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A sensitive assay for simultaneous determination of plasma concentrations of valganciclovir and its active metabolite ganciclovir by LC/MS/MS

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Abstract

A protein precipitation, liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the simultaneous determination of valganciclovir and its active metabolite ganciclovir in human plasma. The solvent system also served as a protein precipitation reagent. The chromatographic separation was achieved on an Aquasil C18 column (50 mm × 2.1 mm, 5 μm). A linear gradient mobile phase between 0.02% formic acid and methanol was used. Detection was by positive ion electrospray tandem mass spectrometry on a Sciex API3000. The standard curves, which ranged from 4 to 512 ng/mL for valganciclovir and from 0.1 to 12.8 μg/mL for ganciclovir, were fitted to a $1/x$ weighted quadratic regression model. The method was proved to be accurate, specific and sensitive enough and was successfully applied to a pharmacokinetic study.

Keywords: Valganciclovir; Ganciclovir; Aciclovir; LC/MS/MS; Protein precipitation

1. Introduction

Ganciclovir (GCV, Fig. 1) is currently the agent of choice for the treatment of cytomegalovirus infection after organ transplantation. Due to its low bioavailability, GCV must be administered intravenously in this indication and is therefore associated with logistical problems and with a risk of intravenous line-related infections. Valganciclovir (VGC, Fig. 1) is an L-valyl ester prodrug of GCV with much improved oral bioavailability (approximately 60%, 10-fold higher compared to GCV), thus providing drug exposure comparable to that achieved with intravenous GCV. It has recently proved successful as a prophylaxis against CMV in high-risk transplant recipients and for the treatment of CMV retinitis in AIDS patients [1].

The occasional monitoring of valganciclovir and ganciclovir concentrations in plasma from HIV or CMV-infected patients may be useful if poor compliance is suspected, or if there is an underlying clinical condition such as renal insufficiency or hepatic impairment. Merodio et al. [2] and Page et al. [3] reported that the limit of quantification of serum ganciclovir using the HPLC method with UV detection was 50 ng/mL. Kishino et al. [4] reported an HPLC method followed by pulsed amperometric detection for quantification of ganciclovir that has a minimum detection limit of 50 ng/mL. Marcus et al. [5] developed a method for the simultaneous determination of valganciclovir and ganciclovir by LC/MS/MS and evaluated the single dose pharmacokinetics of valganciclovir and ganciclovir in a 6-year-old girl after oral valganciclovir. In the method of Marcus et al. [5], trichloroacetic acid used for the precipitation was unacceptable for LC/MS/MS and the analytes had almost no retention. The purpose of this study was to improve the LC/MS/MS method with simple sample preparation for the simultaneous determination of valganciclovir and ganciclovir in human plasma to support a pharmacokinetic study after oral administration of valganciclovir.

Abbreviations: DI, deionized; QC, quality control; LLOQ, lowest level of quantitation

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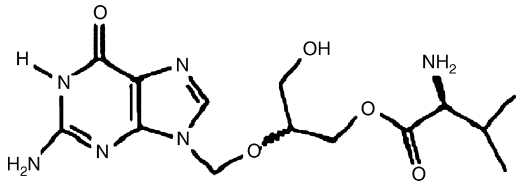
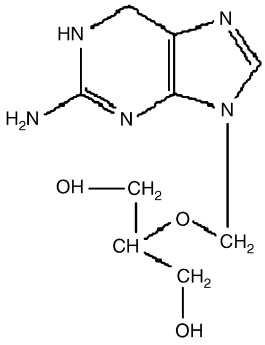
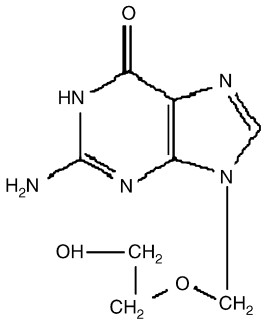


valganciclovir MW: 355.3



ganciclovir MW: 256.2



acyclovir MW: 226.2

Fig. 1. The chemical structures of valganciclovir, ganciclovir (IS).

2. Experimental

2.1. Reagents and chemicals

Valganciclovir (100.0% pure, Lot: BO0403B048) and ganciclovir (99.8% pure, Lot: BO0212B015) were obtained from Roche Co. (Shanghai, China). Aciclovir (98.5% pure) was obtained from Shanghai Institute for Drug Control (Shanghai, China). Formic acid was obtained from Fisher Scientific (Fairlawn, NJ, USA). Methanol and acetonitrile, both HPLC grade, were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Deionized/distilled (DI) water was prepared from tap water in our own department.

2.2. Valganciclovir and ganciclovir standard, QC and IS preparation

Primary stock solutions of valganciclovir and ganciclovir (1 mg/mL) were prepared in methanol. Working standard solutions of valganciclovir and ganciclovir were prepared by combining aliquots of each primary stock solution and diluting with methanol. The working solution for aciclovir (internal standard, 1 μg/mL) was prepared by diluting an aliquot of stock solution with acetonitrile. All standard solutions were stored at 4 °C in the dark when not in use. Human plasma calibration standards of valganciclovir (4, 8, 16, 32, 64, 128, 256, 512 ng/mL) and ganciclovir (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 μg/mL) were prepared by spiking the working standard solutions into a pool of drug-free human plasma.

QC stock solutions of valganciclovir and ganciclovir (1 mg/mL) were prepared from a separate weighing and were also dissolved in methanol. Dilutions were used to prepare three levels of QCs in human plasma: high QC (400 ng/mL valganciclovir and 10 μg/mL ganciclovir), medium QC (200 ng/mL valganciclovir and 5 μg/mL ganciclovir) and low QC (6 ng/mL valganciclovir and 0.15 μg/mL ganciclovir). The QC standards were prepared in plasma like the calibration standards. QCs were stored at −20 °C.

2.3. Sample preparation

50 μL of each standard or QC was added into 1.5 mL labeled microcentrifuge tubes. To each standard or QC sample, 100 μL of the working internal standard solution was added. The tubes were capped and vortexed for 1 min. The samples were then centrifuged in Heraeus Biofuge 28RS at 18,000 rpm and 4 °C for 10 min. Then 100 μL of the supernatant layer and 100 μL distilled water were added into 1.5 mL microcentrifuge tubes and vortexed for 30 s. The dilution was injected into the LC/MS/MS system.

2.4. LC/MS/MS analysis

The liquid chromatography separation system consisted of LC-10AD VP (pump), DGU-14AM and SIL-HTc (autosampler) (Shimadzu Corporation, Kyoto, Japan). The separation column was an Aquasil 5 μm Polar RP C_{18} Column (50 mm × 2.1 mm) (Thermo Electron Corporation, Waltham, MA, USA). An XW-80 vortex was obtained from Shanghai Medical University Apparatus Co. (Shanghai, China). The Biofuge 28RS centrifuge was obtained from Heraeus Sepatech Co. (Osterode, Germany).

The mobile phase consisted of Solvent A (0.02% formic acid in water) and Solvent B (100% methanol). The flow rate was set to 0.25 mL/min. Solvent A was decreased from 99 to 50% in the time range from 0.0 to 0.2 min (linear gradient). From 0.2 to 0.7 min isocratic conditions were run with 50% of each solvent. Solvent A was then increased from 50 to 99% in the time range from 0.7 to 0.8 min (linear gradient). Re-equilibration was performed from 0.8 to 5.5 min at 99% A. The injection volume was 20 μL and the run time was 5.5 min.

A Sciex API 3000 LC/MS/MS system (Foster City, CA, USA) operating under Analyst 1.3 software was used. The electrospray ion source was run in a positive ionization mode for all experiments. The typical ion source parameters were: declustering potential (DP): 26 V (valganciclovir), 22 V (ganciclovir) and 28 V (IS); collision energy (CE): 25 eV (valganciclovir), 30 eV (ganciclovir) and 17 eV (IS); focusing potential (FP) 190 V; collision cell exit potential CXP) 9 V; spray voltage, 2500 V and ion temperature, 500 °C. Nebulizer gas (NEB), curtain gas (CUR)

and collision gas (CAD) were set to 60, 40, 120 psi, respectively. Nitrogen gas was used for CUR, CAD, NEB. The sample were analyzed via selected reaction monitoring (SRM). The monitoring ions were set as *m/z* 355.3 → 151.9 for valganciclovir, *m/z* 256.2 → 135.1 for ganciclovir and *m/z* 226.2 → 151.9 for the IS. The scan dwell time was set at 0.2 s for every channel.

3. Results

3.1. Method validation

3.1.1. Standard curve

After the protein precipitation and LC/MS/MS conditions were defined, a full validation was performed to assess the performance of the method. Eight-point calibration standard curves ranging from 4 to 512 ng/mL of valganciclovir and from 0.1 to 12.8 μg/mL of ganciclovir were used in duplicate in each analytical run. Peak area ratios of valganciclovir or ganciclovir to IS were used for regression analysis. A weighted (1/*x*) quadratic regression model, where *x* is the concentration of valganciclovir or ganciclovir, was fitted to each standard curve. The %CV at each level of valganciclovir varied from 0.75 to 6.62 and that of ganciclovir varied from 1.04 to 8.13. The mean of the absolute value of percent deviation from the theoretical value of valganciclovir and that of ganciclovir varied from 1.32 to 5.65 and from1.80 to 9.95, respectively. The %CV of the five slopes of valganciclovir was 2.56 and that of ganciclovir was 3.43. The lowest coefficient of determination (γ) among the five calibration curves of valganciclovir was 0.9991 (mean = 0.9994) and that of ganciclovir was 0.9983 (mean = 0.9989). Thus, the calibration curves did not exhibit any non-linearity within the chosen range. Based on the standard data presented here, it was concluded that the calibration curves used in this method were precise and accurate for the simultaneous measurement of valganciclovir and ganciclovir in human plasma.

3.1.2. Accuracy and precision

Inter- and intra-batch accuracy and precision for assays were characterized by the four levels of QCs run on three sequential batches in six replicates. The lowest limit of quantification (LLOQ) was 4 ng/mL for valganciclovir and 0.1 μg/mL for ganciclovir (equivalent to the assay limit of quantification), the low-level QC was 6 ng/mL for valganciclovir and 0.15 μg/mL for ganciclovir (less than three times the LLOQ), the medium QC was 200 ng/mL for valganciclovir and 5 μg/mL for ganciclovir, and the high QC was 400 ng/mL for valganciclovir and 10 μg/mL for ganciclovir (within 80% of the top standard). Accuracy was assessed by calculating the percent deviation from the theoretical concentration. Precision was determined by calculating the coefficient of variation for inter- and intra-run replicates.

Table 1 shows a summary of the individual QC data obtained in the four runs for the validation. As can be seen, the assay for valganciclovir and ganciclovir were both accurate and pre-

Table 1
Inter- and intra-assay accuracy and precision of valganciclovir and ganciclovir in plasma

	Valganciclovir				Ganciclovir			
	LLOQ (4 ng/mL)	Low (6 ng/mL)	Middle (200 ng/mL)	High (400 ng/mL)	LLOQ (0.1 μg/mL)	Low (0.15 μg/mL)	Middle (5 μg/mL)	High (10 μg/mL)
Inter-assay accuracy and precision								
Mean	4.00	5.91	198	401	0.097	0.148	5.08	10.1
S.D.	0.32	0.32	9.29	19.4	0.008	0.010	0.227	0.366
%CV	8.08	5.45	4.70	4.84	8.07	6.42	4.46	3.64
%Dev	−0.03	−1.48	−1.24	0.19	−2.79	−1.19	1.63	0.52
n	18	18	18	18	18	18	18	18
Intra-assay accuracy and precision								
Run ID I								
Mean	4.01	5.90	195	409	0.100	0.149	5.06	9.96
S.D.	0.33	0.14	8.74	13.8	0.008	0.010	0.155	0.240
%CV	8.29	2.30	4.47	3.37	7.59	6.90	3.07	2.41
%Dev	0.27	−1.59	−2.32	2.17	0.48	−0.37	1.28	−0.40
n	6	6	6	6	6	6	6	6
Run ID II								
Mean	3.96	5.98	194	393	0.097	0.146	5.16	10.1
S.D.	0.22	0.38	9.03	29.0	0.009	0.010	0.299	0.393
%CV	5.56	6.37	4.67	7.39	8.97	7.02	5.81	3.88
%Dev	−1.04	−0.37	−3.20	−1.70	−3.17	−2.72	3.13	1.10
n	6	6	6	6	6	6	6	6
Run ID III								
Mean	4.03	5.85	204	400	0.094	0.149	5.02	10.1
S.D.	0.44	0.42	8.21	10.1	0.007	0.009	0.223	0.479
%CV	10.9	7.24	4.03	2.52	7.70	6.23	4.44	4.74
%Dev	0.68	−2.49	1.79	0.11	−5.69	−0.47	0.49	0.86
n	6	6	6	6	6	6	6	6

cise between runs and within individual runs for each level. The greatest mean inter-run percent deviations were 8.08% and 8.07% for the LLOQ (valganciclovir = 4 ng/mL, ganciclovir = 0.1 μg/mL), respectively. All QC levels for valganciclovir and ganciclovir had inter- and intra-run percent deviations less than 10% and the LLOQ unaccuracy and unprecision results were within ±10%.

3.1.3. Specificity and matrix effects

Six different lots of control human plasma without standards were analyzed to determine whether any endogenous plasma constituents interfered with the analytes or the IS. The degree of interference was assessed by inspection of SRM chromatograms. No significant interfering peaks from the plasma were found at the retention time or in the ion channel of each analyte or the IS (Fig. 2).

50 μL of blank plasma was added into a 1.5 mL labeled microcentrifuge tube, and then 100 μL acetonitrile was added. The tube was capped and vortexed for 1 min. The sample was centrifuged in the Heraeus Biofuge 28RS at 18,000 rpm and 4 °C for 10 min. 100 μL of the supernatant layer was extracted into a 1.5 mL microcentrifuge tube, and a suitable concentration and volume of the working standard solution for valganciclovir, ganciclovir or IS and distilled water were added. The concentration was equal to the LLOQ for valganciclovir, ganciclovir and IS, respectively. Six replicates for valganciclovir, ganciclovir and IS were prepared, respectively. We replaced blank plasma with distilled water and prepared standard solution in the same way. The matrix effect was assessed by comparing the peak area of the former with the peak area of the corresponding standard solution. The means of the ratios for valganciclovir, ganciclovir and IS were 0.77, 0.80 and 0.92, respectively. Combined with the fact that there was no significant lot-to-lot variation in LLOQ, it was concluded that the low matrix effect did not compromise the performance of the method.

3.1.4. Stability

The stability of valganciclovir and ganciclovir in human plasma was investigated using the low (6 ng/mL valganciclovir and 0.15 μg/mL ganciclovir), medium (200 ng/mL valganciclovir and 5 μg/mL ganciclovir) and high (400 ng/mL valganciclovir and 10 μg/mL ganciclovir) QCs. Test conditions included three freeze–thaw cycles and room temperature (4 h). Stability was also checked by extracting the appropriate QC which had been maintained at specific temperature (4 °C) for the specified time and analyzing the extracts for valganciclovir and ganciclovir concentration. A comparison was made with a control at the same level which had been stored at −20 °C and analyzed in the same analytical run. Additionally, extracted sample stability in the autosampler (4 °C) was tested by comparing the initial results from the QC extract with that determined after 12 h of autosampler storage. Deterioration of valganciclovir and ganciclovir was defined as greater than a 10% difference of tested sample versus control at the sample nominal concentration.

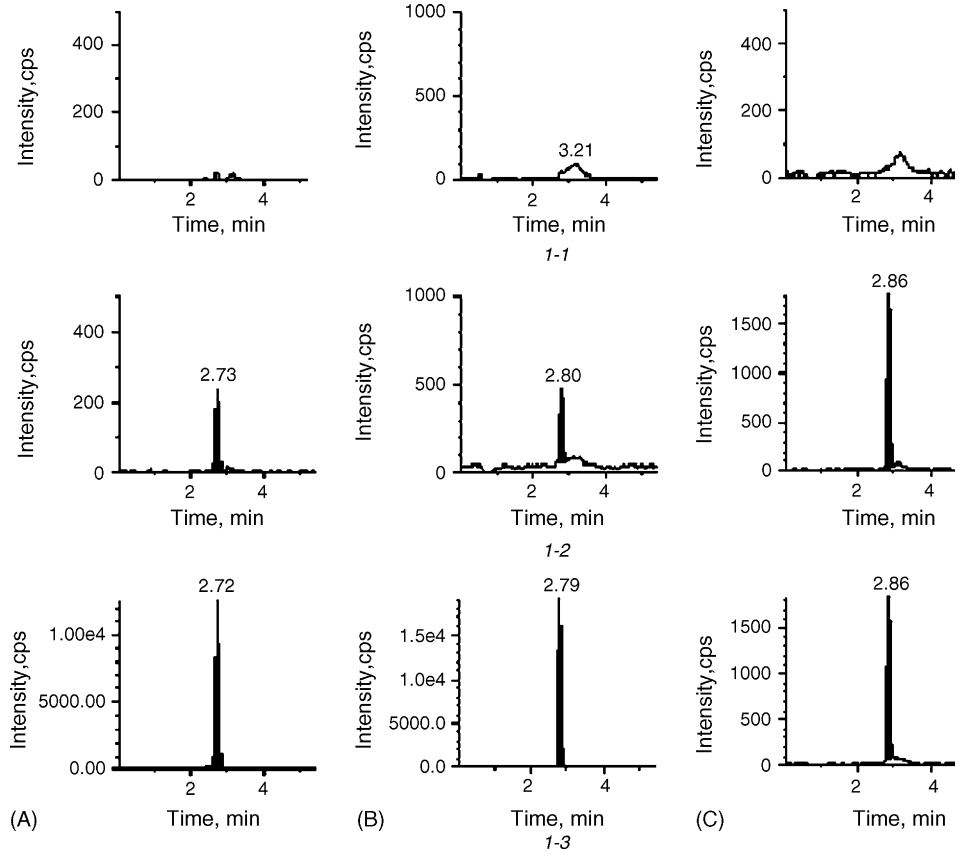


Fig. 2. Chromatography of valganciclovir and ganciclovir and internal standard. (A) Valganciclovir; (B) ganciclovir; (C) acyclovir. I-1: Blank plasma; I-2: plasma spiked with valganciclovir, ganciclovir and internal standard of LLOQ; I-3 plasma obtained from a volunteer after administration.

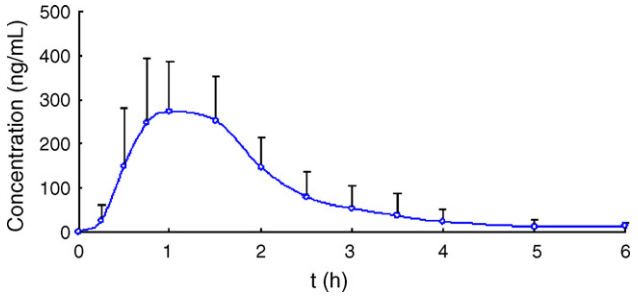


Fig. 3. Mean plasma concentration-time curve of valganciclovir observed after administration of a single 900 mg dose of valganciclovir ($n = 15$).

There was no deterioration in valganciclovir or ganciclovir at any QC level (valganciclovir: 6, 200, 400 ng/mL; ganciclovir: 0.15, 5, 10 μg/mL) for the various freeze–thaw cycles, suggesting that drug concentrations can be confidently determined in samples that had been thawed up to three times prior to analysis or that have been thawed and kept at ambient temperature for up to 4 h. The 4 h stability test at ambient temperature was performed since the plasma sample could stably stand on the bench for up to 4 h after thawing or before freezing.

The stability of valganciclovir and ganciclovir in the extract was also tested after 12 h storage at 4 °C to allow for sample waiting or re-injection. Valganciclovir and ganciclovir were considered stable to storage at 4 °C for 12 h if the relative error (RE) was less than 15% at all QC concentrations after the treatment. The results showed that valganciclovir and ganciclovir were stable following storage at 4 °C for 12 h.

3.2. Clinical application

Plasma samples from 15 healthy volunteers receiving a 900 mg Valcyte tablet were analyzed as described above. The sample time was 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 24 h after administration. The maximum concentrations of valganciclovir ranged from 162.50 to 497.30 ng/mL ($n = 15$) and the mean of the maximum concentration was 337.42 ± 92.32 ng/mL. The maximum concentrations of ganciclovir ranged from 3.10 to 10.19 μg/mL ($n = 15$) and the mean of the maximum concentration was 7.06 ± 2.04 μg/mL. As can be seen from the mean valganciclovir and ganciclovir pharmacokinetic curves (Figs. 3 and 4),

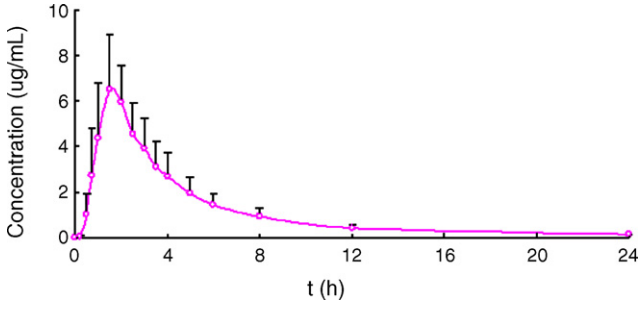


Fig. 4. Mean plasma concentration-time curve of ganciclovir observed after administration of a single 900 mg dose of valganciclovir ($n = 15$).

the times to maximum concentration (T_{max}) of approximately 1.15 ± 0.40 h and 1.82 ± 0.62 h were obtained, respectively. The areas under the concentration–time curve (AUC_{0-t}) for valganciclovir and ganciclovir were 518.75 ± 208.77 ng h/mL and 25.23 ± 7.96 μg h/mL, respectively, and $AUC_{0-\infty}$ were 529.31 ± 213.76 ng h/mL and 26.64 ± 8.00 μg h/mL, respectively. The mean terminal elimination half-life for valganciclovir and ganciclovir were 0.83 and 3.49 h, respectively.

4. Discussion

The quantification of antiviral agents in biological media is difficult because these drugs have a chemical structure that is quite similar to a number of endogenous substances. This fact makes analysis difficult and requires the use of a highly selective analytical methodology, such as HPLC-MS/MS. MS/MS has a greater specificity and higher sensitivity than UV, therefore, the determination of the analogue of the nucleoside by LC/MS/MS, such as the determination of aciclovir [6] and ganciclovir [5], can obtain better results. Marcus et al. [5] removed plasma protein with trichloroacetic acid (15%, 20 μL), which might be harmful to the column and the MS/MS system. In this study, potentially interfering plasma proteins were removed by the acetonitrile precipitation/centrifugation technique before the extracts were injected into the gradient mobile phase stream. Because we used 99% A as mobile phase at the beginning of elution, analytes could be retained on the column for a longer time. The use of 50% A could elute the analytes in a short time, which made the peaks sharp and symmetrical. In the method of Marcus et al. [5], the retention times of ganciclovir, valganciclovir and aciclovir were 1.1, 1.05 and 1.1 min, respectively, which indicates almost no retention on the column.

The results in healthy volunteers are consistent with those of a pharmacokinetic study in HIV-infected patients after 875 mg of oral valganciclovir [7]. The mean values for the AUC_{0-t} of ganciclovir after dosing with 900 mg of oral valganciclovir was 25.23 μg h/mL in our study compared with 24.8 μg h/mL in HIV-infected patients after 875 mg of oral valganciclovir. The value of AUC_{0-t} in healthy volunteers was lower than the mean AUC_{0-t} values of 49.0 and 41.7 μg h/mL obtained in stem cell [8] and liver [9] transplant recipients, respectively. The terminal elimination ganciclovir half-life after oral valganciclovir is also shorter in healthy volunteers (3.49 h) and HIV-infected patients (4.08 h) compared with both stem cell (5.1 h) and liver (5.1 h) transplant recipients [7–9]. The immunosuppressive agents and other drugs with potential nephrotoxicity in transplant patients may decrease the renal clearance of ganciclovir.

The amount and extent of exposure to valganciclovir in this study were high. The mean peak concentration of valganciclovir was 337.42 ng/mL at 1.15 h after dosing. Valganciclovir levels became undetectable 6 h after dosing. In stem cell, liver transplant recipients and HIV-infected patients, lower C_{max} values for valganciclovir were noted after 900 or 875 mg doses of oral valganciclovir with food [7–9]. Administration with food may decrease the C_{max} and increase the T_{max}.

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