Research Article

A Sensitive and Specific Liquid-Chromatographic Assay for Determination of Ganciclovir in Plasma and Urine and Its Application to Pharmacokinetic Studies in the Rabbit

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A liquid-chromatographic assay for the analysis of ganciclovir in plasma and urine is described. This assay involves the use of acyclovir, an antiviral drug structurally related to ganciclovir, as the internal standard. A two-step sample preparation method is used. After protein is precipitated with acetonitrile and the addition of diethyl ether, ganciclovir and the internal standard are back extracted into a small volume of aqueous ammonium phosphate, taking advantage of their relatively high water solubility. This isocratic method is specific and sufficiently sensitive to allow quantification of ganciclovir throughout the entire range of concentrations observed during therapeutic use of this antiviral drug. There was no interference from various over-the-counter and prescription drugs often prescribed to patients most likely to receive ganciclovir therapy. This assay was used to analyze plasma and urine samples obtained after intravenous administration of ganciclovir to rabbits. Biexponential decay of ganciclovir plasma concentration–time and urinary excretion rate–time profiles was observed, with a mean distribution half-life of 15.8 min and an elimination half-life of 96 min. The mean renal clearance, 9.0 ml/min per kg, exceeds the glomerular filtration rate in the rabbit, indicating that ganciclovir is actively secreted in the renal tubule. Similar results were obtained by determining the renal clearance at steady state during constant-rate intravenous infusion of ganciclovir.

KEY WORDS: ganciclovir; analysis; pharmacokinetics; rabbit model; renal secretion.

INTRODUCTION

Ganciclovir {9-[(1,3-dihydroxy-2-propoxy)-methyl]-guanine; DHPG; Fig. 1} is an acyclic nucleoside that is a potent inhibitor of viral replication of the herpes family, including cytomegalovirus (CMV) (1–4). CMV infections are a major cause of morbidity and mortality in individuals with suppressed cellular immunity, such as patients with acquired immunodeficiency syndrome (AIDS), or those on immunosuppressive therapy following organ transplantation. Intravenous ganciclovir has been approved for the treatment of CMV retinitis and has shown promising therapeutic effect in the treatment of other CMV infections (5–7).

The main side effect associated with ganciclovir therapy is suppression of bone marrow function, which appears to be dose dependent (8). Since patients who require ganciclovir therapy are usually on multiple-drug therapy for the treatment of their primary condition, this may lead to pharmacokinetic drug–drug interactions that affect the disposition of ganciclovir. The majority of a dose is excreted by the kidneys, necessitating dosage adjustment in patients with reduced renal function (9–11). These factors demonstrate the importance of monitoring the plasma concentrations during ganciclovir therapy. A simple, specific, and reliable analytical method is needed for pharmacokinetic and drug–drug interaction studies and for routine monitoring of ganciclovir therapy.

Methods for the determination of ganciclovir in biological fluids including high-pressure liquid chromatography (HPLC) (12), radioimmunoassay (RIA) (13), and enzyme-linked immunosorbent assay (14) have been reported. The immunological methods are more sensitive than the HPLC method, which was reported to have a detection limit of 0.1 μg/ml, but they require lengthy procedures for the preparation of assay reagents such as antisera and antibodies.

We describe a simple and reproducible HPLC method for the analysis of ganciclovir. This method, unlike that reported previously (12), utilizes an internal standard and affords a limit of determination of 0.02 μg/ml in 1 ml of plasma. The assay was fully validated in plasma and urine, and 17 over-the-counter or prescription drugs commonly used in treating immunodeficient patients were shown not to interfere with the method. This assay was used to analyze urine and plasma samples obtained after intravenous administration of ganciclovir to rabbits, providing pharmacokinetic data in this animal model in a range of concentrations seen during therapy in humans.

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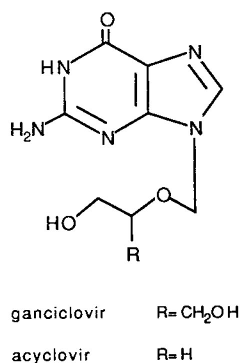

Fig. 1. Structure of ganciclovir and acyclovir.

MATERIALS AND METHODS

Chemicals

Ganciclovir obtained from Syntex (Palo Alto, CA, Lot No. 681-103A) and acyclovir obtained from Burroughs Wellcome Co. (Research Triangle Park, NC) were used to prepare the standard solutions for analysis. Acetonitrile and diethyl ether were supplied by Burdick and Jackson Laboratories, Inc. (Muskegon, MI); monobasic ammonium phosphate and ammonium hydroxide were purchased from Mallinckrodt, Inc. (St. Louis, MO). All solvents were of HPLC grade.

Instrumentation

A high-pressure liquid chromatograph (Model 1084B; Hewlett-Packard, Palo Alto, CA) equipped with a 254-nm fixed-wavelength detector and an automatic sampling system is used. A 15-cm × 4.6-mm (i.d.) C_{18} Supelcosil reversed-phase column with an average particle size of 5 μm (LC-18; Supelco Inc., Bellefonte, PA) is used for chromatographic separation. The flow rate of the mobile phase, 1 ml of acetonitrile per 99 ml of 10 m*M* monobasic ammonium phosphate adjusted to pH 6.8 with ammonium hydroxide, is 1 ml/min. The column temperature is ambient, and the effluent is monitored at 254 nm (the λ_{max} of ganciclovir). Peak areas are measured with an electronic integrator (Model 79850B LC; Hewlett-Packard), using an integrator attenuation of 2^4.

Sample Preparation

Ganciclovir was dissolved in methanol to prepare a stock solution of 10 μg/ml. Acyclovir (the internal standard) was dissolved in methanol to prepare a stock solution at a concentration of 50 μg/ml. The ganciclovir solution was used to add ganciclovir to a series of nine 13-ml ground glass-stoppered tubes (Kontes, Evanston, IL) in amounts of 0 (blank), 0.1, 0.2, 0.4, 1, 2, 4, 6, and 8 μg to prepare the plasma standard curve and to another series of nine tubes in amounts of 0 (blank), 0.2, 0.5, 1, 2, 4, 6, 8, and 10 μg to prepare the urine standard curve. Thirty microliters of the internal standard solution was added to each of these tubes. The same amount of the internal standard was added to another series of 13-ml tubes for the plasma or urine samples. The methanol was evaporated from the standard curve and sample tubes under reduced pressure, with an evaporator (Evapo-Mix; Buchler Instruments, Fort Lee, NJ).

Blank human plasma, 1.0 ml (or 10 μl of blank urine diluted to 1 ml with distilled water), was added to each of the standard-curve tubes, and 1 ml of the plasma sample (or 10 μl of urine sample diluted to 1 ml with distilled water) was added to the appropriate sample tube. Five milliliters of acetonitrile was added to all tubes, which were then stoppered and shaken horizontally at 180 cycles/min on a mechanical shaker (Eberbach Corp., Ann Arbor, MI) for 10 min. After centrifugation for 10 min at 750*g* (Damon, Needham Heights, MA) the supernate was decanted into a clean set of 13-ml tubes, each containing 0.3 ml aqueous solution of 2% (w/v) monobasic ammonium phosphate. Five milliliters of diethyl ether was added, and the tubes were shaken for 10 min and centrifuged for 10 min. The resulting mixture formed a small volume (≈0.3 ml) of aqueous phase in the bottom of the tube and an organic phase on the top. The organic phase was aspirated and discarded. To the aqueous phase was added 5 ml of diethyl ether. After shaking for 5 min and centrifuging for 10 min, the organic phase was aspirated and discarded. The aqueous phase was transferred to microvials for automatic injection. Injections of 20 μl were made, using the chromatographic conditions described above.

Calculations

The peak-area ratios of ganciclovir/internal standard are calculated and the standard curve is constructed by weighted least-squares linear regression of the peak-area ratios vs concentrations. The variances of the peak-area ratios obtained during the analysis of four different plasma standard curves or five different urine standard curves on the same day were used as the weights for plasma and urine standard curves, respectively. Unknown ganciclovir concentrations are determined from the regression equation.

Pharmacokinetic Studies in the Rabbit

Three male New Zealand White rabbits, weighing 3.3 ± 0.2 kg (Birchwood Farm Rabbitry, Birchwood, WI), received a single intravenous bolus dose of 10 mg/kg ganciclovir. Blood samples were collected over 6.5 hr in heparinized tubes through a catheter inserted into the anterior vena cava via the marginal ear vein, without anesthesia. Plasma was obtained by centrifugation. Frequent urine samples were collected over 10 hr using a Foley pediatric catheter, size 8 FR, inserted into the rabbit bladder via the urethra. The bladder was irrigated with four 15-ml aliquots of normal saline maintained at 37°C at 15, 11, 7, and 3 min before the end of the urine collection interval to ensure complete recovery of bladder contents.

On the following day, the same three rabbits received a constant-rate intravenous infusion of 3 mg/hr ganciclovir for 12 hr to achieve steady state. Simultaneous urine and plasma samples were collected hourly as described above, for 5 additional hr. Urine and plasma samples were stored at −20°C until analysis. The method described above was used to analyze 0.5 ml of plasma and 0.1 ml of diluted urine for ganciclovir.

Pharmacokinetic Analysis

After iv administration, the disposition of ganciclovir in the rabbit can be described by a two-compartment pharmacokinetic model with elimination from the central compartment. Ganciclovir disposition is via renal and nonrenal elimination.

$$C_P = \frac{X_0(\alpha - K_{21})}{V_c(\alpha - \beta)} e^{-\alpha t} + \frac{X_0(K_{21} - \beta)}{V_c(\alpha - \beta)} e^{-\beta t} \quad (1)$$

$$\frac{dX_e}{dt} = K_e \left[\frac{X_0(\alpha - K_{21})}{(\alpha - \beta)} e^{-\alpha t} + \frac{X_0(K_{21} - \beta)}{(\alpha - \beta)} e^{-\beta t} \right] \quad (2)$$

Equations (1) and (2) describe the ganciclovir plasma concentration–time profile and the urinary excretion rate-time profile after a single iv bolus dose, where dX_e/dt and K_e are the renal excretion rate and the renal excretion rate constant. The remaining terms are as usually defined (15).

Plasma concentration and urinary excretion rate data for each rabbit were fitted simultaneously to Eqs. (1) and (2) using the reciprocal of the observation squared as the weighting function. PCNONLIN was used to obtain the pharmacokinetic parameters (16).

In the analysis of the bolus data, the renal clearance was calculated from the ratio of the model-predicted urinary excretion rates to the model-predicted plasma concentrations. However, in the analysis of the infusion data, the renal clearance at steady state was calculated from the ratio of the urinary excretion rate and the midpoint plasma concentration during each urine collection interval.

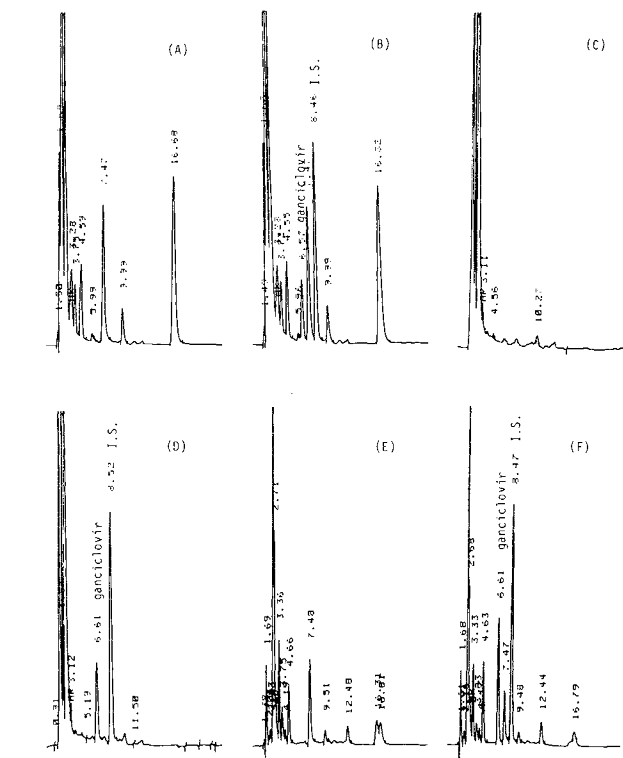

Fig. 2. Representative chromatograms for the analysis of ganciclovir in human plasma and urine and rabbit plasma. (A) Blank human plasma; (B) blank human plasma with added ganciclovir and internal standard (I.S.) (0.4 μg ganciclovir per ml); (C) blank human urine; (D) blank human urine with added ganciclovir and internal standard (40 μg ganciclovir per ml); (E) blank rabbit plasma; (F) rabbit plasma sample obtained after intravenous ganciclovir administration (1.0 μg ganciclovir per ml). Sample volumes: 1 ml of human plasma, 10 μl of human urine, and 0.5 ml of rabbit plasma.

RESULTS AND DISCUSSION

Assay Validation

Ganciclovir is an acyclic nucleoside with a water solubility of 4.3 mg/ml at pH 7.0 and 25°C (1). Because of its high water solubility, ganciclovir was not extractable with organic solvents. A two-step sample preparation is used in the plasma assay. After protein is precipitated with acetonitrile, diethyl ether is added and ganciclovir is extracted in a small volume of aqueous ammonium phosphate. An additional ether extraction of the aqueous phase is performed to obtain cleaner chromatograms.

Table I. Analytical Precision of DHPG Assay in Plasma and Urine

DHPG assay precision in plasma					DHPG assay precision in urine				
	Within run (n = 4)[a]		Run to run (n = 5)[b]			Within run (n = 5)[a]		Run to run (n = 5)[b]	
Conc. (mg/L)	Peak area ratio (mean ± SD)	CV (%)	Peak area ratio (mean ± SD)	CV (%)	Conc. (mg/L)	Peak area ratio (mean ± SD)	CV (%)	Peak area ratio (mean ± SD)	CV (%)
0.000	0.000	—	0.000	—	0.000	0.000	—	0.000	—
0.1	0.071 ± 0.005	6.4	0.065 ± 0.007	11.0	20.0	0.119 ± 0.003	2.3	0.110 ± 0.006	5.0
0.2	0.164 ± 0.008	4.7	0.151 ± 0.013	8.8	50.0	0.273 ± 0.013	4.7	0.252 ± 0.007	2.7
0.4	0.321 ± 0.019	5.8	0.331 ± 0.014	4.1	100	0.525 ± 0.012	2.4	0.506 ± 0.022	4.4
1.0	0.817 ± 0.017	2.0	0.807 ± 0.013	1.6	200	1.043 ± 0.020	1.9	1.005 ± 0.038	3.7
2.0	1.667 ± 0.027	1.6	1.646 ± 0.037	2.3	400	2.064 ± 0.043	2.1	1.986 ± 0.070	3.5
4.0	3.385 ± 0.105	3.1	3.231 ± 0.181	5.6	600	3.125 ± 0.074	2.4	2.977 ± 0.100	3.3
6.0	4.984 ± 0.175	3.5	5.024 ± 0.092	1.8	800	4.147 ± 0.044	1.1	3.946 ± 0.134	3.4
8.0	6.726 ± 0.159	2.4	6.651 ± 0.149	2.2	1000	5.288 ± 0.123	2.3	4.921 ± 0.106	2.1
Slope	0.840 ± 0.022	2.6	0.835 ± 0.017	2.1	Slope	0.0052 ± 0.00008	1.6	0.0491 ± 0.0001	2.4

[a] Analyzed on the same day.
[b] Analyzed on 5 different days.

Table II. Accuracy in the Analysis of DHPG Quality-Control Plasma and Urine Samples

Accuracy in the analysis of plasma samples (n = 6)				Accuracy in the analysis of urine samples (n = 4)			
Nominal conc. (mg/L)	Measured conc., mean ± SD (mg/L)	Percentage of nominal conc.	CV (%)	Nominal conc. (mg/L)	Measured conc., mean ± SD (mg/L)	Percentage of nominal conc.	CV (%)
0.1	0.107 ± 0.012	107	11.1	20.0	20.9 ± 2.28	104	10.9
0.4	0.41 ± 0.016	102	4.0	100	106 ± 2.35	106	2.2
2.0	2.05 ± 0.054	102	2.6	400	413 ± 18.9	103	4.6
6.0	6.06 ± 0.237	101	3.9	800	814 ± 18.5	102	2.2

Specificity and Reproducibility. Figure 2 shows typical chromatograms obtained in the analysis of blank human plasma and urine and of blank human plasma and urine supplemented with ganciclovir and the internal standard. Figure 2 also shows the chromatograms of blank rabbit plasma and a rabbit plasma sample obtained after intravenous administration of ganciclovir. No interfering peaks were observed in these chromatograms. Although the retention times of ganciclovir and the internal standard were 6.6 and 8.5 min, respectively, there was an endogenous compound that showed at a retention time of 17 min. When the present method was used for routine sample analysis, samples were injected every 12 min and the late peak eluted in the following chromatogram without interference.

The chromatographic behavior of a number of over-the-counter drugs and agents commonly prescribed for immunodeficient patients was examined. A solution of each of the drugs was injected onto the HPLC and the retention time was determined. Whenever the observed retention time was similar to that of ganciclovir or acyclovir, the drug solution was taken through the sample preparation procedure, prior to chromatography. Acetaminophen, aspirin, salicylic acid, caffeine, amoxicillin, cephalothin, cefotaxime, streptomycin, tobramycin, trimethoprim, sulfamethoxazole, isoniazide, metronidazole, nystatin, amphotericin B, flucytosine, and zidovudine did not interfere with ganciclovir analysis. For patients receiving acyclovir and ganciclovir, an alternative internal standard must be used. However, concomitant administration of acyclovir and ganciclovir is very unlikely.

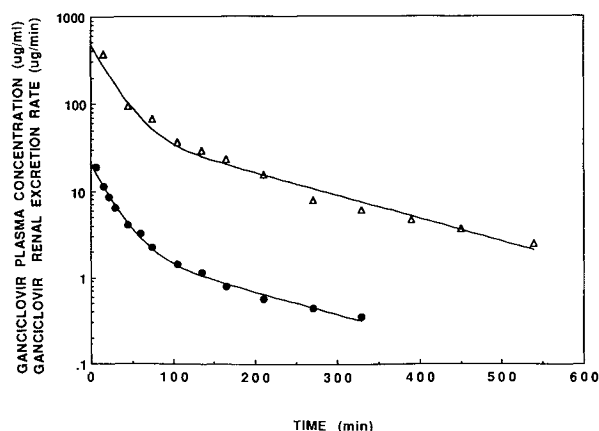

Fig. 3. Ganciclovir plasma concentration–time profile (●) and urinary excretion rate–time profile (△) obtained after administration of a single intravenous bolus dose of 10 mg/kg ganciclovir in one rabbit.

Sensitivity. The sensitivity criteria for five different plasma standard curves (mean ± SD) were calculated using the method described by Oppenheimer *et al.* (17). The critical level (the assay response above which an observed response is reliably recognized as detectable) was 0.011 ± 0.004 μg/ml. The detection limit (the actual net response which may a priori be expected to lead to detection) was 0.022 ± 0.008 μg/ml. The determination limit (the concentration that can be measured with a coefficient of variation of 10%) was 0.076 ± 0.021 μg/ml. The sensitivity criteria determined for five urine standard curves (mean ± SD) were 1.36 ± 0.49, 2.71 ± 0.98, and 4.62 ± 2.55 μg/ml for critical level, detection limit, and determination limit, respectively.

Linearity and Precision. Standard curves obtained during the analysis of ganciclovir in plasma and urine were linear throughout the entire range of concentrations. Within-run precision was determined by analysis of four different plasma standard curves and five different urine standard curves on the same day. Run-to-run precision was determined from standard curves prepared on each of 5 different days over a 45-day period. The precision of the assay determined from the variability in the peak-area ratios at each concentration is summarized in Table I. The coefficient of variation (%) for within-run precision ranged from 1.6 to 6.4 and 1.1 to 4.7 for plasma and urine, respectively, and that for run-to-run precision ranged from 1.6 to 11.0 and 2.1 to 5.0 for plasma and urine, respectively.

Accuracy. Known amounts of ganciclovir were added to blank human plasma and urine to prepare samples of concentrations ranging from 0.1 to 6.0 μg/ml and 20 to 800 μg/ml for plasma and urine, respectively. The samples were stored at −20°C until analysis. Aliquots of these plasma and

Table III. Ganciclovir Pharmacokinetic Parameters Obtained After a Single Intravenous Dose from Compartmental Analysis Using Plasma and Urine Data

Parameter	Rabbit No. 1	2	3	Mean	SD
Vd_c (L/kg)	0.55	0.49	0.61	0.55	0.06
$t_{\alpha 1/2}$ (min)	15.9	16.1	15.3	15.8	0.42
$t_{\beta 1/2}$ (min)	87.7	115.8	84.5	96.0	17.2
AUC (μg-min/ml)	643	795	580	672	110
CL_{TOT} (ml/min/kg)	15.5	12.6	17.3	15.1	2.4
CL_R (ml/min/kg)	9.2	7.3	10.5	9.0	1.6
Urinary recovery (% of dose)	59	58	61	59	1.5

Table IV. Ganciclovir Pharmacokinetic Parameters Obtained at Steady State During Constant-Rate Intravenous Infusion

Parameter	Rabbit No. 1	2	3	Mean	SD
Plasma concentration (μg/ml)[a]	1.24 (0.03)	1.33 (0.04)	1.10 (0.01)	1.22	0.12
Renal clearance (ml/min/kg)[a]	8.8 (0.83)	10.8 (1.45)	9.3 (0.96)	9.6	1.04
Fraction excreted unchanged in the urine (%)[a]	68 (6.4)	70 (9.4)	70 (7.3)	69	1.2

[a] Mean (SD) of five hourly determinations for each rabbit.

urine samples were analyzed for ganciclovir over a period of 45 days. The accuracy of the assay determined by comparing the nominal concentrations with the measured concentrations is summarized in Table II. The coefficient of variation (%) for the assay accuracy ranged from 2.6 to 11.1 and 2.2 to 10.9 for plasma and urine, respectively.

These experiments also confirmed the stability of ganciclovir in plasma and urine stored at −20°C for 45 days, because the measured concentrations in both plasma and urine did not show a decreasing trend over this period.

Analytical Recovery. We compared peak-area ratios determined for five different plasma samples which were carried through the analytical procedure with peak-area ratios for five unextracted samples. The internal standard was added to all samples just prior to injection on the chromatograph. The mean proportion of ganciclovir accounted for, without correction for volume loss during transfer or aspiration of organic phase, was 34%. Despite this relatively modest recovery, the method is reproducible and sufficiently sensitive to cover the entire range of concentrations observed clinically.

Pharmacokinetics of Ganciclovir in the Rabbit

The plasma concentration–time and urinary excretion rate–time profiles after a single intravenous bolus dose of 10 mg/kg were parallel and declined biexponentially. The mean distribution half-life was 15.8 min, and the elimination half-life was 96 min. Sixty percent of the administered dose was excreted unchanged in the urine. Figure 3 provides representative examples of the plasma concentration-time and the urinary excretion rate–time profiles in one of the rabbits. Table III summarizes the pharmacokinetic parameters obtained from the compartmental analysis. The estimated renal clearance of ganciclovir is 9 ml/min per kg, which exceeds the glomerular filtration rate in the rabbit determined in our laboratory (3–5 ml/min per kg; unpublished data), indicating that ganciclovir is actively secreted in the renal tubule as it is in humans (8,10). Similar estimates of the renal clearance were obtained by calculating the renal clearance from the urinary excretion rates and plasma concentrations during each urine collection interval.

At steady state, the fraction excreted unchanged in the urine was 69% of the administered dose, and the renal clearance was 9.6 ml/min per kg, in agreement with the values determined after single intravenous dose administration.

The pharmacokinetic parameters obtained at steady state are summarized in Table IV. The pharmacokinetic parameters for each rabbit estimated after a single intravenous dose from the compartmental analysis were used to simulate the plasma concentration at steady state. The mean predicted steady-state plasma concentration was 1.04 ± 0.19 μg/ml, compared with the mean observed steady-state plasma concentration of 1.22 ± 0.12 μg/ml, indicating good characterization of ganciclovir clearance in the single-dose studies.

Ganciclovir pharmacokinetic studies in man have shown that it follows a two-compartment model with renal clearance exceeding the GFR, indicating active secretion of this antiviral agent in the renal tubule. This high renal clearance results in much greater ganciclovir concentrations in the urine than in plasma. The present analytical method can be used to measure concentrations in the range of 0.1–8.0 and 20–100 μg/ml in human plasma and urine, respectively, which covers the range of concentrations observed during therapeutic dosing of ganciclovir. This method is simple and specific, making it suitable for pharmacokinetic studies and for routine monitoring of ganciclovir therapy.

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