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High-performance liquid chromatographic determination of (−)-β-D-2,6-diaminopurine dioxolane and its metabolite, dioxolane guanosine, using ultraviolet and on-line radiochemical detection

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Abstract

(−)-β-D-2,6-Diaminopurine dioxolane (DAPD) and its metabolite dioxolane guanosine (DXG) have potent activity against hepatitis B virus and HIV, in vitro. A reversed-phase HPLC analytical method using UV and on-line radiochemical detection for the determination of DAPD and DXG in monkey serum and urine is described in this report. Retention times for DXG, DAPD and internal standard (2′,3′-didehydro-2′deoxythymidine, D4T) were 5.0, 6.0 and 13.0 min, respectively. The extraction recovery was greater than 97% for DAPD and 94% for DXG. The limit of quantitation for UV detection was 100 ng/ml and 125 ng/ml for DXG and DAPD in monkey serum. The standard curves were linear from 0.1 μg/ml to 5 μg/ml for DXG and 0.125 μg/ml to 5 μg/ml for DAPD. For radiochemical detection, calibration curves of standard solutions of DAPD and DXG were linear in the range of 3500 Bq to 32 000 Bq and 7500 Bq to 60 000 Bq. The intra- and inter-day relative standard deviations were less than 7.2% using UV and less than 8.6% using on-line radiochemical detection. The HPLC method was applied to serum and urine samples collected from a male rhesus monkey that was administered 33.3 mg/kg DAPD with 200 μCi of [^{3}H]DAPD intravenously.

1. Introduction

(−)-β-D-2,6-Diaminopurine dioxolane (DAPD) is a newly synthesized nucleoside analogue with potent activity against hepatitis B virus and human immunodeficiency virus Type I (HIV-1) in vitro [1,2]. DAPD is susceptible to deamination by adenosine deaminase and the deaminated metabolite, dioxolane guanosine (DXG), also has potent antiviral activity in vitro [1,2]. The chemical structures of DAPD and DXG are illustrated in Fig. 1. Characterization of the pharmacokinetics of these antiviral nucleosides in preclinical studies is an integral part of drug development. Thus, the development of an analytical methodology for the quantitation of

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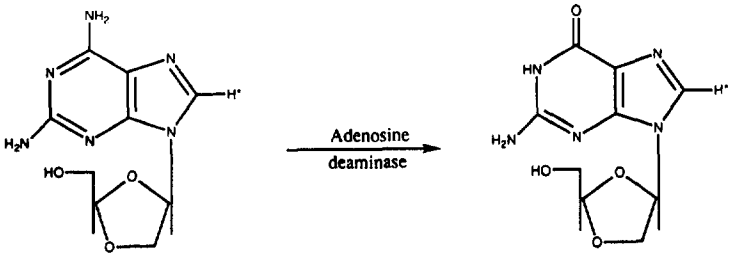

DAPD DXG

Fig. 1. Chemical structures of DAPD and DXG.

DAPD and DXG in biological matrices is essential for conducting preclinical investigations.

High-performance liquid chromatography (HPLC) has been shown to be an efficient technique for quantitating nucleoside analogues in biological samples [3–7]. Typically, ultraviolet detection is employed for the quantitation of nucleosides. However, preclinical pharmacokinetic and drug metabolism studies are also often performed using radiolabelled compounds. Usually effluent fractions from the HPLC are collected using a fraction collector and sample radioactivity is counted in a liquid scintillation counter. This method is time-consuming, tedious and expensive when a large number of samples are to be analyzed. On-line radiochemical detection provides a specific and convenient analytical method for quantitating radiolabelled compounds.

Presently, no analytical method is available for the determination of DAPD and DXG in biological fluids. The objective of this study was to develop an HPLC analytical method for the determination of DAPD and DXG in monkey serum and urine samples using UV absorption and on-line radiochemical detection.

2. Experimental

2.1. Chemicals and reagents

(−)-β-D-2,6-Diaminopurine dioxolane (DAPD, molecular mass 252) and dioxolane guanosine (DXG, molecular mass 253) were synthesized as previously described [1]. 2′,3′-Didehydro-2′-deoxythymidine (D4T), used as an internal standard, was provided by the Developmental Therapeutic Branch, AIDS Program, National Institutes of Health (Rockville, MD, USA). The chemical purity of the nucleosides, as determined by spectral and HPLC analysis, was greater than 98%. Radiolabelled DAPD and DXG were synthesized by Moravek Biochemicals (Brea, CA, USA). Both nucleosides were radiolabelled at the 8-H position of the purine base yielding specific activities of 12 Ci/mmol (Fig. 1). The radiochemical purity of the compounds was 98.8%. HPLC grade mobile-phase solvents and other chemicals (analytical grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). FLO-SCINT III liquid scintillation cocktail (Packard Instrument, Meriden, CT, USA) was used for on-line radiochemical detection.

2.2. Preparation of standards

Standard solutions of DAPD and DXG were prepared in 4% acetonitrile in 50 m*M* phosphate buffer and stored at −20°C. Preliminary studies demonstrated that the compounds were stable at −20°C for at least one month. The standard curve concentrations of DAPD and DXG in monkey serum ranged from 0.125 to 5 μg/ml and from 0.1 to 5 μg/ml, respectively. Standard curves for the analysis of urine samples were prepared in water over the concentration range 1–50 μg/ml for both DAPD and DXG.

2.3. Extraction procedure

Serum samples with expected nucleoside concentrations greater than 5 μg/ml were diluted with blank monkey serum to fall within the concentration range of the standards. Serum samples (50 μl) were placed in 1 ml polypropylene micro-centrifuge tubes followed by 50 μl of internal standard and 75 μl of 2 *M* perchloric acid. Tubes were vigorously mixed for 30 s and centrifuged at 9000 *g* for 10 min. To each tube, 75 μl of 2 *M* potassium hydroxide was added and, after mixing, centrifuged for 10 min at 9000 *g*. One hundred microliters (100 μl) of the clear supernatant were injected onto the HPLC column.

Urine samples were diluted 10- to 200-fold with water. To 50 μl of the diluted urine sample, 50 μl of internal standard was added and 25 μl was injected onto the HPLC column.

2.4. Chromatography

The HPLC system consisted of a Waters Model 510 solvent delivery system, a Model 712 WISP autosampler, a Model 484 tunable wavelength UV absorbance detector and Model 746 data module (Waters Associates, Milford, MA, USA). A Model A-525 on-line radiochemical detector (Packard Instrument, Meriden, CT, USA) was used for determining radioactivity. Chromatograms obtained from the radiochemical detector were analyzed by a PC-based software provided with the instrument. A 25 cm × 0.45 cm C_{18} column packed with 5-μm particles (Jones Chromatography, Lakewood, CO, USA) with a Nova-Pak C_{18} guard column (Waters) was used for the separation of the compounds. Chromatography was performed at ambient temperature.

2.5. Ultraviolet detection

The mobile phase used for detection of the nucleosides by UV absorbance consisted 4% acetonitrile in 50 m*M* phosphate buffer adjusted to pH 2.5 with phosphoric acid (mobile phase A). The flow-rate of the mobile phase was 1.5 ml/min. The wavelength for detection of nucleosides was 272 nm and the detector range was set at 0.005 a.u.f.s.

2.6. Radiochemical detection

The mobile phase for the quantitation of DAPD and DXG by on-line radiochemical detection consisted of 4% acetonitrile in 50 m*M* phosphate buffer, pH 4.5 (mobile phase B). The mobile phase flow-rate was 1.5 ml/min. The liquid scintillation cocktail was pumped at 3 ml/min and was mixed continuously with the HPLC eluate. The mixture was passed through a 500-μl detector flow cell. The lower level and upper level discriminator energies were set at 0.00 and 18.5 keV, respectively. The detector update time was 6 s. Background radioactivity was subtracted from all radio-chromatograms.

2.7. Quantitation

Concentrations of DAPD and DXG in unknown samples using UV detection were determined from the slopes of standard curves of the peak-area ratio (drug/internal standard) versus standard drug concentration. Slopes were determined using linear regression analysis with a weighting factor of $1/x^2$. Use of this weighing factor generated a normal distribution of weighted residuals around the fitted standard curve over the entire range of drug concentrations. Radio-chromatograms were integrated and the dpm under each peak was obtained. From the specific activity and the dilution factor, the original concentrations of radioactive nucleosides were calculated. The total nucleoside concentrations were calculated from the ratio of the radiolabelled to unlabelled drug administered.

2.8. Assay specifications

The extraction recovery of DAPD, DXG and internal standard was determined at concentrations of 0.2 μg/ml and 5 μg/ml. The peak area of the analyte from six extracted serum samples and six direct injections of the same amount of drug in mobile phase was compared to obtain

extraction recoveries. The percentage recovery was calculated from $100 \times$ peak $area_{extract}$/mean peak $area_{direct\ injection}$.

The intra-day and inter-day relative standard deviations (R.S.D.) were assessed for both compounds. The intra-day R.S.D. was determined by assaying six samples at three different drug concentrations. For the inter-day precision, drug concentrations encompassing the entire range of standards were analyzed on three separate days.

2.9. Animal studies

A male rhesus monkey was administered 33.3 mg/kg DAPD with 200 μCi of [^{3}H]DAPD intravenously. Blood and urine samples were collected at selected times following DAPD administration. Serum was obtained by centrifugation of blood samples. Serum and urine samples were stored at −20°C until analysis.

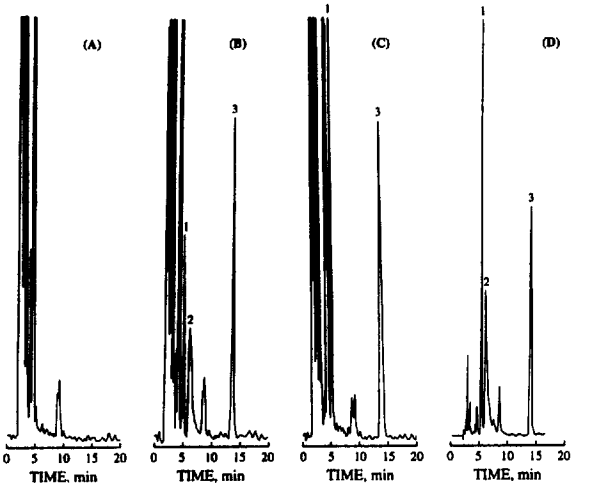

Fig. 2. Typical chromatograms for DXG (1), DAPD (2) and internal standard (3) in (A) blank monkey serum, (B) monkey serum with DXG and DAPD added, (C) a monkey serum sample taken after intravenous administration of DAPD, and (D) a monkey urine sample after administration of DAPD using UV detection.

3. Results and discussion

Chromatograms obtained using UV detection corresponding to the extracts of (a) blank monkey serum, (b) blank serum spiked with DAPD and DXG, and (c) a serum sample collected 0.25 h after an intravenous bolus administration of 33.3 mg/kg of DAPD are depicted in Fig. 2. The retention times of DXG, DAPD and D4T were 5.0, 6.0 and 13.0 min, respectively. There were no interfering peaks from endogenous compounds. An endogenous compound peak did elute at 23 min which increased the sample run time. No interfering peaks from endogenous compounds were visible in urine samples.

The extraction recoveries of DAPD and DXG were greater than 97% and 94%, respectively. The limit of quantitation of the assay using UV detection was 125 ng/ml for DAPD and 100 ng/ml for DXG when a signal to noise ratio of 3 was used as the criterion for a significant response. Standard curves were linear over the range 0.125–5 μg/ml for DAPD and 0.10–5 μg/ml for DXG. The intra- and inter-day precision of the assay was satisfactory. As shown in Table 1, relative standard deviations were less than 7.2%.

A serum concentration versus time profile of DXG after intravenous administration of DAPD obtained using UV detection is given in Fig. 3. After intravenous administration of DAPD, the parent compound was not detected in any of the serum samples. However, the metabolite DXG was found. The maximum serum concentration of DXG was 12.9 μg/ml. From the specific activity and the ratio of ^{3}H-labelled to unlabelled drug administered, the activity of ^{3}H-labelled drug corresponding to 12.9 μg/ml would be 0.59 Bq/ml. The 50 μl of serum sample used for the analysis corresponded to 0.0295 Bq of radioactivity and the 5-fold dilution during sample processing resulted in extremely low quantities of radioactivity. Therefore, it was not possible to analyze the serum samples by radiochemical detection.

A typical chromatogram of a urine sample collected after administration of DAPD using mobile phase A with UV detection is shown in Fig. 2D. A chromatogram obtained using mobile phase B with radiochemical detection corre-

Table 1
Assay precision (UV detection)

Compound	Serum			Urine		
	Concentration (μg/ml)	R.S.D. (%)		Concentration (μg/ml)	R.S.D. (%)	
		Intra-day	Inter-day		Intra-day	Inter-day
DAPD	0.125	3.50	1.69	1.00	3.17	3.80
	0.25	2.07	4.25	5.00	2.70	2.28
	5.00	1.55	2.41	25.00	1.34	5.11
DXG	0.10	5.11	7.17	1.00	6.00	4.87
	2.50	5.75	3.87	5.00	0.37	3.29
	5.00	1.70	1.98	25.00	0.73	6.60

sponding to the same urine sample is illustrated in Fig. 4. DAPD and DXG eluted with sharp peaks. In the case of UV detection, the mobile phase pH was lowered to move interfering peaks from endogenous compounds away from the region of interest. A lower pH also resulted in quicker elution of DAPD and DXG. However, this mobile phase was not suitable for radiochemical detection. A large detector cell volume of 500 μl, commonly used in radiochemical detection, and therefore a longer residence time in the detector cell when compared to UV detection lead to poor resolution of peaks corresponding to DAPD and DXG. Baseline resolution of the two peaks was achieved when phosphate buffer of pH 4.5 was used in preparing the mobile phase.

The retention times of DXG and DAPD under the conditions used for radiochemical detection were 6.0 and 8.0 min, respectively. The calibration curves of standard solutions of DAPD and DXG in mobile phase were found to be linear in the ranges 3500–32000 Bq and 7500–60000 Bq, respectively. Assay precision using

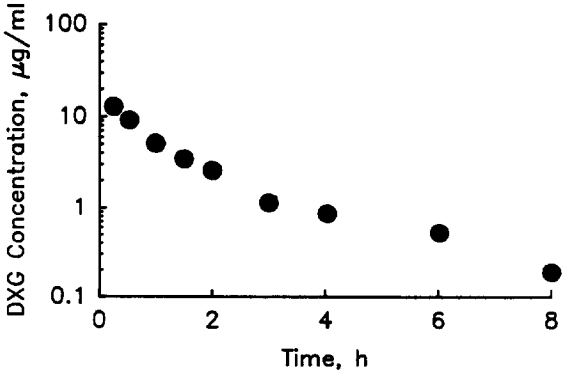

Fig. 3. Serum concentration versus time profile of DXG after intravenous administration of 33.3 mg/kg DAPD to a rhesus monkey.

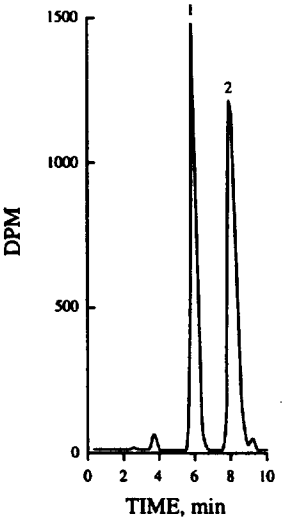

Fig. 4. Typical chromatogram for DXG (1) and DAPD (2) in a monkey urine sample using radiochemical detection.

Table 2
Assay precision (radiochemical detection)

Compound	Mean (Bq)	*n*	C.V. (%)
DAPD	2536	4	8.61
	34713	3	2.33
DXG	5495	4	4.44
	61101	3	1.80

radiochemical detection was performed at two different activities and the relative standard deviation was less than 8.6% (Table 2).

Concentrations of DAPD and DXG in urine samples were high enough to be analyzed by both UV and radiochemical detection. Fig. 5 shows the cumulative amount of the nucleosides in urine samples obtained at various time points after administration of DAPD. Radiochemical detection and UV detection yielded virtually identical results. Differences, if any, in measured concentration values between the two detection methods were smaller than the relative standard deviations of the assay.

By virtue of its clean baseline, it was possible to detect small quantities of unknown metabolites in urine samples using radiochemical detection. Another advantage in using radiochemical detection was increased sensitivity. Due to interfering peaks it was necessary to dilute the urine samples at least 100-fold to determine concentrations of DAPD and DXG using UV detection. In the analysis of urine samples obtained after administration of lower doses a 100-fold dilution could result in nucleoside concentrations below the quantitation limit of the analytical method. Radiochemical detection offers a marked advantage over UV detection in terms of an extremely clean background. When using on-line radiochemical detection, urine samples were diluted only 10-fold. This increased the sensitivity of the analytical method and yielded more reliable data. Furthermore, it is also possible to inject urine samples without dilution, thus increasing sensitivity.

The HPLC method reported here is rapid, reproducible and suitable for the analysis of DAPD and DXG in biological samples. Additional details on the pharmacokinetics of these antiviral agents will be published elsewhere [7].

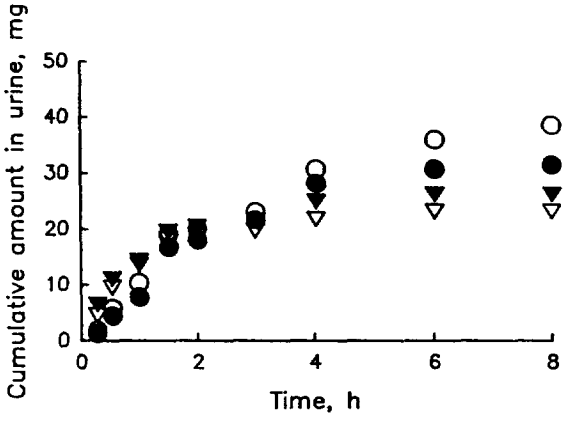

Fig. 5. Cumulative amount of DXG (circles) and DAPD (triangles) in urine after intravenous administration of 33.3 mg/kg DAPD using UV detection (open symbols) and radiochemical detection (closed symbols).

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References

[1] H.O. Kim, R.F. Schinazi, S. Nampalli, K. Shanmuganathan, D.L. Cannon, A.J. Alves, L.S. Jeong, J.W. Beach and C.K. Chu, J. Med. Chem., 36 (1993) 30.
[2] R.F. Schinazi, H.M. McClure, F.D. Boudinot, Y. Jxiang and C.K. Chu, Antiviral Res., 23 (1994) 81.
[3] T. Grune and W.G. Siems, J. Chromatogr., 618 (1993) 15.
[4] N. Frijus Plessen, H.C. Michaelis, H. Forh and G.F. Kahl, J. Chromatogr., 534 (1990) 101.
[5] T. Grune, W.G. Seims, G. Gerber and R. Uhlig, J. Chromatogr., 553 (1991) 193.
[6] T. Grune, W. Seims, G. Gerber, Y.V. Tikhonov, A.M. Pimenov and R.T. Toguzov, J. Chromatogr., 563 (1991) 53.
[7] R.P. Remmel, Y.H. Yeom, M. Hua, R. Vince and C.L. Zimmerman, J. Chromatogr., 489 (1989) 323.
[8] P. Rajagopalan, F.D. Boudinot, H.M. McClure and R.F. Schinazi, Pharm. Res., 11 (Suppl.) (1994) S-381.