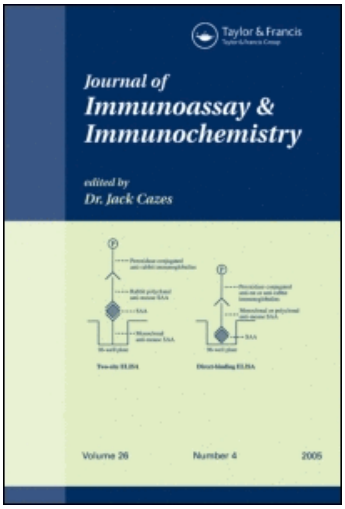


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# Radioimmunoassay for Desciclovir, 2-[(2-Amino-9H-Purin-9-YL) Methoxy] Ethanol, A Prodrug for the Antiviral Acyclovir

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# RADIOIMMUNOASSAY FOR DESCICLOVIR, 2-[(2-AMINO-9H-PURIN-9-YL)METHOXY]ETHANOL, A PRODRUG FOR THE ANTIVIRAL ACYCLOVIR

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## ABSTRACT

A direct radioimmunoassay for the detection of desciclovir (DCV)(2) in biological fluids has been developed. The radioimmunoassay was validated by comparing results obtained from human plasma samples analyzed by both this RIA and a gas chromatographic method. None of the crossreactivities noted interfere with the assay system. Although the succinylated antigen has a slightly higher affinity constant, the non-succinylated tritiated antigen was chosen for routine use. The assay is sensitive with an $I_{50}$ value of 20 nM and with a lower limit of detection of about 3 nM. Intra-assay precision

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gave sample coefficients of variation which ranged from 2.2 to 9.6 % for the standard curve with human plasma and from 2.0 to 8.2 % for the standard curve with human urine. Inter-assay precision and accuracy were within acceptable limits. (KEY WORDS: radioimmunoassay, antiviral, prodrug, desciclovir).

## INTRODUCTION

The oral formulation of acyclovir (ACV) has limited absorption from the gastrointestinal tract, giving lower plasma concentrations of this antiviral agent than are obtained with its intravenous form (1). Because some members of the herpes virus group have relatively high minimal inhibitory concentrations (when compared to Types I and II) they are adequately treated only with relatively large oral doses of ACV. A prodrug of ACV, Desciclovir (DCV), has been developed that has good gastrointestinal absorption (2,3); it is currently undergoing clinical trials. Therefore, it was desirable to develop a rapid and sensitive radioimmunoassay (RIA) for DCV. A preliminary report (4) outlines such an assay. A more recent method of detecting DCV in body fluids is by HPLC, a time consuming method in which only a limited number of samples can be conveniently processed (Krasny, H.C., unpublished data). This paper describes the development of an RIA for DCV, compares it with a gas-chromatographic method and gives details on the inter- and intra-assay variability.

## MATERIALS AND METHODS

### Chemicals and Reagents.

DCV was prepared as described previously (5). 9-[[2-[(3-carboxyproprionyl)oxy]ethoxy]methyl]guanine, (DCV-succinate) and 2-amino-9-[(2-hydroxyethoxy)methyl]-9H-purine, (8-hydroxy-DCV), were synthesized by Lil Beauchamp of these laboratories. 2-Amino-9-[(carboxymethoxy)methyl]-9H-purine, (carboxy-DCV), was synthesized by T. Krenitsky of these laboratories. Rabbit serum albumin (RSA) and labile enzyme-free bovine gamma globulin (LEF BGG) were purchased from Miles Laboratories. Other reagents were described previously (6,7).



### Immunogen.

DCV-succinate was coupled to RSA using the mixed anhydride procedure(6). A typical reaction is as follows: 300 mg of DCV-succinate was dissolved in 3.0 ml of anhydrous dimethylformamide (DMF) with stirring at room temperature. Ten minutes later 300 µl of triethylamine and 320 µl of isobutyl-chloroformate were added. The suspension was stirred at room temperature for one hour; the resulting mixed anhydride solution was

chilled to 4° C. This solution was then slowly added dropwise to a prechilled RSA solution prepared by dissolving 250 mg of RSA in 25 ml of 0.1 N sodium bicarbonate buffer, pH 8.15. The addition took about 2 hours. After the last addition, the solution was held at 4° C overnight. The conjugated product (DCV-succinyl-RSA) was isolated by chromatography on a Sephadex G-25 column (2.5 x 45 cm) using normal saline as the eluent. The first peak, which was the desired product, was identified and then concentrated using an Amicon Model 12 cell fitted with PM10 membrane at 25 psi. The concentrate was filter sterilized (0.22 μ) , analyzed by UV spectroscopy, and then used for immunization. Hapten and protein concentrations were estimated simultaneously using two equations derived from the individual absorption spectra at 254 and 304 nm. The hapten-to-carrier ratio varied from 4 to 10 moles per mole. Protein recovery was generally 75 % or better.



Immunizations and Bleedings.

Eight rabbits were initially immunized i.m. with DCV-succinyl-RSA (2 mg total protein in two sites) in complete Freund's adjuvant. These animals were reimmunized one month later

i.m. with the same quantity of immunogen in incomplete Freund's adjuvant and boosted again one month later s.c. (0.5 ml in 4 sites) with the same material. After this third immunization, the animals were bled and reimmunized s.c. periodically. Blood samples were collected, allowed to clot overnight, the resulting sera were heat-inactivated for 60 min at 60° C to destroy esterase activity and complement, and were frozen until used.



## Preparation of Radioactive Antigens.

$^3$H-DCV was prepared by Dr. John Hill of these laboratories. The antigen was labelled in the side chain and had a specific activity of 19.7 Ci/mMole. This material was succinylated as described earlier (7).

## Binding Assays.

To evaluate the binding characteristics of the antisera, 100 µl of diluted anti-DCV serum was added to 300 µl of buffer (50 mM $KH_2PO_4$, 0.9% NaCl, 10 mM $Na_2EDTA$, and 0.01% ethylmercurithiosalicylic acid, pH 7.5) in 12 x 55-mm polypropylene tubes (W. Sarstedt, Inc.); and

then 100 µl of diluted radioactive antigen was added to each tube (for about 25,000 cpm). After mixing with a vortex apparatus, the tubes were incubated overnight at $4^0$ C. After incubation, 50 µl of cold LEF-BGG (10 mg/ml) was added as the carrier protein and then 550 µl of cold saturated ammonium sulfate in water (pH 7.5 at $4^0$ C) was added. The tubes were mixed and held at $4^0$ C for about one hour. The tubes were then centrifuged for 20 min at 2230 x g and $4^0$ C. The supernatant was decanted and discarded ; any remaining fluid was carefully removed by aspiration. The precipitate was washed once with 1 ml of cold half-saturated ammonium sulfate, and after recentrifugation the supernatant was removed as above. The precipitate was dissolved in 0.1 ml of deionized water; 0.1 ml of 4N HCl was then added to each tube followed by 2.5 ml of Aquasol-2 (NEN Research Products, Boston, Mass.) scintillation counting fluid. The tubes were capped, mixed by Vortex immediately, placed in scintillation vials (capped, caps had a 0.5-in. hole to center the tubes accurately), and then counted. Assays were done in triplicate.



RIA of DCV.

For the assay, 100 µl of diluted sample containing an unknown quantity of DCV was added to a 12 x 55-mm polypropylene tube,

followed by 200 µl of buffer, 100 µl of diluted anti-DCV antiserum and 100 µl of radioactive antigen ($^3$H-DCV or $^3$H-succinate-DCV). Tubes were prepared in triplicate. The tubes were then mixed using a Vortex apparatus and were placed at $4^0$ C for incubation. The procedure from this point was the same as for the binding studies (see above). To obtain a standard curve, known amounts of DCV in buffer and 100 µl of normal plasma, urine or other fluid (diluted to the same extent as the unknowns) were added to the assay tubes, with the final incubation volume of 500 µl being maintained by adjustment of the buffer volume added. From the scintillation-counting data, a standard curve was obtained by plotting concentration versus percentage control binding ($B/B_0$) on log-logit paper, and the unknown concentrations were read directly from the graph. Alternatively, the data were accumulated in a data acquisition system and then directed to a computer where the standards were subjected to the log-logit transformation followed by iterative, weighted least-squares linear-regression analysis; unknown concentrations were calculated using the standard curve parameters obtained.



## RESULTS

### Response of Rabbits to the Immunogen.

The response of one group of rabbits immunized with DCV-succinyl-RSA towards the tritiated antigen, $^3$H-DCV, is shown

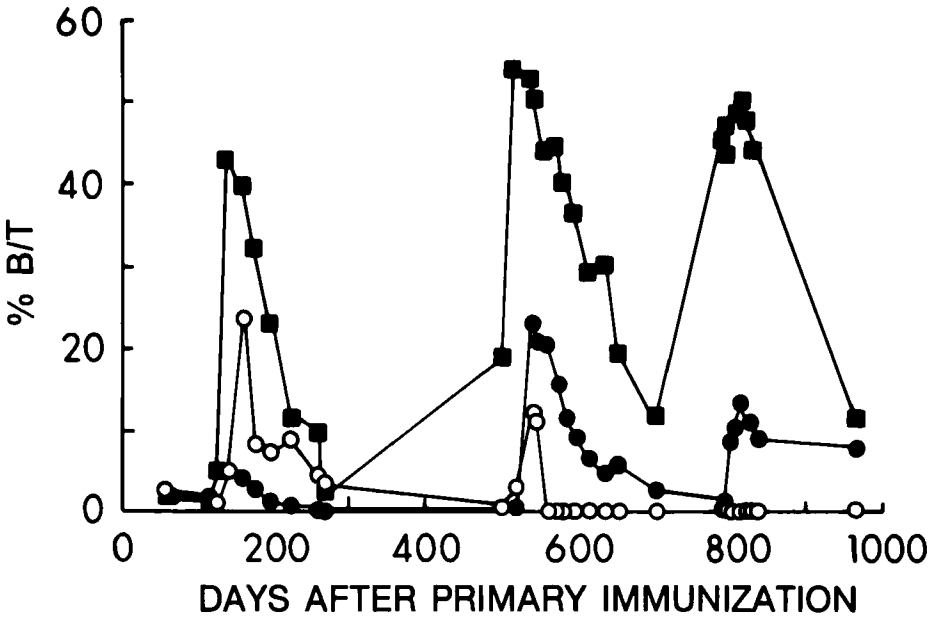


Figure 1. Binding of $^{3}$H-DCV (as % B/T) to Antisera as a Function of Time in Days after Primary Immunization. Antisera diluted 1:10 for Analysis. O-O, Binding of Antisera from Rabbit #6145; ■-■,Binding of Antisera from Rabbit #6149; and ●-●, Average Binding of the remaining six Rabbits in the Group.



in Figure 1. All eight animals in this group responded with antibody; two of these gave only minimal titers. Usable titers were obtained after about 5 months. Restimulation after a prolonged rest period generally gave increased titers. Two animals with the highest initial titers (#6145 and 6149 ) were chosen for further evaluation. Antisera from animal # 6149 bound the tritiated antigen better than did antisera from animal #6145. Therefore, this antisera was chosen for use with the this antigen.

RIA Standard Curve and Comparison with a Gas Chromatographic Method.

A variety of conditions including buffer composition and pH, time of incubation, temperature of incubation and other factors were investigated (data not shown). Results were nearly identical to those reported previously for the radioimmunoassay for acyclovir (7). Lipemic serum and plasma was shown not to interfere in the assay (data not shown). A typical standard curve is given in Figure 2 where antisera from rabbit #6149 was used with tritiated antigen and with dog plasma. A straight line was obtained using the log-logit transformation of the data between 0.5 and 80 nM with an $I_{50}$ at 1.7 nM. A coefficient of correlation (squared) of 0.995 was obtained.

To validate the assay, a range of concentrations of DCV in human plasma and a small number of human plasma samples from a volunteer study were analyzed by RIA and by gas chromatography (9). Samples were coded to avoid bias. After the analyses were complete, results were uncoded by an independent party and subjected to mathematical analysis (6). A comparison of the results obtained using the two methods is given in Table 1.

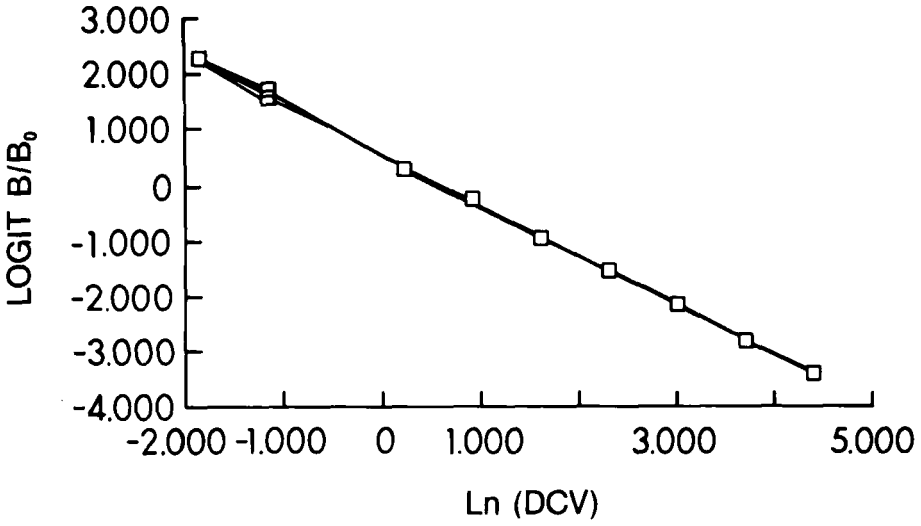


Figure 2. Standard Curve with (Logit $B/B_0$) plotted versus Ln (DCV) using Dog Plasma at a 1:50 Dilution. Antisera #6149 at a 1:100 Dilution; $^3$H-DCV as the Antigen (Specific Activity = 19.7 Curies/millimole). Standard Curve Parameters Obtained: $B_0/T$ = 0.2510; Slope = 0.9114; $R^2$ = 0.99478; and $I_{50}$ = 17.32 µM.



Intra- and Inter-Assay Accuracy and Precision.

Results of a study designed to investigate the intra-assay precision and accuracy of the RIA using antisera from rabbit #6149 and $^3$H-DCV are given in Table 2. Results for DCV in human plasma (Part A) indicate good agreement between the standard concentrations in the standard curve with the fitted values obtained from the log-logit analysis. Precision was also good, as can be judged from the CV values. The agreement (accuracy) for the urine

TABLE 1

Comparison of Results obtained with the Radioimmunoassay using $^{3}$H-Desciclovir and Antisera #6149 and with a Gas Chromatographic Method for Determination of Desciclovir in Human Plasma Samples.

| Nominal Value(μM) | Value Determined by RIA (μM) | Value Determined by GC (μM)[a] |
|---|---|---|
| STANDARDS: | | |
| 0.00 | - | <0.05 |
| 1.10 | 1.29 | 1.09 |
| 3.50 | 4.58 | 3.97 |
| 7.60 | 7.18 | 6.92 |
| 12.00 | 14.70 | 14.22 |
| 26.00 | 25.86 | 30.24 |
| UNKNOWN SAMPLES: | | |
| A | 0.17 | 0.62 |
| B | 16.05 | 11.94 |
| C | 2.95 | 2.27 |
| D | 6.82 | 7.31 |
| E | 2.37 | 2.60 |
| F | 16.05 | 11.94 |
| G | 37.64 | 38.97 |
| H | 27.37 | 26.37 |
| I | 3.66 | 3.45 |
| J | 6.00 | 5.82 |
| K | 2.95 | 2.27 |
| L | 9.32 | 10.84 |

[a] Average of duplicate determinations.

portion of the experiment (Part B) are closer, as are the CV (precision) values.

A similar study was executed to obtain data on the inter-assay precision and accuracy, again using both human plasma and urine

TABLE 2

Desciclovir Radioimmunoassay Intraassay Precision and Accuracy for Human Plasma and Urine Analyses. Antiserum #6149 at 1:100, 3H-DCV as the Antigen. N=10 for both Plasma and Urine Evaluations.

A. HUMAN PLASMA SAMPLES

Standard Curve Parameters:
Bo/T: 0.267+/-0.022
Slope (-): 0.891
I50 (nM): 18.09+/-0.473
Correlation Coefficient: 0.975

Standard Curve Evaluation:

| Standard Conc. | Value Found | Sample CV (%) |
|---|---|---|
| 80.0 pmoles | 77.3 | 7.51 |
| 40.0 pmoles | 39.2 | 9.60 |
| 20.0 pmoles | 16.8 | 6.80 |
| 10.0 pmoles | 10.3 | 4.72 |
| 5.0 pmoles | 4.84 | 6.01 |
| 2.5 pmoles | 2.39 | 3.13 |
| 1.25 pmoles | 1.60 | 9.40 |
| 0.625 pmoles | 0.496 | 4.45 |
| 0.3125 pmoles | 0.216 | 5.96 |

Internal Standard Evaluation:

| Nominal Value | Value Found | Sample CV (%) |
|---|---|---|
| 0.25 pmoles | 0.220 | 7.84 |
| 0.5 pmoles | 0.580 | 8.42 |
| 2.0 pmoles | 2.23 | 3.78 |
| 10.0 pmoles | 8.87 | 2.22 |
| 40.0 pmoles | 35.50 | 3.82 |



(Table 3). The values given for the agreement of the standard curve parameters and the agreement for the internal standard values with the nominal values indicate good accuracy for this system. The CV values obtained for the internal standards in this set of six experiments ranged from 0.5 to 13.2 % .

Table 2 (cont.)

B. HUMAN URINE SAMPLES

Standard Curve Parameters:
Bo/T: 0.217
Slope (-): 0.930 +/- 0.001
I50 (nM): 22.17+/-0.24
Correlation Coefficient: 0.991

Standard Curve Evaluation:

| Standard Conc. | Value Found | Sample CV (%) |
|---|---|---|
| 80.0 pmoles | 60.5 | 6.71 |
| 40.0 pmoles | 32.2 | 5.06 |
| 20.0 pmoles | 17.8 | 5.79 |
| 10.0 pmoles | 9.64 | 5.32 |
| 5.0 pmoles | 5.49 | 6.31 |
| 2.5 pmoles | 2.53 | 2.89 |
| 1.25 pmoles | 1.27 | 2.82 |
| 0.625 pmoles | 0.576 | 2.04 |
| 0.3125 pmoles | 0.315 | 2.19 |

Internal Standard Evaluation:

| Nominal Value | Value Found | Sample CV (%) |
|---|---|---|
| 0.50 pmoles | 0.529 | 2.88 |
| 1.25 pmoles | 1.28 | 1.98 |
| 5.00 pmoles | 5.62 | 3.69 |
| 20.00 pmoles | 18.3 | 4.90 |
| 80.00 pmoles | 68.3 | 8.19 |



## Specificity of the Assay.

The specificity of the antisera chosen for use in this assay is summarized in Table 4. Crossreactivities for antisera # 6149 with $^{3}H$- DCV are listed. The only crossreactivities seen were with the carboxy DCV and with BW A134U. This antigen demonstrated no other crossreactivities with a wide range of naturally occurring

TABLE 3

Desciclovir Radioimmunoassay Interassay Precision and Accuracy for Human Plasma and Urine Analyses. Antiserum #6149 at 1:100, 3H-DCV as the Antigen. N=6 for both Plasma and Urine Evaluations.

A. HUMAN PLASMA SAMPLES

| | Mean | Std. Dev. |
|---|---|---|
| Standard Curve Parameters: | | |
| Bo/T | 0.237 | 0.021 |
| Slope (-) | 0.910 | 0.062 |
| $I_{50}$ (nM) | 13.38 | 2.560 |
| Correlation Coefficient | 0.995 | 0.005 |

| Internal Standard Evaluation: | | |
|---|---|---|
| Nominal Value | Value Found | CV(%) |
| 2.0 pmoles | 2.02 | 4.31 |
| 10.0 pmoles | 9.48 | 0.52 |
| 40.0 pmoles | 33.25 | 8.69 |

B. HUMAN URINE SAMPLES

| | Mean | Std. Dev. |
|---|---|---|
| Standard Curve Parameters: | | |
| Bo/T | 0.217 | 0.012 |
| Slope (-) | 0.939 | 0.147 |
| I50 (nM) | 22.57 | 5.690 |
| Correlation Coefficient | 0.988 | 0.012 |

| Internal Standard Evaluation: | | |
|---|---|---|
| Nominal Value | Value Found | CV(%) |
| 1.25 pmoles | 1.231 | 13.24 |
| 5.00 pmoles | 4.724 | 11.07 |
| 20.00 pmoles | 15.90 | 8.80 |

Table 4

Crossreactivity of various metabolites and Other Materials with DCV in the Radioimmunoassay using $^{3}$H-DCV as antigen with Rabbit Antisera #6149.

| Compound | %Crossreactivity[1] |
|---|---|
| A. Metabolites and Other Analogs: | |
| Acyclovir (ACV) | <0.01 |
| Succinyl-ACV [2] | <0.01 |
| Carboxy-ACV [2] | <0.01 |
| 8-Hydroxy-ACV [2] | <0.01 |
| Carboxy-DCV [2] | 1.38 |
| 8-Hydroxy-DCV [2] | <0.01 |
| BW A134U [2] | 0.047 |
| BW B759U [2] | <0.01 |
| B. Other Materials: | |
| Guanine | <0.01 |
| Guanosine | <0.01 |
| 2'-Deoxyguanosine | <0.01 |
| Azathioprine | <0.01 |
| 6-Mercaptopurine | <0.01 |

[1]Crossreactivity = $\frac{I_{50} \text{ for DCV} \times 100}{I_{50} \text{ for Substance Tested}}$

[2]Compound Names:

| Abbreviation | Chemical Name |
|---|---|
| ire Tables - | 9-[[2-[(3-Carboxyproprionyl)oxy]ethoxy]methyl]guanine |
| Carboxy-ACV | 9-[(Carboxymethoxy)methyl]guanine |
| 8-Hydroxy-ACV | 8-Hydroxy-9-[(2-hydroxyethoxy)methyl]guanine |
| Carboxy-DCV | 2-Amino-9-[(carboxymethoxy)methyl]-9H-purine |
| 8-Hydroxy-DCV | 2-Amino-9-[(2-hydroxyethoxy)methyl]-9H-purin-8-ol |
| BW A134U | 2,6-Diamino-9-[(2-hydroxyethoxy)methyl]-9H-purine |
| BW B759U | 9-[[2-Hydroxy-1-(hydroxymethyl)ethoxy]methyl]-guanine |

purines, other purines, thiopurines, a number of anti-coagulants and other materials. The crossreactivities seen with carboxy-DCV and with BW A134U should not limit the use of this assay for any study.

Affinity Constant Determinations.

Affinity constants for both antigens ( $^{3}$H-DCV and $^{3}$H-succinyl-DCV ) were obtained with the antisera from rabbit #6149. The value was found to be 1.89 x $10^{9}$ L/M for $^{3}$H-DCV and was slightly higher (6.92 x $10^{9}$ L/M) for $^{3}$H-succinyl-DCV.



## DISCUSSION

DCV is rapidly absorbed and converted into ACV (2,3). Thus it was imperative that the antisera for this assay to have little or no crossreactivity with ACV or its two known metabolites. At the time of its development little was known about any other metabolism of DCV, so it was also desirable to try to identify an antisera with low or no crossreactivity to the two corresponding metabolites of DCV. In addition, the assay needed to be very sensitive, because initial observations indicated that this material was extensively and rapidly

converted to ACV, and the clinically relevant samples were expected to have only low concentrations of DCV.

The single-tube RIA for DCV reported here is sensitive with an $I_{50}$ value of about 20 nM and a lower limit of detection of about 3 nM. The affinities of antisera #6149 with the succinylated tritiated antigen was about $7 \times 10^9$ L/Mole while it was only slightly lower at $2 \times 10^9$ L/Mole with the non-succinylated form. For convenience the non-succinylated antigen was chosen for routine use. The assay using these materials gives reproducible results as demonstrated by both the inter-assay accuracy and precision and by intra-assay precision.

This RIA has now been used in both preclinical and clinical studies with DCV. Many sample types have been analyzed including plasma, urine and tissue-homogenate supernatants. Plasma samples from various species including the dog (Krasny, HC, unpublished data), rat and human (3) have been analyzed.



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## Footnotes

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2. Abbreviations Used: DCV, desciclovir, 2-[(2-amino-9H-purin-9-yl)methoxy]ethanol; ACV, acyclovir; RSA, rabbit serum albumin; LEF BGG, labile enzyme-free bovine gamma globulin; DMF, dimethylformamide; TME, tyrosine methyl ester.



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