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Improved fluorometric high-performance liquid chromatographic assay for (−)-carbovir in rat blood and urine

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ABSTRACT

Carbovir is a carbocyclic guanosine analogue with potent *in vitro* activity against the human immunodeficiency virus. All of the activity resides in the (−)-enantiomer. An ion-paired liquid chromatographic assay for (-)-carbovir was developed on a Spherisorb C_8 column with fluorescence detection (275 nm excitation, 345 nm emission) Guanosine nucleosides are fluorescent at a pH < 2.5, and fluorescence detection resulted in a four-fold improvement in the limit of quantitation (0 039 μg/ml) compared to the previously developed assay with ultraviolet detection. Standard curves were processed with an internal standard at (−)-carbovir concentrations of 0 039–40 μg/ml in whole rat blood with a solid-phase extraction technique. Total variability was less than 16% at all concentrations and less than 10% at concentrations >0 3 μg/ml. Within-day variability was less than 7.5% at concentrations >0.3 μg/ml Urine was analyzed directly after dilution and an diethyl ether wash to remove impurities The total coefficients of variation were less than 10% from 0 5–20 μg/ml in urine. The concentrations of (−)-carbovir in rat blood were detectable for as long as 8 h after intravenous and oral doses of 20 and 60 mg/kg, respectively.

INTRODUCTION

Carbovir (carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine, NSC-614846) is a new nucleoside derivative which has been found to be active against the human immunodeficiency virus (HIV) *in vitro* [1]. Carbovir is activated to carbovir triphosphate in cells, and carbovir triphosphate has a high specificity for inhibition of HIV reverse transcriptase as compared to its minimal inhibition of human DNA polymerases α, β, and γ and DNA primase [2]. The (−)-isomer of carbovir (see Fig. 1) is completely responsible for the biological activity [3]. The

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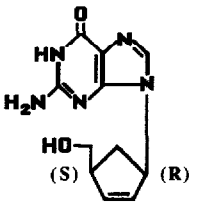

(-)-Carbovir

Fig. 1 Structure of (−)-carbovir

disposition of racemic (±)-carbovir in rats has been previously investigated in our laboratory [4]. The analytical method that was developed for that study was a reversed-phase high-performance liquid chromatographic (HPLC) method with UV detection [5]. With a limit of quantitation of 0.156 μg/ml for 0.2-ml rat blood samples, the concentrations of (±)-carbovir could be determined for approximately 2 h following a 20 mg/kg intravenous dose.

For pharmacokinetic studies with (−)-carbovir, a more sensitive analytical method was desired. Carbovir is a guanosine analogue, and at pH values less than 2.5, guanine becomes fluorescent, presumably because of a tautomeric shift from the 6-keto form to the 6-enol form [6]. In the 6-enol form, the purine ring becomes fully aromatic, resulting in the observed fluorescence. Recently Lake-Bakaar *et al.* [7] described a fluorescent HPLC assay for (*R,S*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine, a guanosine analogue with activity against varicella-zoster virus. In their method, a mobile phase of pH 2.0 with decyl sulfate as an ion-pairing agent was used. In this paper, a more sensitive HPLC assay for (−)-carbovir is described that also employs an acidic, ion-pairing mobile phase. A procedure for the analysis of (−)-carbovir in rat urine is also presented.

EXPERIMENTAL

Chemicals

(−)-Carbovir and the internal standard, (±)-carbocyclic 2′,3′-dideoxyguanosine, were gifts from Dr. Robert Vince, Department of Medicinal Chemistry, University of Minnesota. These compounds were synthesized by a recently published method [1]. Triethylamine was purchased from Aldrich (Milwaukee, WI, U.S.A.). Hexanesulfonic acid, sodium salt, was purchased from Eastman Kodak (Rochester, NY, U.S.A). Sodium dodecylsulfate (SDS) was purchased from Fluka (Ronkonkoma, NY, U.S.A.). Anhydrous diethyl ether was purchased from American Scientific Products (Minneapolis, MN, U.S.A.). *Helix pomatia* β-glucuronidase (Type H2) was from Sigma (St. Louis, MO, U.S.A.). All other chemicals were the same as used previously [5].

HPLC apparatus and conditions

The analytical column was a Spherisorb C_8 cartridge (5 μm particle size, 250 mm × 4.6 mm I.D., Phase Separations, Norwalk, CT, U.S.A.). A reversed-phase Spherisorb guard cartridge, 10 mm × 4.6 mm I.D., 5 μm particle size (Phase-Sep), was coupled directly to the analytical column. The HPLC system was comprised of a Waters Assoc. M45 pump, a Waters 710B WISP automatic injector (Milford, MA, U.S.A.) and a Shimadzu RF-530 fluorescence HPLC monitor (Columbia, MD, U.S.A.). The excitation and emission wavelengths were 275 and 345 nm, respectively. Different mobile phases were used for the assay of blood and urine samples. For blood samples, the mobile phase consisted of methanol–phosphoric acid buffer (50 m*M*) containing 25 m*M* hexanesulfonic acid and 10 m*M* triethylamine, pH 2.2 (14:86, v/v). For urine samples, the mobile phase was methanol–phosphoric acid buffer (30 m*M*) containing 25 m*M* sodium dodecylsulfate and 10 m*M* triethylamine, pH 2.2 (39:61, v/v). The flow-rate for both blood and urine samples was 1.5 ml/min. The analytical and guard columns were maintained at 40°C with an HPLC column water jacket (Alltech, Deerfield, IL, U.S.A.) to increase resolution and shorten retention times.

Sample preparation

Blood samples (200 μl) were extracted by solid-phase extraction on 100-mg C_{18} extraction columns (Burdick and Jackson, Muskegon, MI, U.S.A.) after addition of 1 μg of internal standard, carbocyclic 2′,3′-dideoxyguanosine, as described previously [5]. After evaporation, the extracts were reconstituted in 100 μl of mobile phase and 5–40 μl were injected onto the column with an automatic injector. The urine samples were analyzed directly by an external standard method after a diethyl ether extraction step as follows. In a 13-ml centrifuge tube with a ground-glass stopper, 250 μl of urine and 750 μl mobile phase buffer were mixed, and 3.0 ml anhydrous diethyl ether were added. Blank urine (250 μl) was spiked with an additional 50 μl of (−)-carbovir in water and 700 μl of mobile phase to obtain standards with final concentrations of 0.5–20 μg/ml. The tubes were vortex-mixed for 30 s and centrifuged at 800 *g* for 10 min. The aqueous phase was frozen in a dry ice–acetone bath, and the ether layer was decanted and discarded. The aqueous phase was thawed and transferred to a 1.5-ml polyethylene microcentrifuge tube. After centrifugation at 13 000 *g* for 3 min in a Fisher Model 235B microcentrifuge (Minneapolis, MN, U.S.A.), the supernatant was placed into a 250-μl polyethylene microcentrifuge tube, and 10 μl were injected onto the column by an automatic injector.

To determine if a glucuronide conjugate of (−)-carbovir was present in the urine of rats receiving (−)-carbovir, urine samples (1.0 ml) were diluted 1:1 with 0.6 *M* sodium acetate buffer, pH 5.0. To a 1.0-ml aliquot of diluted urine, 500 U of β-glucuronidase were added (5 μl of crude solution, 100 000 U/ml), and the sample was incubated for 16 h at 37°C. A second 1.0-ml aliquot was incubated under the same conditions without the addition of β-glucuronidase as a control. The diluted urines were processed as described above.

Assay validation

For blood samples, the peak-height ratios (carbovir/carbocyclic 2′,3′-dideoxyguanosine) from triplicate standard curves processed with standard (−)-carbovir concentrations of 0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10, 25 and 40 μg/ml were determined on five different days. The estimated total variability of the blood assay for a single sample measured on different days was determined by one-way analysis of variance (ANOVA) from the peak-height ratios of five replicate standard curves (number of replicates, $r = 3$) [8,9]. The within-day (s^2_{wd}) and between-day (s^2_b) components of variance were determined by the following equations:

$$s^2_{wd} = [MS_{wd}]$$
$$s^2_b = [(MS_b - MS_{wd}/r]$$

where MS_{wd} and MS_b are the within-day and between-day mean squares from the ANOVA table, respectively. Since the total variance of an observation is the sum of the within-day variance and the between-day variance, the total variance of an observation (s^2_{total}) was determined by the following equation:

$$s^2_{total} = s^2_b + s^2_{wd}$$

The percentage coefficient of variation at a given concentration of (−)-carbovir in blood was determined by dividing s_{total}, s_b and s_{wd} by the grand mean of the peak-height ratio values, and then multiplying by 100 [8].

To determine the concentrations of (−)-carbovir in unknown blood samples, a split unweighted linear regression method was used. Regression equations were determined from 0–1.25, 1.25–10 and 5.0–40 μg/ml. For urine samples the total variability and the components of between-day and within-day variation were determined after conversion to concentrations on a single unweighted standard curve at standard concentrations of 0.5, 1, 5, 10 and 20 μg/ml.

RESULTS

The chromatographic separation was performed on a non-chiral column, thus resolution of (+)-carbovir and (−)-carbovir was not possible. The assay was validated with synthetic (−)-carbovir, but similar results would be expected for either (+)-carbovir or (±)-carbovir. The chromatography of the extracts of blank blood, blank blood spiked with (−)-carbovir and blood sample from a rat that received (−)-carbovir are shown in Fig. 2. There were no endogenous peaks interfering with either (−)-carbovir or the internal standard. An endogenous peak that eluted between 33 to 35 min was observed. By correctly spacing the injections, this broad peak appeared in subsequent chromatograms between 4 and 7 min (see Fig. 2). The limit of detection for the whole blood assay was 0.02

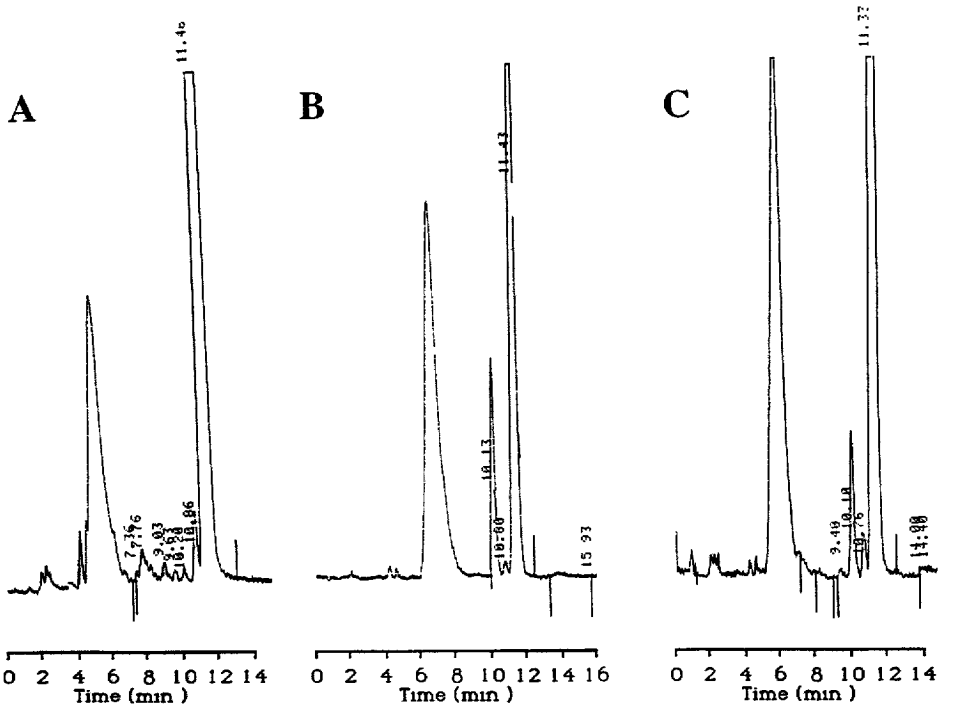

Fig 2. (A) Chromatogram of blank whole blood extracted by solid-phase extraction and spiked with 1.25 μg/ml internal standard (11.4 min) (B) Chromatogram of extracted whole blood spiked with 0.3125 μg/ml (−)-carbovir (10.1 min) and 1 25 μg/ml internal standard (11 4 min) (C) Chromatogram of an extracted whole blood sample from a rat 6 4 h after a 20 mg/kg intravenous infusion of (−)-carbovir Measured concentration = 0.117 μg/ml

μg/ml (signal-to-noise ratio = 3) which is approximately three times lower than the previous HPLC assay with UV detection [5]. The lower limit of quantitation (0.039 μg/ml) was approximately four times lower than that of the UV assay. The total variability and the components of within-day and between-day variation of blood samples over a concentration range of 0.039–40 μg/ml are shown in Table I. The coefficients of variation (C.V.) were generally less than 10% and were randomly distributed throughout the concentration range. The concentrations of (−)-carbovir in whole blood samples were determined by use of split unweighted standard curves, and the overall accuracy of the method is shown in Table II. Fig. 3 shows the blood concentration–time profile after intravenous bolus and oral doses of (−)-carbovir in a rat.

For urine samples, diethyl ether extraction removed most of the endogenous compounds, without extracting (−)-carbovir. There was a small interfering peak under (−)-carbovir in blank urine with the mobile phase used for the analysis of the blood samples. Therefore, SDS was substituted for hexanesulfonate as the ion-pairing agent for the analysis of urine samples to increase the lipophilicity of

TABLE I

TOTAL VARIABILITY AND THE BETWEEN-DAY AND WITHIN-DAY COEFFICIENTS OF VARIATION FOR THE (−)-CARBOVIR ASSAY IN WHOLE BLOOD

Concentration (μg/ml)	Peak-height ratio (mean ± S D) ($n=15$)	s_b	C.V. (%)	s_{wd}	C V (%)	s_{total}	C V. (%)
0.039	0 017 ± 0.002[a]	$1\ 56 \cdot 10^{-3}$	9 0	$1\ 66 \cdot 10^{-3}$	9 6	$2\ 28\ 10^{-3}$	13 2
0 078	0 034 ± 0.004[b]	$3\ 47\ 10^{-3}$	10 2	$2\ 46\ 10^{-3}$	7 2	$4\ 25\ 10^{-3}$	12.5
0.156	0.051 ± 0.008	$5\ 07\ 10^{-3}$	9 9	$6.30\ 10^{-3}$	12.3	$8.09\ 10^{-3}$	15 8
0 313	0.093 ± 0.009	$6\ 39\ 10^{-3}$	6 9	$6.59\ 10^{-3}$	7.1	$9\ 17 \cdot 10^{-3}$	9 8
0 625	0.178 ± 0.015[c]	$1\ 19\ 10^{-2}$	6 7	$1.03\ 10^{-2}$	5.8	$1.57 \cdot 10^{-2}$	8.9
1 25	0.356 ± 0.034	$3\ 33 \cdot 10^{-2}$	9 4	$1\ 42\ 10^{-2}$	4 0	$3\ 62 \cdot 10^{-2}$	10 2
2.50	0.669 ± 0 035[c]	$2\ 23 \cdot 10^{-2}$	3 3	$2\ 74\ 10^{-2}$	4.1	$3.54 \cdot 10^{-2}$	5.3
5.00	1.34 ± 0 121	$9.75 \cdot 10^{-2}$	7.3	$8\ 14\ 10^{-2}$	6 1	$1.27\ 10^{-1}$	9.5
10.0	2 54 ± 0.142	$1.28\ 10^{-1}$	5.0	$7\ 81\ 10^{-2}$	3 1	$1.50\ 10^{-1}$	5.9
25 0	6 15 ± 0 447	$3\ 91 \cdot 10^{-1}$	6 4	$2.62 \cdot 10^{-1}$	4 3	$4\ 71\ 10^{-1}$	7 7
40 0	9 57 ± 0 842	$8\ 33\ 10^{-1}$	8 7	$3\ 38 \cdot 10^{-1}$	3 5	$8\ 99\ 10^{-1}$	9 4

[a] $n = 9$.
[b] $n = 11$
[c] $n = 14$

TABLE II

ACCURACY OF THE (−)-CARBOVIR ASSAY IN WHOLE BLOOD

Concentration added (μg/ml)	Concentration measured (mean ± S D, $n=15$) (μg/ml)	Relative recovery (%)
0 039	0 035 ± 0.007[a]	89 6
0 078	0.093 ± 0.012[b]	119 7
0 156	0.158 ± 0 023	101 4
0 313	0.310 ± 0 030	99 3
0 625	0 615 ± 0 033[c]	98 3
1.25	1 25 ± 0 048	99.8
2.50	2 47 ± 0.134[c]	98.8
5.00	5 17 ± 0 401	103 4
10 0	9 91 ± 0.445	99 1
25 0	25 1 ± 1.788	100 2
40 0	39.2 ± 2 410	98 1

[a] $n = 9$
[b] $n = 11$
[c] $n = 14$

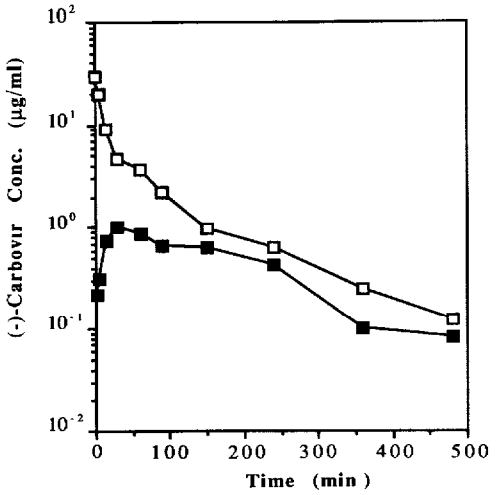

Fig 3 Log concentration–time profile after a 20 mg/kg intravenous dose of (−)-carbovir (open squares) and a 60 mg/kg oral dose of (−)-carbovir (closed squares) in a rat

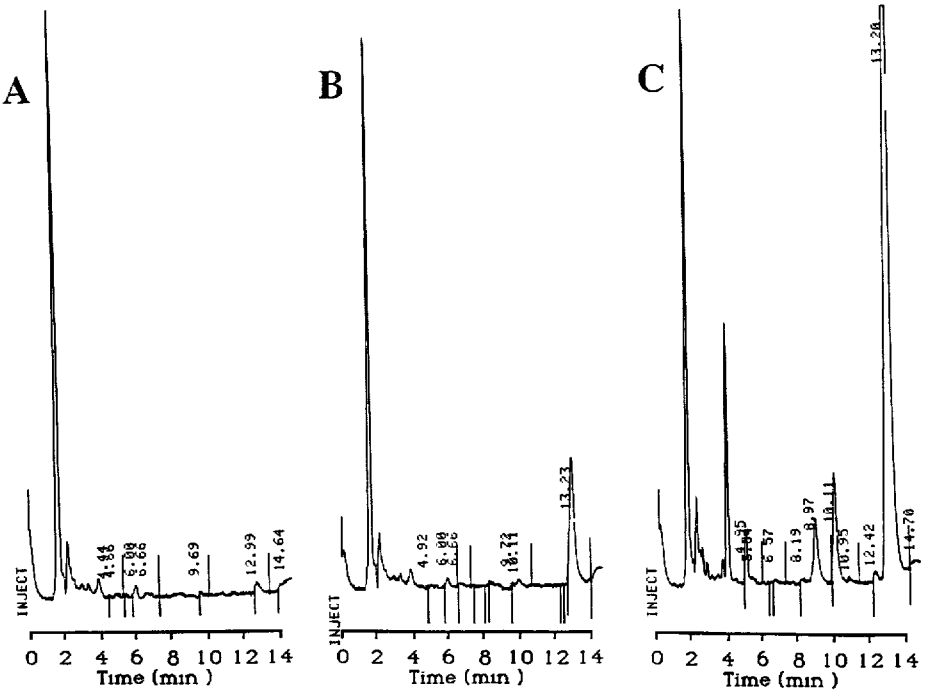

Fig 4 (A) Chromatogram of blank urine (after washing with diethyl ether) (B) Chromatogram of extracted urine spiked with 1 μg/ml (−)-carbovir (13 2 min). (C) Chromatogram of extracted urine sample collected from 2 to 6 h after a 20 mg/kg intravenous infusion of (−)-carbovir Measured concentration = 5 4 μg/ml. Two putative metabolite peaks were observed at 8.97 min and 10.1 min

TABLE III

PRECISION AND ACCURACY OF THE ASSAY FOR (−)-CARBOVIR IN URINE

Concentration added (μg/ml)	Concentration measured (mean ± S D., n = 12) (μg/ml)	R.R [a] (%)	s_b (μg/ml)	C V (%)	s_{wd} (μg/ml)	C.V (%)	s_{total} (μg/ml)	C V (%)
0.5	0 516 ± 0 046	103.1	$3\,93 \cdot 10^{-2}$	7 6	$3\,72 \cdot 10^{-2}$	7 2	$5\,00 \cdot 10^{-2}$	9.6
1 0	0 982 ± 0.053	98 2	$1\,02 \cdot 10^{-2}$	1 0	$5\,75 \cdot 10^{-2}$	5 7	$5.35 \cdot 10^{-2}$	5.3
5.0	4.89 ± 0.264	97.7	0.00	0 0	$2.18 \cdot 10^{-1}$	4.5	$2.22 \cdot 10^{-1}$	4.6
10.0	10.2 ± 0.570	101 9	$5\,40 \cdot 10^{-1}$	5 3	$3.03 \cdot 10^{-1}$	3.0	$5.81 \cdot 10^{-1}$	5.7
20 0	20.3 ± 0.580	101 6	0 00	0 0	$6\,46 \cdot 10^{-1}$	3.2	$5.67 \cdot 10^{-1}$	2.8

[a] R R = relative recovery.

the ion pair. Consequently, the higher percentage of methanol effectively separated the (−)-carbovir from an endogenous compound in urine. Fig. 4 shows the chromatography of processed blank urine, blank urine spiked with (−)-carbovir and a urine sample from a rat treated with (−)-carbovir. There were no interfering peaks. Two additional peaks were observed in rats treated with (−)-carbovir. These peaks are presumed to be metabolites of (−)-carbovir. After treatment with β-glucuronidase, the concentration of (−)-carbovir in rat urine was not significantly different compared to control urine samples, indicating that (−)-carbovir is not metabolized to a glucuronide in rats. The total variability and the within-day and between-day components of variation over a (−)-carbovir concentration range of 0.5–20 μg/ml in urine are shown in Table III. The peak heights were converted to concentrations from unweighted standard curves and the means and standard deviations were calculated. All the coefficients of variation were less than 10% for the urine assay.

DISCUSSION

The high sensitivity of this assay allowed the measurement of (−)-carbovir in rat blood for as long as 8 h after intravenous bolus and oral doses (see Fig. 3). With the previous UV assay [5], the concentrations of (±)-carbovir were measurable for only 4 h after an intravenous bolus dose. The ability to quantitate (−)-carbovir for twice as long is in part due to the enhanced sensitivity of the assay (lower limit of quantitation equals 0.039 μg/ml *versus* 0.156 μg/ml for the UV assay). It should also be noted that the clearance of (−)-carbovir may be different than that of the racemic mixture. The improved sensitivity of the fluorescent assay is important because the terminal half-life of (−)-carbovir can be more accurately determined. A detailed description of the pharmacokinetics of (−)-carbovir will be presented elsewhere, although preliminary results have been reported [10].

The simple diethyl ether washing procedure for urine samples avoided the more laborious solid-phase extraction procedure employed for whole blood samples. Only a relatively minor mobile phase modification was necessary to eliminate interference of contaminants and to insure specificity. The quantitation of (−)-carbovir in rat urine samples was used to calculate the renal clearance and to investigate the renal elimination of (−)-carbovir. Chromatograms of urine samples revealed that there may be at least two metabolites formed after the administration of (−)-carbovir (Fig. 4). The identification of these metabolites is currently underway.

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