Virus Inhibition Assay for Measurement of Acyclovir Levels in Human Plasma and Urine

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A simple microplate virus inhibition assay which measures the levels of acyclovir in plasma and urine samples from patients was developed. The assay is based upon the inhibition of the cytopathic effect of herpes simplex virus type 1 on human fibroblast cells. The extent of inhibition of virus cytopathic effect, caused by dilutions of samples from patients, allowed determination of acyclovir concentrations to be made. The assay, which measured biological activity, could detect acyclovir levels of ≥1.0 μM. Peak and valley levels measured in plasma samples from two patients were comparable to values obtained by radioimmunoassay. The assay is simple, rapid, and quantitative, and it can be adapted to routine use for a large number of specimens.

Acyclovir (9-(2-hydroxyethoxymethyl)guanine [ACV]) has potent in vitro antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 and varicella-zoster virus (1, 3, 4, 13). ACV is phosphorylated by HSV-induced thymidine kinase and preferentially inhibits virus-induced deoxyribonucleic acid polymerase (5, 7). This selective mechanism of action on a virus-specific function makes the compound more active in virus-infected cells with a low potential for toxicity to the host.

ACV has been reported to be effective in treatment of experimental herpes simplex infections in animal models and also in topical therapy of human HSV ocular infections (6, 9, 10, 11, 13). Clinical trials are now under way to determine the potential of parenteral ACV for treatment of human infections caused by herpes-group viruses.

Determination of the pharmacokinetics of ACV in patients is important in establishing both therapeutic and safe dosages and correlating these results with clinical outcome.

In this paper, we describe a virus inhibition assay which is simple, rapid, quantitative, and capable of measuring the biological activity of ACV in plasma and urine samples from patients receiving parenteral ACV.

MATERIALS AND METHODS

Cells. The human Trisomy 21 fibroblast line, CCL-54 (American Type Culture Collection) was used for all assays. Cells were propagated as monolayers in growth medium containing Eagle basal medium with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum. Maintenance medium consisting of BME, antibiotics, and 2% fetal bovine serum was used for virus-infected cultures.

Virus. HSV-1 Linderman was obtained from André Nahmias (Emory University, Atlanta, Ga.). Virus stocks were propagated in CCL-54 cells and stored at −70°C.

Plaque assay. Plaque reduction assays were performed in duplicate petri dishes (60 mm) (Falcon Plastics) containing monolayers of CCL-54 cells. The virus was diluted in Eagle basal medium to contain 60 to 100 plaque-forming units per dish. After a 1-h absorption at 37°C, the inoculum was aspirated and replaced by maintenance medium containing 1% methylcellulose and concentrations of ACV ranging from 0 to 1.0 μM. Plates were incubated for 48 h at 37°C, fixed with methanol, and stained with 1% crystal violet, and the number of plaques was counted.

Drugs. Acyclovir was provided by the Burroughs Wellcome Co. Stock solutions were prepared in sterile distilled water at a concentration of 500 μM.

Virus inhibition assay. Microtissue culture plates (96 wells; Falcon) were plated with approximately 3 × 10⁵ cells per well in growth medium and incubated at 37°C for 5 to 7 days until a confluent monolayer was present. The medium was aspirated, and 100 μl of Eagle basal medium containing 8 × 10⁵ plaque-forming units of HSV-1 Linderman was added to each well, absorbed for 1 h at 37°C, aspirated, and replaced by 50 μl of maintenance medium. The first two wells of each row were aspirated, and 100 μl of patient or control sample suspended in maintenance medium was added. Ten serial twofold dilutions were made in the plate, starting with the second well, by using a micropipette and mixing eight times before transfer.

Fifty microliters of maintenance medium was then added to all wells except the first, resulting in a final dilution of the sample from 1 to 1/1,024. Plates were incubated for 48 h at 37°C in 5% CO₂, fixed in metha-
nol, and stained with 1% crystal violet, and the viral cytopathic effect (CPE) was read at x10 magnification under a dissecting microscope. Scoring of CPE was as follows: no CPE, 0; 1 to 25% CPE, 1+; 25 to 50% CPE, 2+; 50 to 75% CPE, 3+; and 75 to 100% CPE, 4+. Each plate contained uninfected and infected control wells to ensure the presence of 0 and 4+ CPE, respectively, in the absence of test samples. The endpoint of inhibition was defined as the highest dilution of a sample which would produce 1+ CPE.

**Plasma and urine samples.** Whole-blood samples were aseptically collected from laboratory volunteers in heparinized tubes, and plasma was separated by centrifugation and stored at -20°C. Acyclovir was added to urine and plasma samples for use in reconstruction assays. An additional series of blood samples was obtained from each patient receiving parenteral ACV therapy for systemic herpes virus infections. All samples were deproteinized by centrifugation through an Amicon CF-50 ultrafiltration cone, and the filtrate was dried under nitrogen and resuspended to the original volume in maintenance medium before assay.

**RESULTS**

**Plaque reduction assay.** A plaque reduction assay was performed to determine the susceptibility of HSV-1 Linderman in CCL-54 cells to the antiviral effects of acyclovir. The average number of plaques in duplicate drug-treated cultures is presented as a percentage of plaques in control cultures (Fig. 1). The 50% inhibition (50% effective dose) of virus plaque formation was 0.2 μM. This result is within the range of previously reported susceptibilities of other HSV-1 isolates to ACV (4).

**Virus inhibition assay.** To simplify the assay and be able to use small-volume samples available in the clinical setting, we developed a virus inhibition assay for use in microculture plates. This assay is based upon the measurement of the highest dilution of a patient’s sample that will inhibit the cytopathic effect of HSV-1. This value is compared with the virus inhibition endpoint of known standard quantities of acyclovir.

To test the susceptibility and reproducibility of this assay in comparison with those of the plaque assay, we first determined standard dilution endpoints for ACV prepared in maintenance medium. CCL-54 cells grown in microculture were infected with HSV-1, and maintenance medium containing ACV concentrations of 200 μM was added to the first well of each of two rows; serial twofold dilutions were then made in the plates. The plates were incubated and scored for CPE as described above. The results of five initial experiments are tabulated in Table 1. The endpoints of the dilution with 1+ CPE occurred reproducibly at 1.25 and 0.625 μM acyclovir. These values remained constant in this assay and set the lower limit of susceptibility at approximately 1 μM.

To determine the accuracy of the microplate virus inhibition assay over a wide range of ACV concentrations and to construct a standard curve, we performed the assay with concentrations of ACV ranging from 0 to 200 μM diluted in maintenance medium. The reciprocal of the endpoint is plotted as a function of the concentration of ACV in Fig. 2 for triplicate experiments, and a linear regression line was calculated for the data points.

The concentration of ACV in the sample correlated highly with the reciprocal of the dilution of the endpoint. Endpoints did not deviate more than one dilution from the linear regression plot. By using this graph as a standard, it is possible to determine the concentration of ACV in an unknown sample, based on endpoints obtained in the assay.

**Reconstruction assays.** To ensure that deproteinization would not affect the concentration of ACV measured in plasma and urine samples, we performed reconstruction assays with plasma and urine samples containing concentrations of ACV ranging from 0 to 200 μM. The results for plasma samples were almost identical to those for maintenance medium, indicating that deproteinization did not interfere with recovery of acyclovir and that the plasma itself did not affect the assay (Fig. 3).
TABLE 1. Inhibition of HSV-1 CPE by acyclovir

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>CPE score at indicated drug concn (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 100 50 20 10 5 2.5 1.25 0.625 0.312 0.156 VC $^a$ CC $^b$</td>
</tr>
<tr>
<td>1</td>
<td>0 0 0 0 0 0 0 1+ 1+ 2+ 3+ 4+ 0</td>
</tr>
<tr>
<td>2</td>
<td>0 0 0 0 0 0 0 1+ 1+ 2+ 3+ 4+ 0</td>
</tr>
<tr>
<td>3</td>
<td>0 0 0 0 0 0 0 1+ 2+ 2+ 3+ 4+ 0</td>
</tr>
<tr>
<td>4</td>
<td>0 0 0 0 0 0 0 1+ 2+ 2+ 3+ 4+ 0</td>
</tr>
<tr>
<td>5</td>
<td>0 0 0 0 0 0 0 1+ 2+ 3+ 3+ 4+ 0</td>
</tr>
</tbody>
</table>

$^a$ VC, Virus control; no acyclovir added.

$^b$ CC, Cell control; no virus or acyclovir added.

**Fig. 2.** Virus inhibition assay with known amounts of acyclovir in maintenance medium. The endpoint of each sample is graphed as a function of the acyclovir concentration. Symbols: ●, experiment 1; ■, experiment 2; and ▲, experiment 3. Linear regression plot of data points is indicated by the line.

Figure 4 shows an analogous reconstruction experiment for urine samples. The plot is similar to that obtained for plasma and maintenance medium. Urine samples caused cellular toxicity in dilutions under 1/4. This finding proved to be of little practical consequence, because urine samples of patients receiving ACV contain very high concentrations of ACV.

These reconstruction assays indicate that the ACV in patient plasma and urine can be accurately measured by the virus inhibition assay after deproteination. Furthermore, endpoints based on assays with known amounts of ACV in maintenance medium can be used to construct standard curves to be used in assays of plasma and urine.

**Assay of samples from patients.** The levels of ACV in plasma and urine samples of two patients receiving various doses of parenteral ACV were measured. Each study included a standard assay with known amounts of ACV in the maintenance medium. The endpoints of the standard assays were used to construct a curve analogous to that shown in Fig. 2. Each patient sample was assayed in duplicate or triplicate, endpoints were matched to ACV concentrations by using the standard curve, and the results were averaged. Urine samples were diluted 10-fold because of the high concentrations of ACV present in the samples.

The urine and plasma levels of ACV in patients receiving doses of 500 mg/m$^2$ (12 mg/kg) and of 83 mg/m$^2$ (1.8 mg/kg) in 1-h intravenous infusions are presented in Tables 2 and 3, respectively. Portions of the samples were assayed for ACV by using the radioimmunoassay (RIA) technique, and values are included for direct comparison (8). The concentrations of ACV in the samples measured by RIA are graphed in Fig. 5 as a function of the concentration measured by the virus inhibition assay. A linear regression plot of the data has a slope of 0.81.
and a correlation coefficient of 0.989. This indicates that the values obtained by the virus inhibition assay were highly correlated with the values obtained by RIA and generally were 20% higher.

**Fig. 4.** Virus inhibition assay with known amounts of acyclovir in urine. The endpoint of each sample (■) is graphed as a function of the acyclovir concentration. Linear regression plot of data is indicated by the solid line. Linear regression plot of data from assay in maintenance medium presented in **Fig. 2** is indicated by the broken line.

**Table 2.** Plasma and urine levels of acyclovir in a patient receiving doses of 500 mg/m²

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acyclovir levels (µM) determined by:</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Viral inhibition assay</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td>Preinfusion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Postinfusion&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.0</td>
</tr>
<tr>
<td>Preinfusion</td>
<td>2</td>
</tr>
<tr>
<td>Postinfusion</td>
<td>49.0</td>
</tr>
<tr>
<td>Preinfusion</td>
<td>3</td>
</tr>
<tr>
<td>Postinfusion</td>
<td>49.0</td>
</tr>
<tr>
<td>Preinfusion</td>
<td>4</td>
</tr>
<tr>
<td>Postinfusion</td>
<td>49.0</td>
</tr>
</tbody>
</table>

**Urine (24-h collection)**
2,785 2,313

<sup>a</sup> RIA levels courtesy of M. Hintz and J. D. Connor.
<sup>b</sup> Collected immediately before infusion, which was given every 8 h intravenously.
<sup>c</sup> Collected immediately after a 1-h infusion, which was given every 8 h intravenously.

**Table 3.** Plasma levels of acyclovir in patient receiving doses of 83 mg/m²

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acyclovir levels (µM) determined by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viral inhibition assay</td>
</tr>
<tr>
<td>Preinfusion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
<tr>
<td>Immediately post-</td>
<td>12.8</td>
</tr>
<tr>
<td>infusion&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>2 h postinfusion</td>
<td>3.4</td>
</tr>
<tr>
<td>4 h postinfusion</td>
<td>11.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> RIA levels courtesy of M. Hintz and J. D. Connor.
<sup>b</sup> Collected immediately before infusion, which was given every 8 h intravenously.
<sup>c</sup> Collected immediately after a 1-h infusion, which was given every 8 h intravenously.

**Fig. 5.** Acyclovir concentrations in clinical plasma samples determined by RIA and graphed as a function of the concentrations determined by the virus inhibition assay. Linear regression plot of the data points is indicated by the line.

**DISCUSSION**

Assay methods for detecting ACV in plasma samples include high-performance liquid chromatography (4) and RIA, with sensitivities of ≥1.0 and ≥0.1 µM, respectively (12). Both of these methods have limitations in clinical and research use. High-performance liquid chromatography is time consuming and requires expensive equipment, and specific rabbit anti-ACV antibody and radioactive-labeled ACV are necessary for the RIA (12). In addition, these methods do not necessarily measure biologically active drugs.

The virus inhibition assay described here al-
lows rapid and simple determination of acyclovir concentrations in plasma and urine samples from patients. This method requires only small patient samples, 0.6 ml being sufficient for triplicate assays. The equipment necessary to carry out this assay is available in most research and clinical virology laboratories, and the use of microculture plates conserves materials and allows convenient measurement of many samples simultaneously.

The assay is similar in principle to bioassay techniques previously described which measure the levels of adenine arabinoside and ribavirin (2, 14). In contrast to adenine arabinoside, acyclovir has been shown to have minimal cleavage or metabolism in vivo (12). This makes it possible to use the virus inhibition assay presented here with only simple preparation of samples.

This assay technique involves serial twofold dilutions of specimens from patients until an endpoint is reached, analogous to determining an antibody titer by using a complement fixation test which is accurate within one dilution of the endpoint. This potential variability of the test was reduced by including a standard curve of known ACV concentrations from 0 to 200 μM with each assay (Fig. 2). Each sample was run in triplicate or duplicate to ensure an accurate endpoint determination, and the results were averaged.

The concentrations of ACV determined in samples from patients correlated well with the RIA results. The lower limit of sensitivity of our assay, approximately 1.0 μM, is higher than that of the RIA. Because of this, measurements of low levels of ACV should be more accurate if done by RIA. This difference in sensitivity caused a greater percentage of disagreement in values close to the lower limit of sensitivity of the virus inhibition assay.

Overall, the concentrations measured by the virus inhibition assay were 20% higher than those measured by RIA. The difference may be explained in part by a recent discovery that the specific antibody used in the RIA of these particular specimens may not measure both the free base of ACV and its sodium salt (M. Hintz, personal communication). This suggests that the true correlation of the techniques may be even closer than reported. In contrast to RIA and high-performance liquid chromatography, the virus inhibition assay measures the biological activity of ACV. The fact that ACV concentrations measured by RIA and the virus inhibition assay were similar indicates that the majority if not all of the ACV measured by RIA retained biological activity.

The lowest ACV concentration measurable by the assay is limited by the susceptibility of the virus to ACV. The HSV-1 strain used in this assay was inhibited by a relatively low concentration of ACV; however, it is possible that the use of a more susceptible HSV-1 strain or another cell line may increase the sensitivity of the assay. In addition, the assay can be adapted to a 24-h procedure with the use of an HSV-1 virus stock with a higher titer. This compares favorably with the 2 to 3 days required for obtaining results with the RIA technique.

This assay has proved to be reliable and clinically useful in following patients receiving parenteral acyclovir. The principle of this technique may also be applied to other antiviral agents for the study of pharmacokinetics.

ACKNOWLEDGMENTS

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LITERATURE CITED


