A Simple, Sensitive Determination of Ganciclovir in Infant Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

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Abstract

This study developed a simple and sensitive method using reversed-phase high-performance liquid chromatography (HPLC) for ganciclovir (GCV) plasma concentrations in cytomegalovirus infectious infants with hearing loss. The method involves a simple protein precipitation procedure that uses no solid-phase or liquid–liquid extraction. The HPLC separation was carried out on a Cadenza CD-C_{18} column (3 µm, 4.6 mm × 150 mm) with phosphate buffer (pH 2.5, 25 mM) containing 1% methanol–acetonitrile mixture (4:3, v/v) as a mobile phase at a 0.7 mL/min flow rate. GCV was detected using a fluorescence detection (λex/em: 265/380 nm). The quantification limit was 0.025 µg/mL for 100 µL of plasma sample at which good intra- and inter-assay coefficient of variation values (< 4.96%) and recoveries (94.9–96.5%) were established.

Introduction

Ganciclovir [(9-(1,3-dihydroxy-2-propoxymethyl) guanine, GCV, Figure 1)] is a synthetic acyclic nucleoside analogue closely related to acyclovir, which has shown activity against herpes simplex viruses (type 1 and 2), varicella-zoster virus, Epstein-Barr virus, and cytomegalovirus (CMV). GCV has good in vitro activity and is considerably more potent than acyclovir against human CMV (1), so GCV plays an important role in the prevention of CMV disease after bone marrow and liver transplantation and is likely to have wider clinical use in other transplant recipients (2,3). It has been known in recent studies that CMV infection sometimes causes hearing loss in newborns (4,5). GCV treatment is also applied to patients with hearing loss (6). On the other hand, GCV has been associated with serious toxic side effects, such as haematological toxicity (6). Elimination of GCV is mainly renal, thus monitoring plasma level is useful in renal failure or in a premature and/or newborn baby.

Some high-performance liquid chromatographic (HPLC) methods have been described for the analysis of GCV in plasma (7–14), serum (11,12,15), and other biological fluids (15,16). These methods have used ion-pairing agents (10,11,13,14), UV detection (7,8,10,15,16), amperometric detection (9), fluorescence detection (13,14) with no internal standard (11,15), and with pre-column derivatization step (12). In sample preparation, liquid–liquid (7,8) or solid-phase extraction (9,12) have been adopted. In addition, large (250–1000 µL) quantity of plasma and serum samples (7–9,11–13,15) or time-losing procedures were applied in these literatures.

Particularly from small infants, it is not easy to obtain a large volume of blood. Inadequate blood volume was needed by these methods for therapeutic drug monitoring of GCV in small infants. It is necessary to develop the method, which has sensitivity from small quantities of blood sample.

Using fluorescence detector, GCV in plasma or serum samples has been sensitively detected in comparison with the UV detector (13,14). However, the former method (QOD 0.1 µg/mL) required higher quantities of plasma samples (1000 µL) and an HPLC run time (42 min) with a treatment of ion-pairing agent. The latter study using 200 µL of plasma with fluorescence detection has adopted a direct injection of acid supernatant to the HPLC system after perchrolic acid deproteinization, which contributes significantly to the reduction of analytical column lifetime even when the injection volume is low.

Here this study developed a simple method for the analysis of GCV in smaller volume of plasma samples using fluorescence detection with internal standard, and involving a simple protein precipitation procedure that uses no liquid–liquid and solid-phase extraction. This method can be applied to therapeutic drug monitoring and further pharmacokinetic studies in infants with CMV and related virus infections.

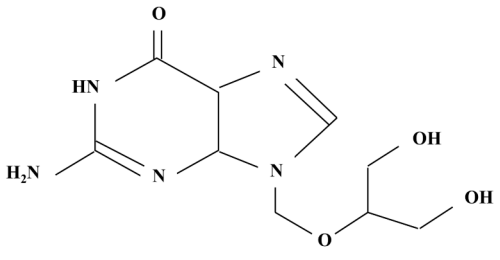

Figure 1. Chemical structure of ganciclovir.

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Experimental

Reagents

GCV and acycloguanosine (ACV) as an internal standard (IS) were purchased from Sigma (St. Louis, MO). Acetonitrile, methanol, distilled water of HPLC-grade, and other chemicals of analytical-grade were supplied by Kanto Chemical (Tokyo, Japan). Samples of human plasma from healthy volunteers were obtained from the Japan Red Cross (Tokyo, Japan).

Stock solutions

Stock standard solution of GCV was prepared by dissolving 50 mg in 50 mL of 25 mM phosphate buffer (pH 6.8) and was diluted with phosphate buffer as working standard solutions at seven respective concentrations of 0.25, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 µg/mL. ACV was also dissolved in phosphate buffer, thereby preparing a solution of 50 µg/mL. No changes in GCV and ACV concentrations were detected in working standards and IS solutions after two months of storage at 4°C.

Calibration curves and quality control samples

For preparation of the calibration curves, working standards were further diluted in healthy human plasma to give plasma concentrations of GCV (0.025, 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 µg/mL).

Quality control (QC) samples were also prepared by spiking the stock solutions into blank human plasma, as described for the preparation of the calibration standards. The concentrations used for the QC were 0.025, 0.10, 1.0, and 5.0 µg/mL. The QC samples were stored at –25°C and analyzed every time along with patient samples. The QC samples were stable at –25°C for at least five months.

Apparatus and chromatographic conditions

Analyses of standards and sample extracts were conducted using a 1200 Series Agilent HPLC system (Waldbronn, Germany) including a binary pump, a degasser, a column thermostat, a fluorescence detector, and an automatic sample injector. The Cadenza CD-C_{18} analytical column (3 µm particle size, 150 mm × 4.6 mm i.d., Imtakt, Kyoto, Japan) was heated at 40ºC during the analyses. A guard column cartridge (10 mm × 4.6 mm i.d.) of the same material was used with the column. The mobile phase was sodium phosphate buffer (pH 2.5; 25 mM) containing 1% mixed solvent of methanol–acetonitrile (4:3, v/v) at a flow rate of 0.7 mL/min under isocratic condition. After each ten analyses, the HPLC column was washed by phosphate buffer containing 50% of methanol–acetonitrile (4:3, v/v) at a flow rate of 1.0 mL/min for 30 min followed by mobile phase described previously for 15 min. The injection size was 10 µL; GCV was detected by a fluorescence detector (λex/em: 265/380 nm). The extracts of plasma samples, calibration standards, and QC samples were allowed to stand at 4°C through the analyses.

Sample preparation

A 100 µL aliquot of plasma samples, calibration standards, and QC samples in a 1.5 mL centrifuge tube were added to 10 µL of IS solution, and 50 µL of 1% perchloric acid as a deproteinization solvent. The solution was vortex-mixed twice for about 5 s and kept at room temperature for 5 min. The mixture was centrifuged for 10 min at 13500 rpm. A 100 µL of the clear supernatant was pipetted and transferred into an HPLC vial, followed by addition of 20 µL of 0.1 M sodium hydroxide solution. Subsequently, 10 µL of the prepared solution was injected into the HPLC system.

Precision and accuracy

The intra-assay precision and accuracy of the proposed method were evaluated by performing six replicate analyses of control samples with four different concentrations. Performing analyses of the same control samples assessed the inter-assay precision and accuracy. The procedures were repeated on eight different days.

Sample collection

According to a study protocol previously approved by the Ethics Committee of our institute, blood samples were taken from CMV infectious infants with hearing loss at the seventh day under intravenous treatment with GCV during their stay. Blood samples were collected with heparin as anticoagulant and immediately centrifuged at 3000 rpm for 10 min. The plasma was transferred to a centrifuge tube and stored at –25°C up to analysis. No differences in GCV quantitation were produced by –25°C storage of plasma samples for at least four months and three cycles of freeze-thaw. In each case, the variation was < 3.3% (data not shown).

Results and Discussion

Chromatography

The retention times were approximately 8.1 and 10.7 min for GCV and ACV, respectively. Figure 1 shows the chromatograms from the analysis of blank human plasma sample (A), from one patient's plasma obtained just after intravenous infusion over 1 h at a dose of 5 mg/kg weight every 12 h (B), and from the same patient's at 10 min pre-infusion (trough) level (C). No chromatographic interference from endogenous substances or other administrated drugs such as prednisolone, phenobarbital, cyproheptadine, and their metabolites were observed.

Chromatographic resolution of endogenous substances from GCV and ACV (IS) was sensitive to changes in a ratio of methanol and acetonitrile because the chemical structure of this molecule, being a nucleoside analogue of guanine, is quite similar to endogenous substances. When a volume ratio of methanol and acetonitrile was 4:3 (v/v), good chromatographic resolution was obtained (Figure 2).

After each ten analyses, the HPLC column was washed by phosphate buffer containing 50% of methanol–acetonitrile (4:3, v/v) because it appeared in more than ten continuous analyses that the retention times of the drugs became faster and faster.

Linearity

Calibration curves were obtained by plotting the ratio of the peak area of GCV to peak area of IS against the corresponding

concentrations of GCV in spiked plasma. Calibration curves were linear in the concentration ranges of 0.025–10.0 µg/mL for GCV. The linear-regression equation of the curve for GCV was $y = 0.1669x + 0.0025$, where y and x are the peak-area ratio (GCV/IS) and the GCV concentration (µg/mL in the plasma), respectively. The correlation coefficient (r^2) was higher than 0.999 throughout all assays.

Precision and accuracy

The intra- and inter-assay precision and accuracy of the proposed method were evaluated. The results are shown in Table I. Precision is expressed as coefficients of variation (CV) of 1.61–4.28% for the intra-assay and 2.31–4.96% for the inter-assay. Accuracy is defined as (found value/added value) × 100%, which was 94.9–102.7% throughout examinations for the four different concentrations.

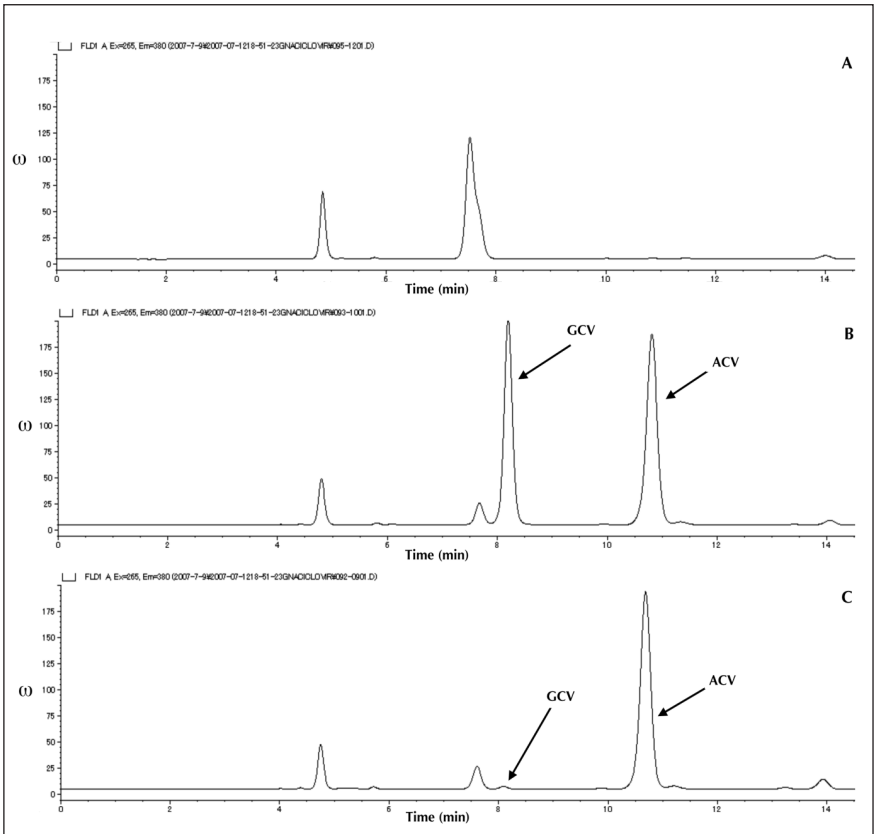

Figure 2. Chromatogram of (A) blank plasma from human volunteer, (B) a patient's plasma taken just after intravenous infusion over 1 h at a dose of 5 mg/kg weight every 12 h (GCV 4.71 µg/mL), (C) a patient's plasma taken before intravenous infusion (GCV 0.07 µg/mL: trough level).

Table I. Intra- and Inter-Assay Precision and Accuracy for Plasma Samples with GCV

		Intra-assay (n = 6)			Inter-assay (n = 8)		
Drugs	Added (µg/mL)	Found (mean ± SD) (µg/mL)	Precision CV (%)	Accuracy (%)	Found (mean ± SD) (µg/mL)	Precision CV (%)	Accuracy (%)
GCV	0.025	0.024 ± 0.001	2.87	96.5	0.024 ± 0.001	4.72	94.9
	0.100	0.096 ± 0.003	3.00	96.3	0.102 ± 0.005	4.96	102.2
	1.000	1.006 ± 0.043	4.28	100.6	1.027 ± 0.044	4.36	102.7
	5.000	4.960 ± 0.065	1.61	99.2	4.951 ± 0.114	2.31	99.0

Limit of quantification

The quantification limit (LOQ) was 0.025 µg/mL for GCV at which the signal-to-noise ratios of the peaks were at least 8:1, and good intra- and inter-assay CV values (< 4.96%) and recoveries (94.9–96.5%) were achieved for calibration standards (Table I).

The LLOQ of 0.02 µg/mL for GCV has been reported using fluorescence detection, which required 200 µL of human plasma (14). However, our method attained nearly the same level, requiring only 100 µL of plasma.

Absolute recovery

Absolute recoveries for GCV were calculated at four different concentrations by comparing the peak area of the plasma standard solution with the mean peak area obtained from direct injection of the corresponding stock standard solutions added to the IS and perchloric acid followed by sodium hydroxide solution as described previously. Table II lists the sufficient results of recovery studies.

Sample preparation

Most previously published methods for determination of GCV (LOQ: 0.04–0.10 µg/mL) need a large volume (250–1000 µL) of serum and plasma (7–9,11–13,15), but particularly from small infants, it is not easy to obtain a large volume of blood. Thus these methods for therapeutic drug monitoring of GCV in small infants were actually difficult to conduct at clinical site.

In the present study, to get more sensitivity from a small quantity of plasma and to avoid losing time, simple protein precipitation procedures such as using acetonitrile and perchloric acid were examined in sample preparation. In the case of acetonitrile, after protein precipitation, the extracts containing organic solvents had to be removed with nitrogen gas because GCV was slightly retained with a much polar mobile phase such as presented chromatographic condition. Furthermore, low GCV recoveries (about 85–90%) were detected from plasma, which were at the same level reported in the study of Cociglio et al. (8). On the points of using perchloric acid, after protein precipitation, direct injections to the HPLC system was too difficult because of shortened column life by the strong acidity of perchloric acid. A direct injection was tried in the same way by Teshima et al. (10). But as a result, column life has been shortened.

On the other hand, an appreciate concentration of perchloric acid was examined. A 0.8 M (4.8%) perchloric acid was tried, but numerous impurities

containing plasma decompositions were observed in chromatograms (15). That resulted in low sensitivity and selectivity for GCV analysis. Consequently, the use of 1% perchloric acid as a deproteinization solvent markedly improved them. After protein precipitation, the pH of the extracts was adjusted to approximately pH 2.5 with 0.1 M sodium hydroxide solution. Moreover less injection size (10 µL) to the HPLC system in this study kept the deterioration of the column to a minimum.

Application to patient sample

In three CMV infectious infants (5 months, 11 months, and 1 year 2 months old, respectively) with hearing loss, the application for this method was demonstrated. GCV (5.0 or 6.0 mg/kg body weight) was administrated for intravenous infusion over 60 min every 12 h for seven days. GCV of plasma concentration at the seventh day as determined by the described analytical methods is shown in Table III. These results were nearly the same concentration levels reported by Burri et al., where a six-old girl had been administrated 4.4 mg/kg of GCV by intravenous infusion (17).

Conclusions

A simple and more sensitive HPLC method was developed for determination of GCV for 100 µL of plasma sample using fluorescence detector with an internal standard. Results obtained by this study demonstrate its usefulness for therapeutic monitoring or further pharmacokinetics studies of GCV concentrations in infants with CMV or other relative infections.

Table II. Absolute Recoveries of GCV from Human Plasma

Drugs (*n* = 6)	Concentration (µg/mL)	Recovery (mean ± SD, %)	CV (%)
GCV	0.025	92.8 ± 3.0	3.19
	0.100	97.7 ± 3.9	3.99
	1.000	100.5 ± 6.6	6.62
	5.000	95.9 ± 1.7	1.75

Table III. Plasma GCV Concentrations (µg/mL) of the Seventh Day with GCV Treatment*

Pt No.	Year	Intravenous dosage† mg/kg weight	GCV concentrations (µg/mL) –10 min	60 min	120 min
1	11M	5.0	0.07 ± 0.01	4.71 ± 0.08	NA‡
2	5M	5.0	0.06 ± 0.01	NA	2.15 ± 0.04
3	1Y2M	6.0	0.09 ± 0.01	NA	3.66 ± 0.05

* Data are given as mean ± SD (*n* = 3). ‡ NA = not analyzed.
‡ Receiving intravenous infusion over 60 min every 12 h for seven days.

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