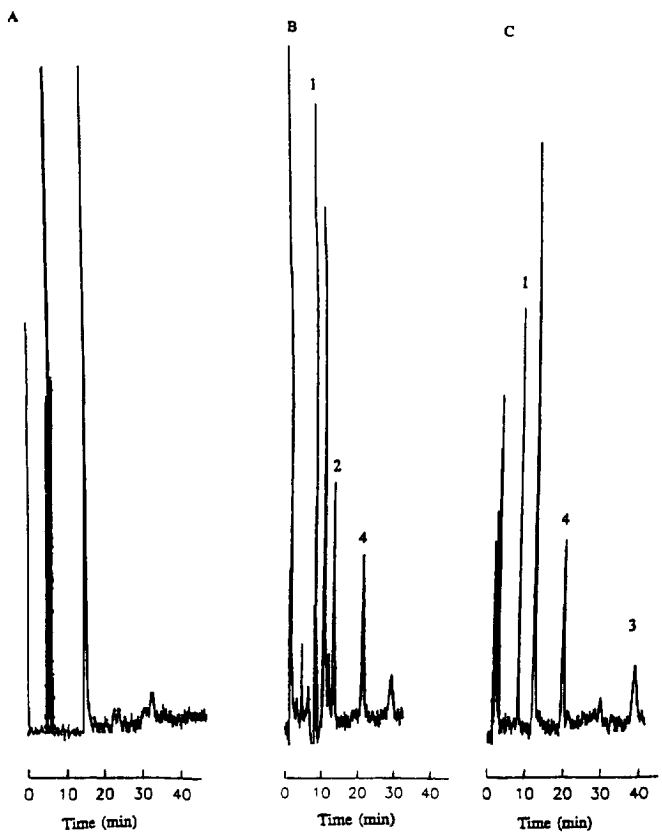

Fig. 4. Chromatograms of (A) blank monkey urine and urine samples collected after oral administration of 33.3 mg/kg (B) APD (4.0 μg/ml DXG and 6.8 μg/ml APD) and (C) ACPD (2.5 μg/ml DXG and 2.9 μg/ml ACPD). Numbers represent (1) DXG, (2) APD, (3) ACPD and (4) internal standard, FDDI.

Table 2
Assay specifications for the determination of APD, ACPD and DXG in monkey urine

Compound	Concentration (μg/ml)	Precision		Accuracy	
		Intra-day	Inter-day	Intra-day	Inter-day
APD	1.0	5.3	5.1	90.8	94.0
	5.0	5.6	6.4	99.1	94.9
	50.0	6.1	8.9	95.3	97.9
ACPD	0.1	5.5	6.5	92.5	93.8
	5.0	7.7	8.0	91.9	96.2
	50.0	3.6	3.7	98.1	99.6
DXG	1.0	8.9	9.0	94.3	97.8
	5.0	6.1	6.8	97.4	95.3
	50.0	5.3	4.4	96.4	95.4

and inter-day relative standard deviations for each compound at low, medium and high nucleoside concentrations were less than 9.0%. The accuracy of the analytical method was greater than 90% for prodrug and parent compound. Further studies showed virtually identical results using water, rather than blank urine, for standard curve preparation.

A single analytical method was also suitable for

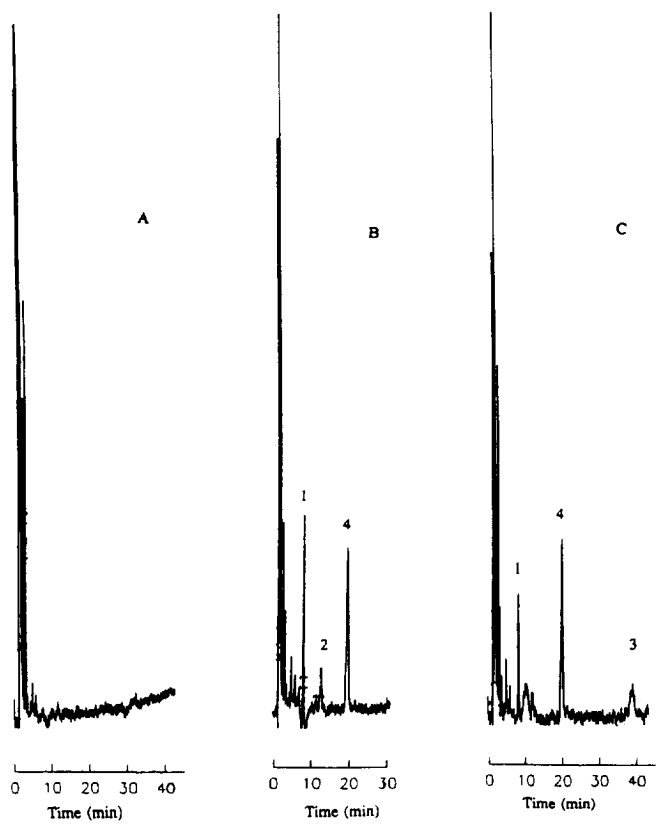

Fig. 5. Chromatograms of (A) blank monkey CSF and CSF samples collected 1 h after oral administration of 33.3 mg/kg (B) APD (0.7 μg/ml DXG and 0.6 μg/ml APD) and (C) ACPD (0.5 μg/ml DXG and 1.0 μg/ml ACPD). Numbers represent (1) DXG, (2) APD, (3) ACPD and (4) internal standard, FDDI.

Table 3
Assay specifications for the determination of APD, ACPD and DXG in monkey cerebrospinal fluid

Compound	Concentration (μg/ml)	Precision		Accuracy	
		Intra-day	Inter-day	Intra-day	Inter-day
APD	0.5	3.5	4.0	95.6	94.5
	5.0	5.0	6.8	98.0	97.5
	50.0	2.0	3.5	93.8	95.0
ACPD	0.5	2.4	2.8	98.0	99.2
	5.0	2.9	4.8	95.3	92.5
	50.0	6.0	6.2	97.5	95.4
DXG	0.5	5.0	5.2	99.8	97.9
	5.0	2.5	3.6	98.5	95.4
	50.0	4.1	5.0	94.3	93.5

the determination of APD, ACPD and DXG in CSF samples. Chromatograms of blank monkey CSF and CSF samples collected after oral administration of APD and ACPD are shown in Fig. 5. There were no interfering peaks from endogenous compounds (Fig. 5A). The retention times of DXG, APD and FDDI were 8.0, 12.7 and 19.6 min, respectively (Fig. 5B). The retention times of DXG, FDDI and ACPD were 8.0, 19.6 and 37.8 min, respectively (Fig. 5C). The assay methodology for the compounds in CSF samples was linear over the concentration range of 0.5 to 50 μg/ml for each compound. The assay specifications for the quantitation of prodrugs and parent compound are shown in Tables 2 and 3. The intra- and inter-day precision and accuracy of the assay methodology were satisfactory. The intra- and inter-day relative standard deviations for each compound at low, medium and high nucleoside concentrations were less than 7.0%. The accuracy of the analytical method was greater than 92% for prodrug and parent compound.

HPLC analytical methodologies for the determination of APD, ACPD, and DXG in monkey serum, urine and CSF are described in this report. These methods provide sensitive, accurate and reproducible determination of the prodrugs and parent nucleoside in biological samples. Further, the analytical methods are suitable for characterizing the pharmacokinetics of the nucleoside analogues and the bioconversion of prodrugs to parent compound. The pharmacokinetics of APD, ACPD, and DXG in monkeys utilizing these assay methodologies are reported elsewhere [6].

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