ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1982, p. 44–50
0066-4804/82/010044-07$02.00/0

Vol. 21, No. 1

Uptake, Distribution, and Anabolism of Acyclovir in Herpes Simplex Virus-Infected Mice

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Received 11 August 1981/Accepted 21 October 1981

The uptake, distribution, and anabolism of the nucleoside analog 9-(2-hydroxyethoxymethyl)guanine (acyclovir) were compared in herpes simplex virus-infected and uninfected mice. Analyses of tissue distribution and the concentration of acyclovir after either a single dose or multiple doses failed to reveal significant differences between drug levels in infected and uninfected animals. Extracts of tissues from [8-^{14}C]acyclovir-treated animals were examined by high-performance liquid chromatography to detect the presence of any phosphorylated forms of the drug. The sensitivity of this method did not allow a reproducible demonstration of acyclovir anabolism in herpes simplex virus-infected tissues owing to the low numbers of infected cells per organ.

The acyclic nucleoside analog 9-(2-hydroxyethoxymethyl)guanine (acyclovir [ACV]) has been shown to be a selective inhibitor of herpes simplex virus (HSV) both in vitro and in vivo in several animal models (1, 3–5, 10, 12, 14). The in vitro antiviral activity of ACV is associated with the anabolism of the drug to its monophosphate form by virus-coded thymidine kinase (4, 7, 11) and its subsequent conversion to the di- and triphosphate forms by host enzymes (11). ACV triphosphate functions as both a substrate for and a preferential inhibitor of the virus-specified DNA polymerase and thereby inhibits viral DNA replication (6, 14). Vero cell cultures infected synchronously with HSV type 1 (HSV-1) and treated with various concentrations of [8-^{14}C]ACV have been shown to accumulate higher levels of drug when compared with uninfected cells by high-performance liquid chromatography (HPLC) analysis (P. Furman, unpublished data). The intracellular level of ACV is related to the concentration of the drug in the medium.

In view of the increased uptake and accumulation of ACV in cells infected with HSV in vitro, studies were designed to evaluate the tissue uptake and distribution of ACV in HSV-infected mice. The distribution of ACV in infected brain was examined to determine whether lethal infection in the encephalitic animals affected the integrity of the blood-brain barrier. The intraperitoneal (i.p.) injection of newborn mice with HSV provides a systemic infection model which involves the gut, spleen, liver, kidney, lung, and brain and eventually terminates in encephalitis and death of the untreated animals (9). The efficacy of ACV in this animal model has been demonstrated (E. Kern, personal communication). The distribution of ACV in the plasma and tissues of uninfected CD-1 mice has been reported (13). In the present study, the uptake and distribution of ACV in the tissues and plasma of HSV-infected mice were compared with those of uninfected mice after single- or multiple-dose regimens. Extracts of both infected and uninfected tissues were examined by HPLC for evidence of phosphorylation of ACV in an attempt to correlate in vivo antiviral activity with the known in vitro mechanism of action. In other groups of mice, the distribution of infectious virus in the brain, kidney, liver, and spleen was determined during the course of infection.

MATERIALS AND METHODS

Virus and cells. The MS strain of HSV-2 was obtained from Earl Kern, University of Utah College of Medicine, Salt Lake City. The virus was propagated and titrated on Vero cells (American Type Culture Collection, Rockville, Md.) and stored as a cell-free virus stock at −70°C. Vero cells were grown in monolayer cultures in Eagle minimal essential medium supplemented with 5% fetal calf serum (Sterile Systems, Salt Lake City, Utah) and antibiotics (50 U of penicillin and 50 μg of streptomycin per ml).

Virus assays. Virus titrations were carried out by plaque assay on Vero cells (3) by using 0.6% Seaplaque agarose overlays (Marine Colloids, Rockland, Maine). To quantitate virus levels in the organs of infected mice, animals were sacrificed on various days after infection, individual organs were removed and weighed, and 10 or 20% homogenates were made in minimum essential medium containing 2% fetal calf serum by using a Brinkmann Polytron homogenizer. Organ homogenates were clarified by centrifugation, and serial 10-fold dilutions of the supernatant were titrated on Vero cells as previously described.

Animal infection and drug dosing. Male and female CD-1 mice (Charles River Breeding Laboratories, Inc., Downington, Maine), weighing 18 to 20 g (3

weeks of age), were infected i.p. with 5×10^5 PFU of virus in a 0.1-ml volume, which resulted in 95 to 100% mortality in untreated animals. The mortality and the median day of death were determined. ACV was reconstituted in phosphate-buffered saline at the appropriate stock concentration and was administered i.p. in a volume of 0.1 ml/10 g of body weight. [8-^{14}C]ACV, synthesized by J. Scharver of these laboratories, was administered subcutaneously (s.c.) in the scruff of the neck. Infected ACV-treated or control animals were observed for a 21-day period for determinations of efficacy.

Tissue distribution of ACV. Animals treated with [8-^{14}C]ACV were sacrificed at the indicated times, and individual organs were removed, weighed, and homogenized in a phosphate-buffered saline suspension. Samples were solubilized with NCS (Amersham Corp., Arlington Heights, Ill.) and decolorized with benzoyl peroxide when necessary. Toluene-based scintillation cocktail was added, and radioactivity was determined with a scintillation counter. Since the drug is largely unmetabolized in mice (13), tissue concentrations were determined from the specific activity. The concentrations of ACV in tissue extracts of animals treated with non-radiolabeled ACV were determined by radioimmunoassay (15).

HPLC for analysis of ACV anabolism. Tissues for analysis of ACV anabolism were removed from animals under ether anesthesia and were immediately freeze-clamped in a liquid nitrogen bath. Tissue pieces were weighed while frozen and ground to a powder in a cold mortar with a pestle. The powder was extracted with 2 volumes of ice-cold 15% perchloric acid and allowed to thaw before the addition of 3 volumes of ice-cold deionized water. After centrifugation, the supernatants were neutralized with freshly made, saturated KOH and centrifuged. The supernatants were evaporated to dryness under reduced pressure, and the residue was redissolved in deionized water. Samples were analyzed with a Varian Aerograph LCS-1000 HPLC fitted with a Partisil-10 SAX column (0.46 by 25 cm; Whatman Inc., Clifton, N.J.). The resolution of ACV and its anabolized forms was achieved by a linear gradient of KH_2PO_4 (0.015 to 1.0 M at pH 3.5), with a flow rate of 30 ml/h (13). Fractions were collected, and radioactivity was determined in a liquid scintillation counter. The UV absorption of the eluate was monitored at 254 and 280 nm and was recorded with a dual-pen Honeywell electronic 194 recorder. Results are expressed as picomoles of ACV or its phosphorylated derivatives per milligram of tissue.

Statistical analysis. Data were analyzed by using Student's *t* test, except where otherwise noted.

RESULTS

Effect of ACV on mortality of CD-1 mice infected i.p. with HSV-2. Preliminary experiments were performed to characterize the infection in 3-week-old CD-1 mice and to confirm the efficacy of ACV administered by the systemic route. CD-1 mice were infected i.p. with 5×10^5 PFU of HSV-2. Animals were treated with ACV (20 or 80 mg/kg per day) administered i.p. in two divided doses, 12 h apart. Treatment was initiated 24 h postinfection and continued for 5 days.

Treatment with 80 mg of ACV per kg per day resulted in a significant decrease in mortality and a significant increase ($P < 0.05$) in survival time (Table 1). The mortality of mice which received 20 mg of ACV per kg per day was only slightly reduced, whereas the effect on the median day of death was more apparent ($P < 0.05$).

Distribution of infectious virus in CD-1 mice after i.p. infection with HSV-2, with or without ACV treatment. CD-1 mice were infected and treated with ACV (80 mg/kg per day) as described previously. The course of the infection was monitored by removing organs at the indicated times and quantitating the levels of infectious virus by plaque assay on Vero cells (Fig. 1). Peak virus titers in untreated animals were observed 4 or 5 days postinfection. Virus levels were often undetectable in extracts of organs from ACV-treated animals under these experimental conditions. The limits of virus detection of this method were 10^2 PFU/g of tissue, and values graphed in Fig. 1 represent average titers from five animal organs per sample.

Virus recovered from the kidneys and brains of ACV-treated mice on days 4 and 6, respectively, was found to be as sensitive to ACV as the starting virus population by plaque reduction assay in vitro (data not shown).

Tissue distribution of ACV in HSV-2-infected and uninfected CD-1 mice after a single dose of [8-^{14}C]ACV. The tissue distribution of [8-^{14}C]ACV in mice was studied after a single s.c. dose of 100 mg/kg (specific activity, 7,500 dpm/nmol) to groups of three normal or three HSV-infected mice on day 5 postinfection. The levels of ACV were determined by measuring the total radioactivity, and the results were graphed as the mean concentration of ACV for each group ± the standard error of the mean (Fig. 2). The tissue concentrations of ACV reached peak levels in both infected and uninfected animals within 15 min after the administration of the drug and then rapidly declined within the first 2 h. A slower disappearance of the drug from tissues and plas-

TABLE 1. Effect of treatment with ACV on the mortality of 3-week-old CD-1 mice inoculated i.p. with HSV strain MS

Treatment[a]	Mortality[b]		
	No.	%	MDD[c]
Virus control	40/41	98	7.0
ACV (20 mg/kg per day)	27/30	90	9.2[c]
ACV (80 mg/kg per day)	14/27	52	15.3[c]

[a] Treatment (i.p.) initiated 24 h postinfection and continued twice daily for 5 days.

[b] Animals monitored for 21 days.

[c] With normal distribution, median days of death (MDD) are significantly different at $P < 0.05$.

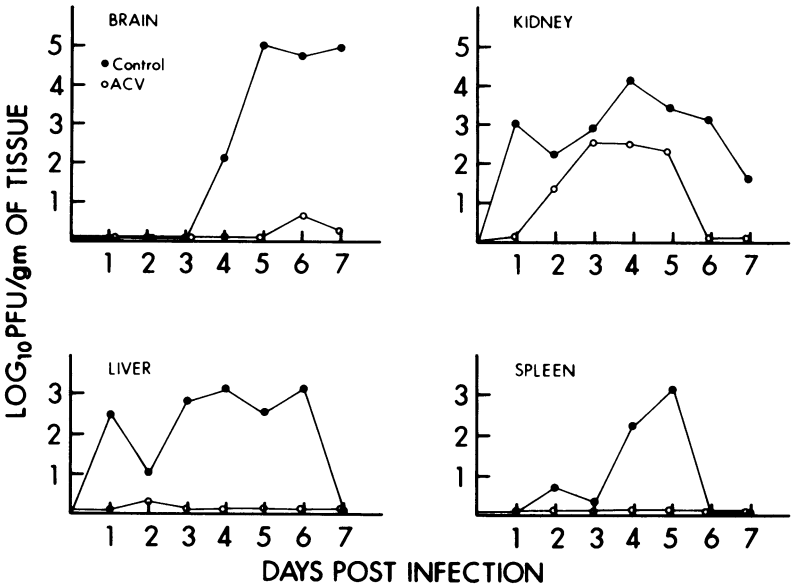

FIG. 1. Distribution of infectious virus in 18 to 22 g of CD-1 mice, five animals per sample, inoculated i.p. with 5×10^5 PFU of HSV-2 strain MS. The course of the infection was monitored by removing the organs at the indicated times and quantitating the levels of infectious virus by plaque assays. Symbols: ●, control; ○, ACV (80 mg/kg/per day).

ma occurred over a 24-h period. The concentrations of the drug in the kidneys and livers of infected and uninfected animals were higher than the corresponding plasma levels in both groups. ACV levels in the lungs, spleens, and hearts were similar to plasma levels in both groups. Drug concentrations in the brains of either group were significantly lower than those in the plasma and in all other tissues, similar to results previously reported for uninfected animals (13).

Effect of multiple dosing of mice with ACV on tissue levels of drug in HSV-infected and uninfected animals. An analysis of the tissue distribution of ACV after a single dose failed to reveal significant differences in the ACV levels between infected and uninfected animals. In an attempt to determine whether multiple doses might result in a significant difference between the two groups, the drug was administered in three consecutive s.c. doses (100 mg/kg per dose) at 3-h intervals to sustain plasma levels over a longer period of time. Such conditions might allow the detection of any selective uptake or the accumulation of ACV in the asynchronously infected tissues. The concentrations of ACV in the plasma, cerebrum, cerebellum, brain stem, and liver were determined by radioimmunoassay (Fig. 3). A comparison of tissue/plasma ratios between infected and uninfected animals revealed the same trend as was seen after a single dose of ACV. Significant differences ($P < 0.05$) were apparent between ACV concentrations achieved in tissues of infected and uninfected animals only at 1 h after the third dose of ACV (Fig. 3). No other significant differences were observed.

Anabolism of ACV in tissues of uninfected and HSV-infected mice. Uninfected or HSV-infected mice were dosed s.c. with [8-^{14}C]ACV on day 4 or 5 postinfection. Animals were sacrificed at 0.5, 2.0, and 6.0 h after dosing, and the organs were quickly removed and processed as previously described. Results are presented in Tables 2 and 3. The profiles of ^{14}C radioactivity from HPLC analysis of extracts of infected and uninfected tissues indicated very little anabolism of ACV to its phosphorylated forms. There were no significant differences in the anabolism of ACV in the liver or kidney tissues from infected or uninfected animals. One brain, obtained from a mouse 0.5 h after dosing on day 4 postinfection, showed anabolism of the drug to the mono-, di-, and triphosphate forms. In fact, 18.7% of the total radioactivity (2.83 pmol/mg) in the acid-soluble extract was present as the triphosphate form of the drug. Subsequent attempts to detect phosphorylated forms of ACV in brain extracts from other HSV-infected animals were unsuccessful.

Additional experiments were performed utilizing younger animals (8 to 10 g, 2 weeks of age) whose organs (except the brain) reproducibly support the replication of 1 to 2 logs more (than

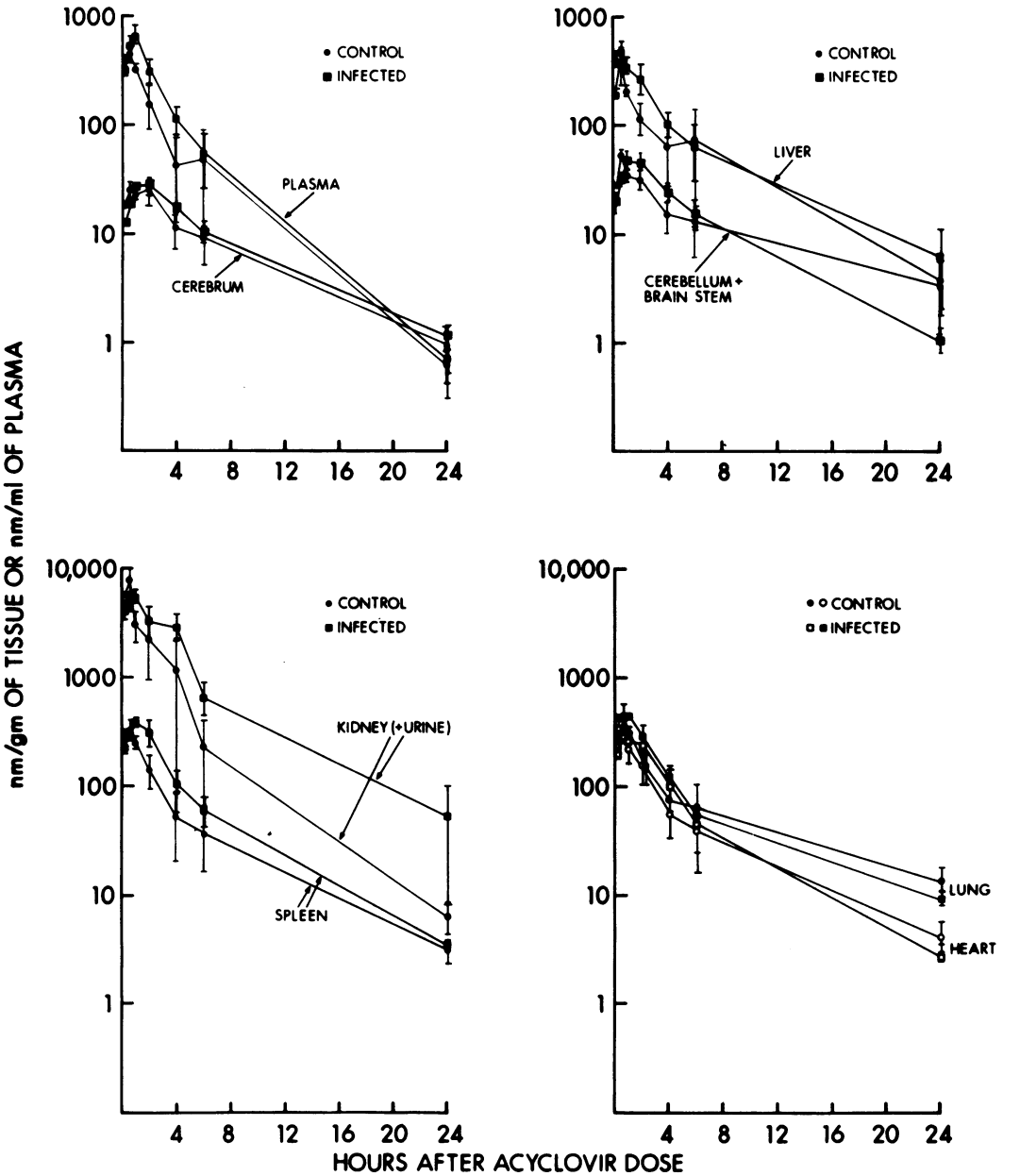

FIG. 2. Mean concentrations ± standard error of ACV in CD-1 mice tissues at various times after a single s.c. administration [8-^{14}C]ACV (100 mg/kg) to groups of three normal or three infected mice. ACV was administered i.p. on day 5 postinfection with HSV-2 strain MS. Symbols: ○, control; □, infected.

the older animals) of infectious virus per g of tissue. Organ excision and extraction procedures were modified for a more rapid temperature stabilization of excised organs. Despite these efforts to increase the sensitivity of detection of ACV anabolism, HPLC analysis showed that extracts of both infected and uninfected organs contained >95% unchanged [8-^{14}C]ACV (Table 4). Low levels (often less than 0.3% of total radioactivity) of the di- and triphosphate forms of ACV were found in >90% of the infected kidney and liver samples. Similar trends were observed in organs from uninfected animals (Table 2).

DISCUSSION

The i.p. infection of weanling mice with HSV-2 provided a systemic infection model with

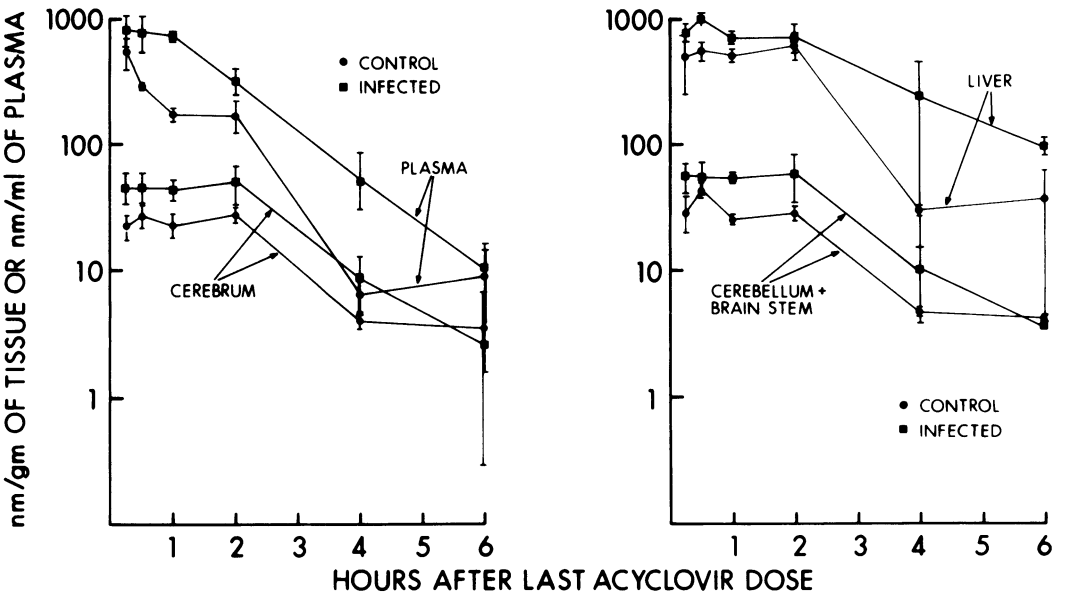

FIG. 3. Mean concentrations ± standard error of ACV in tissues of uninfected CD-1 mice and HSV-2-infected mice, strain MS, on day 5 postinfection i.p. ACV was administered s.c. in three doses (100 mg/kg/per dose) at 3-h intervals to groups of three mice, and tissue concentrations were determined by radioimmunoassay at various times after the third dose. Symbols: ●, control; ■, infected.

TABLE 2. Metabolism of [8-^{14}C]ACV in normal CD-1 mice (5 to 6 weeks of age, three mice per group) weighing 22 to 25 g after a single s.c. dose of 50 mg/kg

Organ	Time post-dose (h)	Unconverted ACV		ACV-MP[a]		ACV-DP[a]		ACV-TP[a]	
		pmol/mg[b]	Mean % of total radioactivity	pmol/mg	Mean % of total radioactivity	pmol/mg	Mean % of total radioactivity	pmol/mg	Mean % of total radioactivity
Brain	0.5	8.64	98.1	0.06	0.22	0.04	0.23	0.02	0.16
	2	10.64	96.6	0.05	0.6	0.06	0.8	<0.01	<0.1
Liver	0.5	112.9	96.5	0.34	0.26	0.06	0.1	<0.01	<0.1
	2	11.3	90.4	0.04	0.4	0.14	1.3	0.01	<0.1
Kidney	0.5	1,792.7	97.3	0.35	<0.1	0.24	<0.1	0.03	<0.1
	2	90.8	95.6	0.2	0.2	0.13	0.1	0.02	<0.1

[a] ACV-MP, ACV monophosphate. ACV-DP, ACV diphosphate. ACV-TP, ACV triphosphate.
[b] Mean picomoles per milligram = micromolar concentration.

sufficient organ involvement to assess the efficacy of ACV. Treatment of these HSV-infected mice with ACV at 80 mg/kg resulted in a significant antiviral effect. The observed reduction in mortality and the increased survival time correlate well with the findings of E. Kern for this dose of ACV (personal communication).

The present studies on the distribution of ACV in various tissues of uninfected and infected mice after single or multiple doses of ACV were in agreement with earlier studies performed with normal mice (13). In the earlier disposition studies with CD-1 mice given [8-^{14}C]ACV, it was shown that over 95% of the ^{14}C excreted in the 24-h urine was unchanged ACV, indicating that there was little biotransformation of the drug and making it possible to determine approximate ACV levels in the tissues from ^{14}C measurements. The experiments described here have indicated few significant differences (within the range of variations observed) in the uptake and concentration of ACV in tissues of infected mice when compared with those of uninfected mice. Although the mean levels of ACV, between 1- and 24-h postdosing, in the kidneys of infected mice were higher than the correspond-

TABLE 3. Metabolism of [8-^{14}C]ACV in HSV-2-infected CD-1 mice (3 to 4 weeks of age, three mice per group) weighing 15 to 20 g after a single s.c. dose of 50 mg/kg

Organ	Day p.i.[a]	Time post-dose (h)	Unconverted ACV		ACV-MP[b]		ACV-DP[b]		ACV-TP[b]	
			pmol/mg[c]	Mean % of total radioactivity	pmol/mg	Mean % of total radioactivity	pmol/mg	Mean % of total radioactivity	pmol/mg	Mean % of total radioactivity
Brain	4	0.5[d]	16.1	89.6	0.05	0.33	0.23	1.53	0.99	6.5
		2	12.0	98.5	0.02	<0.1	0.02	0.7	0.03	0.6
	5	0.5	11.4	99.9		0	0.05	<0.1	0.03	<0.1
		2	21.9	99.9		0	0.05	<0.1	0.16	<0.1
Liver	3	0.5	112.8	98.1	0.09	0.1	0.17	0.1	0.04	<0.1
		2	17.2	91.4	0.07	0.26	0.15	1.2	0.03	0.23
	4	0.5	182.8	98.5	0.03	<0.1	0.07	<0.1	0.01	<0.1
		2	62.3	92.5	0.10	0.53	0.22	1.2	0.1	0.15
Kidney	3	0.5	1,281.3	97.7	0.87	<0.1	0.15	<0.1	0.04	<0.1
		2	159.6	96.9	0.09	<0.1	0.24	0.13	0.05	<0.1
	4	0.5	3,698.3	96.9	3.76	0.13	0.78	<0.1	0.02	<0.1
		2	2,845.8	96.4	3.4	0.15	0.25	0.1	<0.01	<0.1

[a] p.i., Postinfection.
[b] ACV-MP, ACV monophosphate. ACV-DP, ACV diphosphate. ACV-TP, ACV triphosphate.
[c] Mean picomoles per milligram = micromolar concentration.
[d] Includes the brain extract with high levels of ACV mono-, di-, and triphosphate.

ing levels in uninfected mice (Fig. 2), the differences were not statistically significant ($P > 0.05$). The higher ACV levels in the plasma and tissues of infected mice, when compared with those of uninfected mice after multiple dosing, may indicate a trend for greater uptake and concentration of ACV in infected tissues. These differences were statistically significant only between the 1-h samples. Alternatively, they could have resulted from an impaired renal function in terminally ill animals. It is apparent that there was limited penetration of ACV into the brain when compared with other tissues. In all of the tissues, however, ACV trough levels were above the 50% effective dose for HSV and varicella-zoster virus (2, 3). In the brain tissue, they were above the 50% effective dose values for at least 6 h after dosing. The results also suggest that the ability of ACV to penetrate the blood-brain barrier was not significantly increased by virus infection. Although not apparent in this study, it is possible that a localized breakdown of the blood-brain barrier in the area of virus involvement could occur, since a relatively small proportion of the cells in an organ is infected (8). The brain can support the replication of 10^5 to 10^6 PFU of virus per g of tissue; other tissues had even lower viral titers. Assuming 10^8 to 10^9 cells per g of tissue, only 0.01 to 0.1% of the cells produce infectious particles at any one time. Thus, biochemical events specific to the virus-infected cells would be difficult to measure by methods involving whole tissue extraction. This may account for the inability to demonstrate increased uptake or anabolism of ACV to its phosphorylated forms in tissues of HSV-infected mice. The conditions in vitro were quite different when selective uptake and anabolism of ACV to its triphosphate form were demonstrated (4). In those studies, cells were synchronously infected with 5 to 10 PFU per cell and maintained in the continuous presence of the drug for 7 h during the period of optimal HSV-thymidine kinase activity.

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