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LIQUID CHROMATOGRAPHIC ASSAY OF CARBOVIR, A CARBOCYCLIC NUCLEOSIDE ACTIVE AGAINST HUMAN IMMUNODEFICIENCY VIRUS[a]

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SUMMARY

Carbovir is a novel carbocyclic guanosine derivative that has potent in vitro activity against human immunodeficiency virus, the causative agent of acquired immunodeficiency syndrome (AIDS). Two methods of sample preparation were developed for the analysis of carbovir in rat blood. Solid-phase extraction on C_{18} extraction columns proved to be the most effective. Whole rat blood (200 μl) was diluted with 0.8 ml of distilled water containing the internal standard. After two freeze–thaw cycles to lyse the red blood cells and subsequent centrifugation at 13 000 *g*, the supernatant was loaded on the C_{18} extraction columns. Carbovir and the internal standard were eluted with methanol–water (60:40). The extract was evaporated and reconstituted in mobile phase and the samples were injected onto a high-capacity reversed-phase column. The compounds were detected at 252 nm. Other nucleosides that could be used in the treatment of AIDS such as zidovudine and acyclovir did not interfere. Standard curves were linear over the concentration range 0.156–28.0 μg/ml ($r^2 > 0.99$). The within-day coefficient of variation was $< 7.6\%$ at all concentrations ($n = 4$). The between-day coefficient of variation ranged from 16.7 to 2.0% ($n = 14$). The limit of sensitivity was 0.05 μg/ml

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with a 200-μl blood sample and the average extraction recovery was 74%. Carbovir was stable in rat blood for at least 4 h at 37°C. The assay was used to determine the blood levels of carbovir in a rat after a 20 mg/kg intravenous dose.

INTRODUCTION

Carbovir (carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine, NSC-614846) is a novel carbocyclic guanosine derivative (see Fig. 1 for structure) that has potent in vitro activity against human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) [1,2]. Carbovir inhibited HIV replication and HIV-induced cytopathic effects in human T-lymphoblastoid cells (cell lines ATH8, MT-2, and CEM) with an EC_{50} (effective concentration, 50%) range of 0.15–0.19 μg/ml [1,2]. Cytotoxicity was observed at IC_{50} (inhibitory concentration, 50%) values ranging from 35.0 to 60.5 μg/ml in the three cell lines [2]. Thus carbovir appears to be a promising agent for the treatment of AIDS. For pre-clinical testing a sensitive and selective assay was needed to determine preliminary disposition and bioavailability of the drug in animals. In this paper we report the first quantitative assay for carbovir in biological fluids.

EXPERIMENTAL

Chemicals

Racemic (±)-carbovir and the internal standard, carbocyclic 2′,3′-dideoxyguanosine (Fig. 1), were synthesized in the laboratory of Dr. Robert Vince [2]. Stock solutions of carbovir and the internal standard were prepared at a concentration of 50 μg/ml in water or methanol. For the solid-phase extraction procedure, a solution containing 1.0 μg of the internal standard in 0.8 ml of distilled water was prepared. These solutions were stable for at least three months at 4°C. Triethylamine (Gold label) was purchased from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade ammonium phosphate was from J.T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade methanol and acetonitrile were obtained from Mallinckrodt (Paris, KY, U.S.A.). All other chemicals were reagent grade or better.

High-performance liquid chromatography (HPLC) apparatus and conditions

Octadecylsilane solid-phase extraction columns (Baker spe®, 1 ml size) were obtained from J.T. Baker. The analytical column was a 5-μm, 250×4.6 mm I.D.

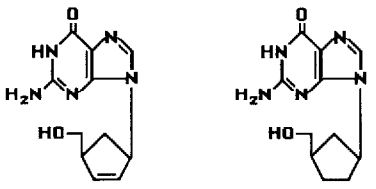

Fig. 1. Structure of carbovir and the internal standard, carbocyclic 2′,3′-dideoxyguanosine

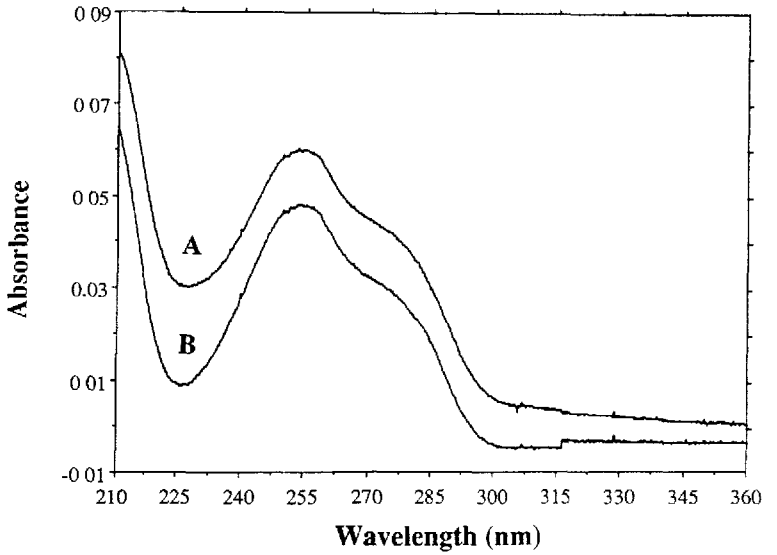

Fig. 2. UV spectra of 1 μg/ml solutions of carbocyclic 2′,3′-dideoxyguanosine (A) and carbovir (B) dissolved in methanol. Spectra were obtained on a Beckman DU-70 scanning spectrophotometer at a scan speed of 300 nm/min and a 2-nm slit width.

Adsorbosphere HS C_{18} cartridge (Alltech, Deerfield, IL, U.S.A.) protected by a 10 mm × 4.6 mm guard cartridge packed with the same material. The HPLC system consisted of a Waters Assoc. M45 pump, a Waters 710B WISP automatic injector (Milford, MA, U.S.A.) and a Shimadzu SPD-6A UV detector (Columbia, MD, U.S.A.) set at 252 nm and 0.05 a.u.f.s. The optimal wavelength for detection was 252 nm as determined by a UV scan of carbovir and the internal standard in methanol (Fig. 2). Peak heights were determined with a Waters data module. The mobile phase was composed of methanol–0.01 *M* ammonium phosphate buffer containing 0.005 *M* triethylamine, pH 7.0 (22:78, v/v). The flow-rate was 1.0 ml/min.

Sample preparation

Two methods of extraction for whole blood were examined. The first method was a liquid–liquid extraction method. In a 1.5-ml polyethylene microcentrifuge tube, 1.0 ml of acetonitrile and 20 μl of a 50 μg/ml methanolic solution of the internal standard were added to 200 μl of whole rat blood. The tubes were vortexed for 1 min and centrifuged at 13 000 *g* for 10 min. The supernatants were transferred to clean 15-ml centrifuge tubes with ground-glass stoppers containing 200 μl of 2% ammonium phosphate. Diethyl ether (1.0 ml) was added, the tubes were shaken for 10 min on a reciprocating shaker, centrifuged at 2000 *g* for 10 min, and the organic layers were aspirated and discarded. The aqueous layers were reextracted with 1.0 ml of fresh diethyl ether, the organic layers aspirated, and 20–50 μl of the aqueous ammonium phosphate layers were injected directly onto the column.

The second sample preparation procedure studied was a solid-phase extraction

method. Whole blood (200 μl) was mixed with 0.8 ml of distilled water containing 1 μg of internal standard in polyethylene microcentrifuge tubes. The samples were frozen in a dry ice–isopropanol bath and thawed two times to insure lysis of the red blood cells. The samples were centrifuged at 13 000 *g* for 10 min in a Fisher Model 235B microcentrifuge (Minneapolis, MN, U.S.A.) and the supernatant was loaded onto a C_{18} sample preparation column which had been previously washed with 2 ml of methanol and 2 ml of water. After loading of the sample, the cartridge was washed with 1 ml of distilled water, and carbovir and the internal standard were eluted with two 0.5-ml portions of methanol–water (60:40) on a twelve-position vacuum manifold (Supelco, Bellefonte, PA, U.S.A.). The 60% methanol washes were pooled and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and 10–50 μl were injected onto the HPLC column. In general, a 25-μl aliquot was injected, however, for the highest and lowest standard curve samples, 10- and 50-μl aliquots were injected, respectively.

Assay validation

After comparison of the two sample preparation procedures, the accuracy and precision of the solid-phase extraction was further investigated. To determine the within-day variability, 180 μl of rat blood were spiked with 20 μl of aqueous solutions of carbovir to give the following final concentrations: 0.156, 0.312, 0.625, 1.25, 2.50, 5.00, 10.0 and 28.0 μg/ml. The samples were prepared in quadruplicate and extracted by the solid-phase extraction procedure. In general, for low concentrations of carbovir in the blood a low end standard curve (0–1.25 μg/ml) was used. For higher concentrations the entire range was used to determine the amount of carbovir in the unknown samples. The between-day variability was determined by processing multiple duplicate standard curves on different days. From the standard curve processed for that day, the peak-height ratios of the standards were converted to concentrations and the means and standard deviations were calculated for each spiked concentration value. Extraction recovery was determined by comparison of the absolute peak heights of the extracted samples to the peak heigths of standards injected directly onto the HPLC system.

Stability

The stabilities of carbovir and the internal standard dissolved in water were determined at 4 °C. Every two weeks, aliquots of the refrigerated solutions were removed, equilibrated to room temperature and injected onto the HPLC system. The stability of carbovir in rat blood was also examined. Carbovir was incubated with fresh rat blood (taken from the abdominal aorta of a rat under diethyl ether anesthesia) at a final concentration of 5 μg/ml at 37 °C for 4 h. The samples were extracted by the acetonitrile–diethyl ether extraction procedure and the concentration of carbovir was determined by comparison to a standard curve which ranged from 0.625 to 10.0 μg/ml.

RESULTS

Carbovir and the internal standard were well separated in the methanol-based mobile phase with retention times of 11.6 and 13.2 min, respectively (see Fig. 3). Mobile phases made with the identical buffer, but with acetonitrile or tetrahydrofuran as the organic solvent displayed poor resolution of the two compounds. Triethylamine prevented tailing, although the compounds only tailed slightly on this column. The liquid–liquid extraction method was reasonably simple and provided sample extracts that were relatively free of contamination. Several large endogenous peaks were observed as late as 8 min in the chromatograms. The extraction recovery by this method was $\geq 95\%$. This method was deemed acceptable, however, a cleaner extract was desired. In addition, the procedure also diluted the sample by as much as two-fold, thus reducing sensitivity. Therefore, a second sample preparation procedure, solid-phase extraction, was investigated. By taking advantage of the relatively high lipophilicity of carbovir compared to endogenous nucleosides or antitumor nucleosides such as cytarabine, which normally elute with mobile phases containing less than 10% methanol on HPLC, an extraction method with C_{18} sample preparation columns was attempted. After washing with water, carbovir was successfully eluted with 50–60% methanol in water. This procedure provided cleaner extracts, removed highly lipophilic substances, and the extraction recovery was acceptable. The extraction recoveries of carbovir at various concentrations are presented in Table I. The extraction recovery of the internal standard was $65 \pm 5.8\%$ at a blood concentration of 5 μg/

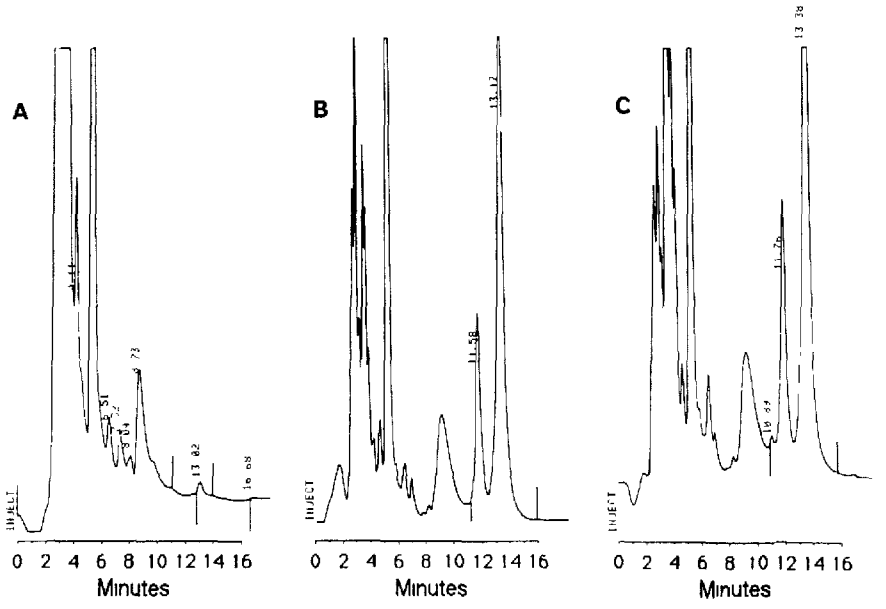

Fig. 3. (A) Chromatogram of blank whole blood extract by solid-phase extraction method. (B) Chromatogram of extracted whole blood spiked with 1 25 μg/ml carbovir (11.6 min) and 5 μg/ml of internal standard (13.2 min) (C) Chromatogram of extracted whole blood sample from a rat 45 min after a 20 mg/kg intravenous dose of carbovir. Measured concentration: 2.25 μg/ml.

TABLE I

RECOVERY OF CARBOVIR FROM RAT BLOOD

Spiked concentration (μg/ml)	Recovery (mean ± S.D.) (%)	Coefficient of variation ($n=4$) (%)
0.156	81.1 ± 4.5[a]	5.5
0.312	87.5 ± 17.7	20.2
0.625	71.1 ± 6.8	9.6
1.25	65.3 ± 8.0	12.3
2.50	57.4 ± 7.3	12.7
5.00	71.8 ± 9.5	13.2
10.0	69.8 ± 1.9	2.7
28.0	89.9 ± 9.2	10.2

[a] $n=3$.

TABLE II

HPLC CAPACITY FACTORS (k') FOR CARBOVIR AND OTHER COMPOUNDS OF POTENTIAL USE IN AIDS

Determined relative to uracil, a void volume marker.

Compound	k'
Foscarnet	0.02
Cytosine arabinoside	0.02
Acyclovir	0.13
Vidarabine (Ara-A)	0.57
Sulfamethoxazole	0.79
Carbovir	2.25
2′,3′-Dideoxycarboxyclic guanosine (internal standard)	2.66
Zidovudine (AZT)	4.44
Trimethoprim	22.20
Pentamidine	> 30.0
Amphotericin B	–[a]

[a] Not detectable at 252 nm.

ml ($n=32$). No observable peaks were detected under carbovir when blank whole blood extracts were injected with the solid-phase procedure. A small, but insignificant endogenous peak was observed under the internal standard (see Fig. 2). There were also fewer problems with late-eluting peaks than with the acetonitrile–diethyl ether extraction procedure. Several nucleosides and antibiotics were chromatographed to check for possible interference. A list of possible interfering drugs and their respective capacity factors (k') are presented in Table II. No interference was found with these drugs that are commonly used in AIDS patients.

The solid-phase extraction procedure was both precise and accurate. Table III shows the within-day and between-day variability over a concentration range of 0.156–28.0 μg/ml. The coefficients of variation were less than 10% at all but the

TABLE III

WITHIN- AND BETWEEN-DAY VALIDATION FOR THE ANALYSIS OF CARBOVIR IN RAT BLOOD

Spiked concentration (μg/ml)	Within-day ($n=4$)		Between-day ($n=14$)	
	Measured concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Measured concentration (mean ± S.D.) (μg/ml)	C.V. (%)
0.156	0.155 ± 0.003[a]	1.9	0.132 ± 0.022[b]	16.7
0.312	0.326 ± 0.007	2.1	0.338 ± 0.029[c]	8.6
0.625	0.673 ± 0.034	5.1	0.698 ± 0.052	7.4
1.25	1.21 ± 0.051	4.2	1.23 ± 0.078	6.3
2.50	2.11 ± 0.086	4.1	2.16 ± 0.109	5.0
5.00	4.71 ± 0 173	3.7	4.88 ± 0.159	3.3
10.0	10.2 ± 0.774	7.6	10.1 ± 0.287	2 8
28.0	28.4 ± 0.667	2.3	28.7 ± 0.575	2.0

[a] $n=3$.
[b] $n=15$.
[c] $n=16$.

lowest concentrations tested. Two standard curves were normally used. One standard curve was used for low concentrations (0–1.25 μg/ml) and a second standard curve that included all concentrations was used for the higher concentrations.

Carbovir and the internal standard appear to be relatively stable compounds. No degradation of the compounds dissolved in water was observed when the compounds were stored at 4 °C for three months. The compounds eluted as single peaks with a constant peak area throughout this time period. The stability of carbovir in rat blood was also studied. When carbovir was incubated for 4 h at 37 °C with fresh rat blood, there was no discernable loss of compound when compared to samples spiked with various concentrations of carbovir and immediately extracted by the acetonitrile–diethyl ether extraction procedure.

DISCUSSION

The chromatography of the compound was relatively straightforward. A methanol-based solvent provided the best resolution. Several other compounds were investigated as potential internal standards. Both cytosine arabinoside and vidarabine (Ara-A) were obscured by impurities in the sample at retention times shorter than carbovir. An analogue of carbovir with a reduced double bond was chosen for its similar chemical properties and chromatographic characteristics. Several compounds that could potentially interfere with carbovir in patients were also chromatographed. Neither acyclovir and zidovudine, two drugs that are likely to be used along with carbovir for treatment of AIDS patients, interfere with the assay. In the development of a sensitive, accurate and precise assay for carbovir in small quantities of blood for pharmacokinetic and toxicologic evaluation, we

compared two sample preparation methods. The first method was a liquid–liquid extraction procedure that involved precipitation of proteins with acetonitrile, followed by diethyl ether extraction of the supernatant to remove fats and other lipophilic impurities. This procedure was effective and would have been useful if the nucleoside was metabolized rapidly in blood, because the proteins were immediately precipitated. Later studies showed no degradation of carbovir in rat blood. The disadvantage of the procedure was the relative impurity of the extracts and the dilution of the sample. Consequently, a solid-phase extraction procedure with C_{18} columns was chosen because carbovir lacks two hydroxyl groups on the sugar moiety and thus would be expected to be more lipophilic than other nucleosides.

The solid-phase extraction procedure was relatively fast and easy. Attempts at extracting diluted whole blood directly by solid-phase extraction caused some difficulty. Most samples eluted slowly with the vacuum manifold and required centrifugation at 2000 *g* for 10–30 minutes to elute the diluted blood through the columns. Occasionally some columns became plugged and the columns could not be reused after washing with methanol. Consequently, dilution of the blood with water and two cycles of freeze–thawing were included to lyse the erythrocytes. When the 13 000 *g* supernatant was used for analysis, no significant problems were encountered. The somewhat lower extraction recovery of this procedure can be largely accounted for by the loss of the compound in the red blood cell pellet. This procedure also allowed for a two-fold concentration of the sample after an evaporation and reconstitution step. If high sensitivity is not required, then the 60% methanol eluate could be injected directly.

The assay has proven to be reliable. Typical within-day coefficients of variation are less than 10% throughout the concentration range. The lowest standard curve concentration that we measured was 0.156 μg/ml which is close to the reported EC_{50} of 0.15–0.19 μg/ml for carbovir against HIV-infected cells [1]. A desired therapeutic concentration would be in the range of 5–10 μg/ml to insure maximal therapeutic effect. Thus the assay has more than the necessary sensitivity for therapeutic monitoring and has sufficient sensitivity to determine the pharmacokinetics for five half-lives. We estimate the lower limit of sensitivity to be approximately 0.05 μg/ml with a signal-to-noise ratio of 5:1.

The assay was also used to determine the stability of carbovir in solution and in rat blood. Carbovir appears to be relatively stable and was not degraded in rat blood over 4 h at 37 °C. Therefore special precautions concerning the storage of the compound do not appear to be necessary.

A whole blood assay was developed because of limited sample in small animals and for pharmacokinetic considerations. The total body clearance of a drug is commonly compared to an organ blood flow (e.g. hepatic flow) in order to determine the ability of the organ to remove the drug [3]. It is thus more appropriate to base clearance on blood concentrations rather than on plasma concentrations. Furthermore, the blood-to-plasma ratio of carbovir is greater than 1.0 and is concentration-dependent [4]. Therefore, measuring plasma concentrations of carbovir may not directly reflect concentrations of carbovir in the blood that is perfusing the eliminating organs. These considerations aside, the assay should also

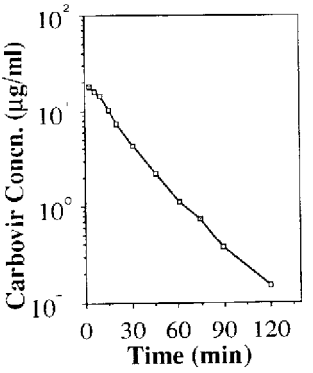

Fig. 4. Log concentration–time profile after a 20 mg/kg intravenous dose of (±)-carbovir in a rat.

work well for plasma or serum samples. With plasma or serum samples, the 13 000 *g* centrifugation step would not be necessary. In addition larger sample sizes (up to 1.0 ml) could be extracted with these columns to improve sensitivity when working with larger animals or with human samples. The solid-phase extraction procedure is currently being employed to investigate the pharmacokinetics of carbovir in rats. To demonstrate the applicability of the assay, the concentration–time profile of carbovir in a rat after an intravenous dose of 20 mg/kg is shown in Fig. 4.

In conclusion, we have developed a sensitive and reliable assay for carbovir in whole blood. The solid-phase extraction procedure had significant advantages over a liquid–liquid extraction. No interference with endogenous nucleosides or nucleoside analogues that are used in AIDS patients was observed. The assay has sufficient sensitivity for both routine monitoring and for the investigation of pharmacokinetics of carbovir in small animals.

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