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Report

A Radioimmunoassay Procedure for the Determination of the Antiviral Nucleoside DHPG (9-[(1,3-Dihydroxy-2-propoxy)-methyl]guanine) in Plasma or Serum

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A procedure is described that is suitable for the radioimmunoassay (RIA) of 9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine (DHPG) in plasma or serum at concentrations as low as 0.7 ng/ml (2.75×10^{-9} *M*). Antiserum was prepared by coupling DHPG monohemisuccinate to bovine serum albumin and immunizing rabbits with the resulting conjugate. The antibodies did not show significant cross-reactivities with structurally related endogenous compounds. For RIA, tritium-labeled DHPG was used as the tracer and charcoal–dextran was used to separate the free and bound fractions. No purification of samples was required prior to RIA. The accuracy of the method was assessed by adding known quantities of DHPG to DHPG-free plasma and determining the ratio of measured to added analyte. Linear regression analysis for the concentration range 0.0007 to 15.0 μg/ml yielded the following equation; $y = 0.90\,x + 0.033$ ($r = 0.999$). Additional validation was obtained from studies in which DHPG was administered to a monkey, mice, dogs, and rats, and plasma-clearance profiles were determined by RIA and high-performance liquid chromatography (HPLC). The results obtained by RIA were in good agreement with those obtained by HPLC.

KEY WORDS: DHPG; antiviral nucleoside; RIA.

INTRODUCTION

The nucleoside 9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine (DHPG; Fig. 1) is a new antiviral compound that has been found to be active against herpes simplex 1 and 2, cytomegalovirus, and Eppstein–Barr virus (1–3). It is currently being evaluated for several possible clinical applications. The radioimmunoassay procedure described here was developed for use in pharmacokinetic and toxicity studies and for the evaluation of various formulations of DHPG.

MATERIALS AND METHODS

Materials and Equipment. The following compounds were supplied or synthesized by the Institute of Organic Chemistry, Syntex Research: DHPG, DHPG monohemisuccinate, DHPG monophosphate, 2′-deoxyguanosine, guanine, guanosine, thioguanine, thioguanosine, adenosine, 2′-deoxyadenosine, Ara-A, acyclovir, ^{3}H-DHPG, and ^{3}H-DHPG monohemisuccinate.

The sources of the following materials are given in parentheses. Bovine serum albumin, tris-(hydroxymethyl)aminomethane, and EDTA disodium salt (Sigma Chemical Co., St. Louis, Mo.); 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (Pierce Chemicals, Rockford, Il.); sodium azide (J. T. Baker Chemical Co., Phillipsburg, N.J.); oxyfluor liquid scintillation fluid (New England Nuclear, Boston); Norit A charcoal (Matheson, Coleman and Bell, Norwood, Ohio); Spectrapor 2 membrane dialysis tubing (Spectrum Medical Industries, Los Angeles); gelatin (Knox, Englewood Cliffs, N.J.); dextran T-70 (Pharmacia, Inc., Piscataway, N.J.); and Freund's

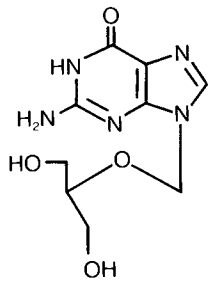

Fig. 1. Structure of DHPG.

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complete and incomplete adjuvant (Difco Laboratories, Detroit, Mich.).

The manufacturers or suppliers of the following equipment are given in parentheses. A Model DPR-6000 refrigerated centrifuge with swing-out head (IEC/Damon, Needham, Mass.), a Model 3330 liquid scintillation counter (Packard Instruments, Downers Grove, Ill.), a Model 559 spectrophotometer (Perkin–Elmer, Norwalk, Conn.), and a Model 2600 multitube vortex mixer (Scientific Manufacturing Industries, Emeryville, Calif.).

Synthesis of DHPG Monohemisuccinate. A mixture of DHPG (7.6 g, 30 mmol), *p*-anisylchlorodiphenylmethane (22.3 g, 72 mmol), triethylamine (13.7 ml, 98 mmol), and 4-dimethylaminopyridine (0.08 g) in dimethylformamide (100 ml) was magnetically stirred for 4 hr at 40°C, quenched with methanol (10 ml), and then evaporated to dryness. The residue was taken up in ethyl acetate, washed with saturated aqueous sodium bicarbonate and then water, dried over sodium sulfate, and evaporated to dryness. The resulting yellow foam was chromatographed over silica gel (1:19 methanol/dichloromethane) and the product recrystallized from ethanol to give 12.1 g (49%) of N^2-(*p*-anisyldiphenylmethyl)-9-[(1-(*p*-anisyldiphenylmethoxy)-3-hydroxy-2-propoxy)methyl]guanine (I): mp 159–160°C; λ_{max} (methanol) 260 nm (ε 12,000), 279 nm (ε 13,000). *Anal.* Calcd. for $C_{49}H_{45}N_5O_6 \cdot 0.5\ H_2O$ (808.94): C, 72.75; H, 5.73; N, 8.66. Found: C, 72.48; H, 5.56; N, 8.55.

A solution of I (600 mg, 0.73 mmol), succinic anhydride (163 mg, 1.6 mmol), and 4-dimethylaminopyridine (10 mg) in dimethylformamide (6 ml) was kept at room temperature for 4 days and then evaporated to dryness. The residue was dissolved in 80% aqueous acetic acid (20 ml), and the resulting solution heated at 80°C for 3 hr and then evaporated to dryness. The residue was recrystallized from ethanol to give 181 mg (70%) of 9-[(1-hydroxy-3-succinyloxy-2-propoxy)methyl] guanine (DHPG monohemisuccinate): mp 191–192°C; λ_{max} (methanol) 270 nm (ε 10,100), 254 nm (ε 13,900); NMR (300 MHz, Me_2SO-d_6) 7.80 (s, 1H, H-8), 6.50 (s, broad, 2H, NH_2), 5.43 (s, 2H, H-1′), 4.12, 3.94 (ABX, $J_{A,B}$ = 11 Hz, $J_{A,X}$ = 3 Hz, $J_{B,X}$ = 6 Hz, 2 H, CO_2CH_2), 3.41, 3.35 (ABX, $J_{A,B}$ = 11 Hz, $J_{A,X}$ = $J_{B,X}$ = 5 Hz, 2 H, $HOCH_2$). *Anal.* Calcd. for $C_{13}H_{17}N_5O_6$ (339.31): C, 46.02; H, 5.05; N, 20.64. Found: C, 45.98; H, 5.06; N, 20.51.

Production of Antiserum. The DHPG hapten–protein conjugate was synthesized by a water-soluble carbodiimide method (4). Since DHPG lacks a free carboxyl group, DHPG monohemisuccinate was employed for the conjugation reaction. The coupling reaction was carried out by dissolving 24.7 mg of the DHPG monohemisuccinate in a mixture of 0.2 ml of dimethylformamide (DMF) and 0.5 ml of water. Tritiated DHPG monohemisuccinate (sp act, 16 Ci/mmol) was also added at this stage. This solution was added to 59.1 mg of bovine serum albumin (BSA) dissolved in 1.0 ml of water. The 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (100.0 mg) was dissolved in a mixture of 0.3 ml of DMF and 0.5 ml of water and added dropwise (over a 5-min period) with stirring to the BSA solution. The mixture was stirred for an additional 5 min, then the pH was adjusted to 7.4 with 0.5 *M* phosphate buffer, and stirring was continued overnight at room temperature. The entire reaction mixture was transferred to a dialysis bag and dialyzed exhaustively against saline (9.0 g/liter). After dialysis was complete, the entire volume (6.3 ml) was subdivided into 0.2-ml aliquots (2.0 mg protein) and frozen.

Six New Zealand white rabbits were immunized with the hapten–protein conjugate. The immunization emulsion was prepared by dissolving 2.0 mg of the hapten–protein conjugate in 7.0 ml of saline (9.0 g/liter) and emulsifying in 8.0 ml of Freund's adjuvant. Freund's complete adjuvant was used for the initial sensitization of the animals; Freund's incomplete adjuvant was used for subsequent booster shots. Typically, 2.0 ml of this emulsion was injected subcutaneously into each animal at four different sites, one in each hind thigh (inside) and two at lateral sites in the intrascapular area. Each site received a cluster of five injections of 0.1 ml per injection. Booster shots were given at intervals of 3–4 weeks, and titers were monitored at the same time. After 6–8 months, the antisera were of sufficiently good quality to be used in the assay.

Radioimmunoassay. Standard solutions for the standard curve were prepared from a primary stock solution containing 1.0 mg/ml of DHPG in 0.1 *N* HCl. This stock solution can be stored at 4°C for periods of 1–2 months. The exact concentration of this primary standard was determined by ultraviolet absorption at 255 nm (absorptivity ε = 10,950). The primary standard was diluted in RIA buffer [tris(hydroxymethyl)aminomethane–HCl buffer, 0.1 *M*, pH 7.4, with 0.1% gelatin, 0.001 *M* EDTA, and 0.001% sodium azide] to yield a series of standards containing 50, 100, 200, 400, 600, 1000, and 1500 pg/0.1 ml. These standards were stored at 4°C, and the unused portions were discarded after 3 weeks. Antiserum was diluted in RIA buffer such that at least 35–40% tracer binding was achieved. Depending on the quality of the antiserum, this dilution ranged from 1:50 to 1:100. The diluted antiserum was stored at 4°C for periods of 3–4 months without any evidence of deterioration. Radiolabeled DHPG for the assay was prepared by diluting ^{3}H-DHPG (sp act, 16 Ci/mmol) in RIA buffer such that a 0.1-ml aliquot contained 3500 cpm. The procedure for setting up the assay is as follows: add 0.1 ml of label, standards, unknowns, and antiserum to appropriate 12 × 75-mm disposable glass tubes; add RIA buffer to adjust the final volume in each tube to 0.6 ml; cover the tubes with parafilm; vortex briefly to mix the contents of the tubes; and then incubate the tubes overnight at room temperature.

The separation of bound from free radioactivity was carried out using 0.2 ml of a charcoal–dextran suspension (2% charcoal, 0.75% dextran; diluted with three parts of water). After the addition of the charcoal, the entire rack of tubes was vortexed vigorously for 15 sec. The tubes were then placed in an ice bath for 30 min and centrifuged (5 min at 1500*g*), and a 0.5-ml aliquot was removed for liquid scintillation counting (5 to 10 min). All counters were equipped with paper punch-tape outputs for transmittal of data to computers for automated calculation of RIA results by means of a logistic program (5). Cross-reactivities were determined in accordance with the method described by Abraham (6).

Optimization of Charcoal Separation of Bound from Free Radioactivity. To determine the optimum conditions for the separation of bound from free radioactivity, experiments were carried out in which the concentrations of char-

Table I. Cross-Reactivity Data

Compound	Cross-reactivity (%)
DHPG	100.0
DHPG monophosphate	1.2
2'-Deoxyguanosine	<0.02
Guanine	<0.02
Guanosine	<0.02
Thioguanine	≤1.2
Thioguanosine	≤1.2
Adenosine	≤0.02
2'-Deoxyadenosine	≤0.02
Ara-A	≤1.2
Acyclovir	18.4

coal and dextran were varied. It was found that diluting the charcoal–dextran stock suspension (2% charcoal and 0.75% dextran T-70) with three parts of water and adding 0.2 ml per tube yielded consistently good results, for both accuracy and precision. Under these conditions the nonspecifically bound fraction averaged between 3 and 5% in buffer and from 5 to 8% in the presence of plasma or serum.

Precision and Accuracy. Precision and accuracy were assessed by determining the recovery of different quantities of DHPG that had been added to human plasma or serum. In addition, a comparison of RIA and high-performance liquid chromatography (HPLC) assay data was done using data for specimens collected serially from a monkey, dogs, rats, and mice after either iv or oral dosing with the drug. For the HPLC procedure partial purification of the analyte in the plasma was achieved by precipitating plasma proteins with acetonitrile and then passing the resolubilized acetonitrile residue through a reverse-phase J. T. Baker C_{18} column. Recoveries during the partial purification before HPLC were monitored with ^{3}H-DHPG. HPLC was carried out on a reverse-phase microparticulate column (Regis Spherisorb Octadecyl, 5 μm) using water and 0.1% H_3PO_4 as the mobile phase. The sensitivity of the method was 100 ng/ml when using 1.0 ml of plasma.

RESULTS AND DISCUSSION

Initially the preparation of the monohemisuccinate of DHPG was carried out by direct succinylation of DHPG with succinic anhydride. This approach, however, yielded substantial amounts of the bis-hemisuccinate. As an alternative approach, DHPG was first monomethoxytritylated, which protected one of the hydroxyl groups but left the other free for reaction with succinic anhydride. The desired DHPG monohemisuccinate was obtained in a satisfactory yield by this approach.

The BSA conjugate of DHPG monohemisuccinate (molar ratio of DHPG to BSA, approximately 7.6) yielded antisera that were usable for RIA at dilutions of 1:50 to 1:100. Conjugates of DHPG with keyhole limpet hemocyanin yielded similar antibody titers, but conjugates with bovine γ-globulin did not.

The limit of sensitivity of the assay was assessed to be 0.7 ng/ml on the basis of the accuracy and precision data summarized in Table II. The average intraassay coefficient of variation calculated from the results of three separate experiments ($N = 6$ for each of 12 levels of added analyte which ranged from 0.00070 to 15.0 μg/ml) varied from 1.9 to 18.0%. The interassay coefficient of variation calculated from the three mean values ranged from 1.0 to 7.7% over this concentration range. Linear regression analysis of the overall means yielded the following equation; $y = 0.90\,x + 0.033$ ($r = 0.999$).

The assay was designed as a direct serum or plasma assay, and no preliminary purification of the sample is required. If the samples are analyzed without any dilution or

Table II. Accuracy and Precision Data for the Concentration Range 0.0007–15.0 μg/ml[a]

DHPG added (μg/ml)	DHPG measured (μg/ml) Expt A mean[b] (% CV)[c]		Expt B mean[b] (% CV)[c]		Expt C mean[b] (% CV)[c]		Group mean (% CV)[d]		Ratio measured/added
0.00070	0.00058	(17.0)	0.00056	(17.0)	0.00061	(11.0)	0.00058	(4.3)	0.83
0.00200	0.00190	(4.8)	0.00190	(3.7)	0.00170	(6.5)	0.00183	(6.3)	0.92
0.00600	0.00580	(4.1)	0.00570	(1.9)	0.00560	(4.0)	0.00570	(1.8)	0.95
0.01500	0.0133	(3.0)	0.0131	(5.6)	0.0128	(6.2)	0.0131	(1.9)	0.87
0.0700	0.065	(8.0)	0.061	(9.1)	0.071	(18.0)	0.066	(7.6)	0.93
0.2000	0.200	(6.2)	0.190	(6.7)	0.190	(3.6)	0.193	(3.0)	0.97
0.6000	0.610	(4.6)	0.570	(3.7)	0.570	(2.7)	0.580	(4.0)	0.97
0.7000	0.580	(16.0)	0.610	(13.0)	0.670[e]	(9.0)	0.620	(7.4)	0.89
1.5000	1.40	(6.4)	1.30	(5.5)	1.20	(4.0)	1.30	(7.7)	0.87
2.000	1.90	(3.3)	1.80	(5.9)	1.80	(5.0)	1.83	(3.2)	0.92
6.000	5.90	(4.9)	5.90	(4.0)	5.80	(4.6)	5.87	(1.0)	0.98
15.000	13.0	(6.6)	13.0	(6.2)	14.0	(4.1)	13.3	(4.3)	0.89

[a] Known amounts of DHPG were added to blank plasma and assayed by this method.
[b] $N = 6$.
[c] Intraassay variability.
[d] Interassay variability.
[e] $N = 5$.

Table III. Comparison of RIA and HPLC Assays for the Determination of DHPG in Plasma in a Monkey, Dogs, Rats, and Mice Given Either Oral or IV Doses of the Drug

Species	Time (postdose)	DHPG plasma conc. (μg/ml) RIA	HPLC
Monkey (oral dose)	1 hr	0.15	0.14
	2 hr	0.30	0.31
	3 hr	0.55	0.51
	5 hr	0.57	0.54
Mice (iv dose)	15 min	6.77	5.40
	30 min	3.28	3.31
	1 hr	2.11	2.19
	2 hr	0.46	0.67
	3 hr	0.07	0.13
	5 hr	0.13	0.20
Mice (oral dose)	30 min	1.30	1.44
	1 hr	1.10	1.11
	2 hr	1.10	1.03
	3 hr	1.08	1.17
	5 hr	0.52	0.69
	7 hr	0.18	0.30
Dogs (oral dose)	15 min	2.32	2.12
	30 min	4.01	3.97
	1 hr	5.43	5.36
	2 hr	4.98	5.42
	3 hr	4.00	4.05
	5 hr	2.90	3.00
	7 hr	1.61	1.80
Rats (oral dose)	15 min	1.03	0.87
	30 min	1.38	1.49
	1 hr	2.33	2.29
	2 hr	2.49	—
	3 hr	1.28	1.14
	5 hr	0.43	0.30
	7 hr	0.22	0.20
	10 hr	0.10	0.09

at low dilutions, it is necessary to add an equivalent aliquot of DHPG-free plasma or serum to all standard curve tubes in order to minimize differences in the RIA incubation media for standards and unknowns. If the sample dilutions exceed 50-fold, it is not necessary to do this.

The data on cross-reactivities are summarized in Table I. Endogenous substances with structures closely related to that of DHPG that could be present in blood at fairly high concentrations did not show significant cross-reactivities. Comparative data obtained by RIA and HPLC showed very good agreement (Table III). Linear regression analysis of the data yielded the following equations: monkey, $y = 0.82\,x + 0.015$ ($r = 0.997$); mice, $y = 0.80\,x + 0.28$ ($r = 0.991$) and $y = 0.91\,x + 0.15$ ($r = 0.979$) for iv and oral doses, respectively; dogs, $y = 1.02\,x - 0.02$ ($r = 0.990$); and rats, $y = 1.00\,x - 0.064$ ($r = 0.993$). This agreement between the results obtained by the two methods indicates relatively little interference from metabolites or other substances that are present in plasma or serum.

The primary advantage of RIA over HPLC for the determination of DHPG is the increased sensitivity (0.7 vs 100 ng/ml for HPLC). Furthermore, in order to achieve the HPLC sensitivity of 100 ng/ml, an entire 1.0 ml of plasma or serum must be processed, and this relatively large sample volume often is not available in studies involving small animals. The relative simplicity of the RIA procedure gives it a further advantage in that a higher sample throughput is achieved.

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