Pharmacokinetics and Distribution over the Blood Brain Barrier of Two Acyclic Guanosine Analogs in Rats, Studied by Microdialysis

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The free extracellular concentrations of acyclovir and (*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (H2G) in the blood and in brain tissue of rats were measured in microdialysis samples by high-pressure liquid chromatography with UV detection. The half-life of acyclovir in blood was 2 h, while that of H2G was 28 min. The concentration attained in the brain compared with the concentration in blood was slightly lower for H2G than for acyclovir. The results show that the pharmacokinetic profiles of acyclic nucleosides may vary as a consequence of the structure of the acyclic chain corresponding to the sugar moiety.

Various strategies are used in the development of antiviral drugs in the form of structural analogs of nucleosides. In particular, modifications of the 3' substituent on the sugar moiety or acyclic sugar substitutes are used. The pharmacokinetic properties of these antiviral drugs are important for their in vivo effects. Microdialysis (18) offers a method for continuous monitoring of drug concentrations when drugs are in their unbound form in the extracellular space of several organs in the same experimental animal (11, 12, 15–17). This is achieved by inserting a thin dialysis tube into a tissue and collecting dialysates, which contain small molecules which diffuse from the extracellular fluid to the medium used to perfuse the dialysis probe. The method allows the collection of many samples without disturbing the blood volume, and the samples are sufficiently clean to allow direct analysis by reversed-phase high-pressure liquid chromatography (HPLC) without purification steps. In previous studies we have used microdialysis to study the distribution and pharmacokinetics of methylxanthines (11, 12, 15, 17) and the 3'-substituted thymidine analogs zidovudine and 3'-fluorothymidine (8, 13, 14), with particular emphasis on the distribution over the blood-brain barrier.

The aim of the present study was to study the distribution and pharmacokinetics in rats of two acyclic nucleoside analogs, acyclovir and (*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (H2G), over the blood-brain barrier. In addition, we estimated the binding of H2G to plasma proteins, since this information is not available in the literature. The choice of compounds was based on the detailed knowledge about the clinical effects and pharmacokinetic properties of acyclovir (4, 5, 7) and the unique effect profile of H2G, which has a considerable selectivity for varicella-zoster virus (1) and is highly efficient in monkeys infected with simian varicella virus (6, 9).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (weight, 180 to 250 g) were used throughout this study (Alab, Stockholm, Sweden). The rats had free access to tap water and standard lab chow and were housed at five rats per cage. Each rat was used only once.

Microdialysis. Detailed accounts of the microdialysis method can be found elsewhere (11, 12, 15–18). In the present study, rats were anesthetized with halothane during the entire experiment and were placed in a David Kopf stereotaxic instrument, with the bite bar 2.5 mm below the interaureal line. Dialysis probes (0.50-mm-diameter, 3.0-mm-long membrane) of the concentric type (CMA Microdialysis, Stockholm, Sweden) were implanted into the corpus striatum on one side of the brain (sterotaxic coordinates, 2.2 mm lateral, 1.3 mm anterior, and 6.5 mm ventral to the brain surface), the gastrocnemius muscle, and the jugular vein. The dialysis probe was perfused with a Ringer solution at a rate of 2 µl/min, and samples of 40 µl were collected at 20-min intervals. After implantation and a 40-min washout period, the perfusion medium was switched to a solution containing a 10 µM concentration of the drug that was later injected systemically (i.e., acyclovir was used in the perfusion medium of rats later treated with acyclovir). Three samples were collected, and the perfusion medium was switched back to the Ringer solution. After a second washout period of 40 to 60 min, no drug was detectable in the dialysates. The proportion of drug lost over the dialysis membrane was taken as an estimate of the in vivo recovery over the dialysis membrane (11). The procedure used to determine recovery was applied to each probe at each implantation site.

Either acyclovir or H2G was injected subcutaneously, and nine samples were collected postinjection (i.e., for 180 min.). The concentration measured in the dialysate was converted to estimates of the free extracellular concentration by dividing by the recovery, which was estimated as described above.

Plasma protein binding. Protein binding of H2G was determined by microdialysis as described in detail elsewhere (8, 16). In short, H2G was added to plasma (25°C) from untreated rats to a total concentration of 50 µM. The recovery of the dialysis probe was determined by using a stirred Ringer solution containing a known concentration of H2G (C_m) into which the dialysis probe was inserted and dialysis samples were collected, and the concentration was measured (C_d). The recovery is the ratio $R = C_d/C_m$. Then, the dialysis probe was inserted into the plasma with 50 µM H2G, which was stirred in the same way as the Ringer solution, and the dialysate concentration was measured. The

free concentration was then calculated as the dialysate concentration divided by the recovery R. The ratio of the free concentration to the total concentration was then the fraction unbound (fu), and $1 - fu$ was the fraction bound to plasma protein.

Antiviral drugs. Acyclovir was obtained from Burroughs Wellcome, Dartford, United Kingdom, and H2G (previously abbreviated 2 HM-HBG [1, 6]) was obtained from Medivir AB, Huddinge, Sweden. H2G was dissolved in Ringer solution to a concentration of 25 mg/ml. Acyclovir was difficult to dissolve and was injected as a suspension of 25 mg/ml. The systemic injections were 25 mg of acyclovir per kg of body weight ($n = 3$) and 25 mg of H2G per kg ($n = 4$) and were given subcutaneously.

Analysis of acyclovir and H2G. Analysis of acyclovir and H2G was made by isocratic HPLC separation and UV detection. A C_{18} column (100 by 4.0 mm; particle size, 5 μm) was used. The mobile phase was 0.05 M ($NH_4)H_2PO_4$ with 5 to 10% methanol, and a flow rate of 0.8 to 1.0 ml/min was used. H2G (retention time from the solvent front, 4.0 min) was easily separated from endogenous compounds, but acyclovir (retention time from the solvent front, 4.8 min) coeluted with an endogenous compound in muscle samples. The within-day coefficients of variation were 0.3% for acyclovir and 0.6% for H2G.

Partition coefficient of octanol-water. Equal volumes of octanol and a 100 μM water solution were mixed with a Vortex mixer, and the phases were separated by centrifugation (Wifug) for 2 min at 5,700 rpm. The concentrations of acyclovir ($n = 4$) and H2G ($n = 4$) in each phase were determined by HPLC.

pK$_a$ of H2G. Equal volumes (5 ml) of 0.02 M H2G and 0.01 M NaOH were thoroughly mixed, and the pH of the mixture was measured with a pH electrode which was carefully calibrated by using the standard calibration buffers of the manufacturer. The pH was taken as a measure of pKa since, in the equation pH + log $[A^-]/[HA]$ = pKa (where HA is the nonionized form and A^- is the ionized form), the term log $[A^-]/[HA]$ vanishes because the strong base NaOH forces the degree of ionization to be 50% (half-molar method).

Experimental design and statistics. The experimental design was a simple time-concentration curve. Because the samples were collected for 20 min, they are represented in Fig. 1 and 2 as the midpoint of the collection period (i.e., 10, 30, 50, . . . 170 min), which is an acceptable approximation (10). Data from individual rats are plotted in time-versus-log (concentration) diagrams. The half-lives of acyclovir and H2G were compared by Student's t test. An α value of 0.05 was chosen for hypothesis testing.

RESULTS

The levels of acyclovir varied considerably between individuals rats, as shown in Fig. 1. It was possible to estimate recovery of blood and brain dialysis probes in all animals, while an interfering peak in the HPLC assay made it impossible to estimate recovery of muscle in two of three rats. In the third rat, the extracellular concentrations in muscle and blood were virtually identical (Fig. 1A). The concentrations in the brain were approximately 30% of those in blood 1 h after administration. The half-life of the drug in the brain could not be determined, while the half-life in blood was approximately 2 h.

The plasma protein binding of H2G was below the level of detection (<5%). The maximum concentration of H2G in muscle and blood was attained 20 to 40 min after the
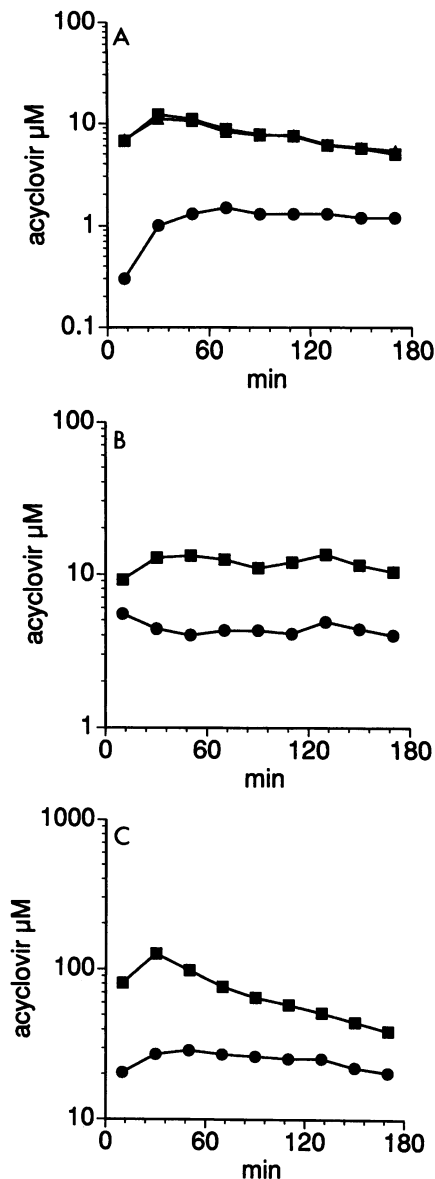

FIG. 1. Time-log (concentration) curves for free extracellular concentrations of acyclovir in blood (■), muscle (only in one rat) (▲), and brain (●) from three rats (A to C) treated subcutaneously with acyclovir at 25 mg/kg.

subcutaneous injection, and H2G was then rapidly eliminated, with half-lives of 28 and 25 min, respectively (Fig. 2). The distribution to the brain was slower, in that the peak concentration was maintained during 20 to 60 min after injection, suggesting that distribution and elimination balanced each other during this time period. In two rats, the concentration gradient was maintained during the 180-min observation period, while in two rats, the concentrations of H2G in the brain approached the concentrations in blood. The half-life in the brain was 49 min. The changes in distribution after 120 min should be interpreted with caution because the measurements were close to the limit of detection in the HPLC assay.

The partition coefficient of octanol-water for acyclovir was found to be 0.023 ± 0.001, and for H2G the partition
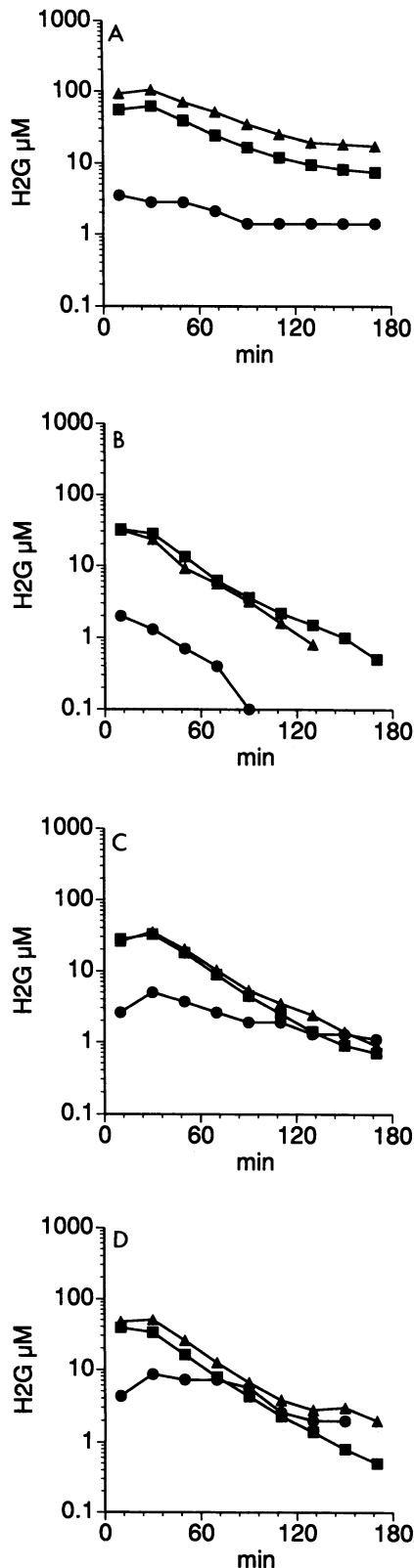
FIG. 2. Time-log (concentration) curves for free extracellular concentrations of H2G in blood (■), muscle (▲), and brain (●) from four rats (A to D) treated subcutaneously with H2G at 25 mg/kg.

coefficient was 0.041 ± 0.002 (values are means ± standard errors of the means). The pKa of H2G was 9.8.

DISCUSSION

Before discussing the findings particular to acyclovir and H2G, we want to comment on the microdialysis method. Microdialysis of blood is a way to sample the concentration of unbound drug in plasma. Similar information can also be achieved by repeated blood sampling, but not without considerably disturbing the blood volume when many samples are collected. The new type of information that can be obtained by microdialysis is a continuous registration of the concentration of unbound drug in the extracellular fluid in different tissues simultaneously in one animal in vivo. In particular, this bridges in vitro and in vivo data, in the sense that the concentration in the extracellular fluid represents the amount of drug available to the cells for uptake and phosphorylation, in the same way as the concentration in the medium does in cell culture test systems. However, microdialysis cannot measure the intracellular concentrations for obvious reasons. It is important to estimate the recovery in vivo for each probe, since the tissue contributes strongly to the recovery and recovery varies between tissues (11, 12). It should be noted that transients in concentrations may, in theory, also cause transients in recovery, although this seems to be a problem of little practical importance (12).

The pharmacokinetic properties of acyclovir in rats found in the present study are similar to those previously reported by other workers who used different sampling techniques (4). When acyclovir was given to rats intravenously, deMiranda et al. (4) found a half-life of 1.2 h, and when it was given orally, the half-life was 1.9 h in the early elimination phase. This suggests that the microdialysis method is appropriate for the study of pharamacokinetics in blood, as we have found with other nucleoside analogs (8, 14) and methylxanthines (17). Furthermore, the similarities between the present results and those of deMiranda et al. (4) indicate that the fact that a suspension of acyclovir is injected does not significantly affect the results.

The relatively high concentrations of acyclovir attained in the brain (20 to 30% of the levels in blood) correlate well with the levels of acyclovir in the brain tissues of rats, which increase from 20% at 0.5 h after subcutaneous administration to 30% at 6 h (4). It should be noted that the blood-brain barrier is often less permeable in rats than it is in primates, including humans (3).

The pharmacokinetic properties of H2G have not been investigated previously, except for a recent study in cynomolgus monkeys (2) and a previously published study of the racemic form (R,S-H2G), also in monkeys (6). In the present study, we found a short half-life in blood and muscle (25 to 28 min). This is in accordance with the results of Böttiger and Vrang (2), but it is considerably shorter than what was found with the racemic from in monkeys (6). It is possible that the metabolism of H2G is enantioselective, but we have no direct evidence for this at present.

The concentration of H2G in the brains of rats was approximately 10 to 20% of those in blood and muscle during the first hour after administration. In two rats this difference was maintained, while in the other two rats the levels in the brain approached those in blood. The changes in distribution found later than 2 h after administration must be interpreted with caution since the levels of H2G are small and close to the limit of detection in the HPLC assay. This is also reflected in the nonlinearity of the blood elimination curve at

the latest time points. Further studies are under way to examine the kinetics of H2G after prolonged administration.

An interesting observation is that the concentration of H2G in the brain declines at the same time as the concentration in blood declines, even though the concentration in blood is up to 5 to 10 times higher than the concentration in the brain. According to elementary pharmacokinetic compartment models with distribution by means of passive diffusion along concentration gradients, this is impossible. Hence, such models are inappropriate and active processes must participate to maintain the concentration gradient over the blood-brain barrier. The relatively small octanol-water partition coefficient of 0.041 suggests that the transport into the brain over capillaries may be slow, but this does not explain why a concentration gradient is maintained, the pKa of H2G (9.8 as determined by adding half the concentration of NaOH and measuring the pH), also fails to explain this, because an ion-trapping phenomenon cannot occur, since H26 will not be ionized at the physiological pH. Thus, the elementary physicochemical properties of H2G do not explain the findings of this study. Several mechanisms, such as nucleoside transport proteins, uptake and metabolism in brain cells, as well as transport from the brain via the cerebrospinal fluid, may contribute to an explanation of the present findings.

Acyclovir and H2G act as inhibitors of varicella-zoster virus and herpes simplex virus after phosphorylation by viral and cellular kinases to acyclovir and H2G triphosphates, which selectively inhibit the viral DNA polymerase (1). It has been found in vitro that the intracellular H2G triphosphate is more stable than the acyclovir triphosphate (1). Therefore, a comparison of the kinetics of the free nucleoside underrates the in vivo therapeutic potential of H2G compared with that of acyclovir in terms of half-lives.

We conclude that the microdialysis method is a convenient method of continuously monitoring levels of drugs in different organs, and further studies on H2G in monkeys are indicated in view of the excellent in vivo antiviral effects of H2G against simian varicella virus (6, 9). The findings of this study also indicate that therapeutic levels of H2G may be attained in the brain.

ACKNOWLEDGMENTS

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