BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **17**: 500–503 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.258

ORIGINAL RESEARCH

A simple and simultaneous determination of acyclovir and ganciclovir in human plasma by high-performance liquid chromatography

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Received 19 December 2002; revised 15 January 2003; accepted 24 January 2003

ABSTRACT: A simple high-performance liquid chromatographic method was developed for the simultaneous determination of the therapeutic levels of acyclovir and ganciclovir in human plasma. After precipitation of plasma proteins with 6% perchloric acid, acyclovir and ganciclovir were simultaneously determined by reversed-phase chromatography with spectophotometric detection at 254 nm. The peak heights for acyclovir and ganciclovir were linearly related to their concentrations ranging from 0.063 to 2.080 μg/mL. The recovery was 100.48–102.84% for acyclovir and 99.26–103.07% for ganciclovir. The intra- and inter-day relative standard deviation values were in the range 0.186–8.703% for acyclovir and 0.137–6.424% for ganciclovir. The detection limits for both compounds were 0.01 μg/mL determined as the signal-to-noise ratio of 3. The present method is applicable to therapeutic monitoring during antiviral medication. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: antiviral agents; nucleoside analogues of guanosine; reversed-phase chromatography; therapeutic monitoring; UV detection

INTRODUCTION

Viral infection is the most serious problem during immunosuppressant treatment after transplantation. Latent herpes simplex virus (HSV) (Bustamante *et al*., 1991), varicella-zoster virus (VZV) (Locksley *et al*., 1985; Stover *et al*., 1998) and cytomegalovirus (CMV) (Prentice *et al*., 1994; Jong *et al*., 1998) are the major causes of morbidity and mortality in immunocompromized hosts. Acyclovir (ACV) and ganciclovir (GCV), both of which are nucleoside analogues of guanosine, are potent anti-viral agents widely used for the prevention or cure of such infectious diseases. The former is efficacious as acute therapy or prophylaxis of HSV and VZV (Wagstaff *et al*., 1994), being most effective in suppressing the growth of HSV type 1 (IC_{50}; 0.01–2.7 μg/mL) and type 2 (IC_{50}; 0.01–4.4 μg/mL), followed by VZV (IC_{50}; 0.17–26 μg/mL), Epstein–Barr virus (IC_{50}; 1.5–8.8 μg/mL) and human herpes virus 6 (IC_{50}; 3–25 μg/mL; Wagstaff *et al*., 1994). On the other hand, GCV is considered to be effective for the treatment of CMV (Crumpacker, 1996; Piketty *et al*., 2000; Tornatore *et al*., 2001). This agent is virustatic with the IC_{50} for most clinical isolates of CMV ranging from 0.2 to 1.6 μg/mL (Crumpacker, 1996; Tornatore *et al*., 2001). It is postulated that the determination of ACV and GCV in serum or plasma is required for the successful prevention or therapy for viral infection without inducing the emergent resistant viral strains.

Although the high-performance liquid chromatographic determination of ACV or GCV in human plasma or urine has been reported by a number of investigators (Page *et al*., 1996; Boulieu *et al*., 1997; Chu *et al*., 1999; Pham-Huy *et al*., 1999; Bangaru *et al*., 2000; Olsen, 2001), there have been few reports on the simultaneous determination of these anti-viral agents (Campanero *et al*., 1998), except for the simultaneous assay for teicoplanin and ganciclovir (Cociglio *et al*., 1998).

Therefore, we report in the present study a simple and simultaneous determination of ACV and GCV in human plasma.

EXPERIMENTAL

Chemicals and reagents. ACV, GCV and HPLC-grade methanol were obtained from Wako Pure Chemicals Industries (Osaka, Japan). Zovirax® 200 mg tablets and Denosine® 250 mg vials were purchased from Glaxo-Smithkline (Tokyo, Japan) and Tanabe & Co. Ltd (Osaka), respectively. Blood was taken from young adult healthy volunteer using Venoject® heparinized collection tubes coated with silicon (Terumo, Elkton, MD, USA) and centrifuged at 3000 rpm for 10 min. The resultant

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Abbreviations used: ACV, acyclovir; CMV; cytomegalovirus, CV; coefficient of variation; GCV, ganciclovir; HPLC; high-performance liquid chromatography, HSV; herpes simplex virus, MRE; mean relative errors, QC; quality control, RSD; relative standard deviation, SD; standard deviation; SE; standard error, VZV; varicella-zoster virus.

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drug-free human plasma was stored at –20°C. Other reagents used in the present experiment were all of analytical grade. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

HPLC apparatus. The HPLC system consisted of a solvent delivery system (model LC-10AS, Shimadzu Scientific Instruments Kyoto, Japan), a guard column (4.6 mm i.d. × 5 mm) packed with 5 μm ODS resin, a separation column (TSK gel ODS-80 Tm, 5 μm, 4.6 mm i.d. × 150 mm, Tosoh, Tokyo), a UV–vis detector (SPD-10A, Shimadzu) and a data processing apparatus (Chromatopack CR-4A Data System, Shimadzu).

Sample preparation and chromatographic conditions. Blood was taken from a patient, who orally took 200 mg ACV (Zovirax®) tablets four times daily and injectable 250 mg GCV (Denosine®) twice daily for 3 days, at 12 h after the last treatments. For the precipitation of plasma protein, 6% perchloric acid (40 μL) was added to a 100 μL plasma sample and vortexed for 1 min. After centrifugation at 20,000 rpm for 10 min, 50 μL aliquots of the supernatant were directly injected into the HPLC system. The mobile phase was a mixture of acetonitrile and 20 mM phosphate buffer (pH 3.0), in which the mixing ratio was 2:70 (v/v), and delivered at a flow rate of 0.6 mL/min. The UV detection was set at 254 nm.

Calibration curves and quality control samples. The solutions of ACV (400 μg/mL) and GCV (400 μg/mL) were prepared by dissolving in water. The ACV and GCV solutions were mixed together (1:1, v/v), followed by preparation of various concentrations (1.25–40.00 μg/mL) of the standard solution for ACV and GCV. The resultant solutions were stored at –20°C until assayed. Calibration standards (0.063–2.080 μg/mL) were prepared every time before assaying by diluting the stock solutions with drug-free human plasma. Plasma samples obtained from a patient were stored at –20°C until assay.

Quality control (QC) samples were prepared by spiking the stock solutions into drug-free human plasma, as descried for the preparation of the calibration standards. The concentrations used for the QC were 0.36–1.49 μg/mL. The QC samples were stored at –20°C and analyzed every time along with each batch of samples to monitor the performance of the method during routine use. Linear least-squares regression analysis was used to characterize calibration curves. The equation $Y = aX + b$ was fitted to the data, where Y represents the peak heights of ACV and GCV, X is the spiked concentrations of ACV or GCV, a is the slope of the regression, and b is the Y intercept. Calibrations were made for ACV and GCV in the concentration range between 0.063 and 2.080 μg/mL. The concentrations in plasma were calculated from the peak height on the basis of the standard curve equation.

Recovery, detection limit, precision, stability and selectivity. The concentrations used for the recovery tests were 0.36–1.49 μg/mL. The detection limit was defined as the concentration corresponding to the peak height that was 3-fold higher than the baseline noise at 0.064 AUFS. The precision of the method based on within-day repeatability was determined by five replication analyses of samples spiked with six different concentrations ranging from 0.063 to 2.080 μg/mL. The reproducibility (day-to-day variation) of the method was established using the same concentration range as described above, but only a single determination of each concentration was made on five different days. The coefficient of variation (CV) was calculated from the ratio of the standard deviation (SD) to the mean. The precision was determined by replicate analyses of six different concentrations ranging from 0.063 to 2.080 μg/mL and comparing the differences between the spiked values and the real concentrations.

The stability of ACV and GCV in QC samples was tested after leaving the standard solutions for 24 h at room temperature (25°C) or storing at –20°C for 2 months. The selectivity of the method was checked by injecting the mixture of ACV and GCV with conventionally co-administered drugs, including cyclosporin A, tacrolimus, mycophenolic acid, predonisolone, sulfamethoxazole, trimethoprim, fluconazole and itraconazole.

RESULTS AND DISCUSSION

Typical chromatograms obtained from the blank plasma, drug-free plasma spiked with ACV and GCV and patient's plasma were shown in Fig. 1. GCV and ACV appeared as well-separated peaks with retention times of 8.4 and 10.9 min, respectively, and the analysis was completed in approximately 15 min. The selectivity of the assay was checked by injecting blank plasma. The chromatographic interferences derived from endogenous substances or drugs such as cyclosporin A, tacrolimus, mycophenolic acid, predonisolone, fluconazole and itraconazole, all of which were administered in combination with ACV and GCV, were completely separated from ACV and GCV.

The calibration curves for ACV and GCV were linear within the concentration ranges of 0.063–2.080 μg/mL. Although no internal standard was used here, there was an excellent correlation between the peak heights (Y) and

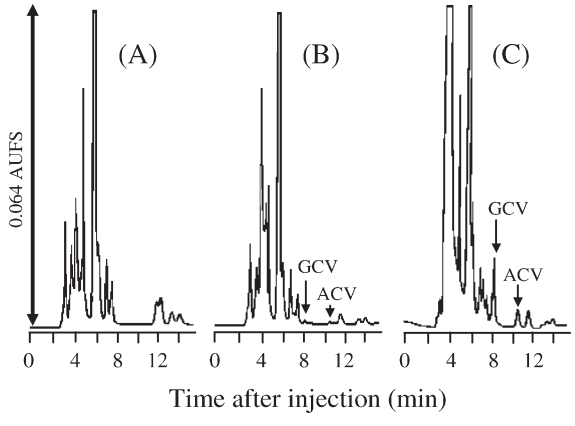

Figure 1. Typical chromatograms showing (A) blank plasma, (B) plasma spiked with ACV (0.063 μg/mL) and GCV (0.065 μg/mL) and (C) the patient's plasma taken at a trough point of 3 days after administering oral Zovirax® 200 mg four times daily and injectable Denosine® 250 mg twice daily.

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Table 1. Recovery of ACV and GCV added to the drug-free human plasma

	Concentration (μg/ml)(added)	Recovery[a] (%, mean ± SD)	CV (%)
ACV	0.36	100.48 ± 3.01	3.00
	0.72	102.84 ± 3.01	2.92
	1.44	101.82 ± 2.26	2.19
GCV	0.37	99.26 ± 4.25	4.28
	0.75	102.67 ± 3.06	2.98
	1.49	103.07 ± 3.69	3.58

[a] Average of five determinations.

the concentrations (X) as follows: $Y = 4203.2(\pm 40.7)X - 25.7(\pm 15.8)$ (mean ± SE, $n = 5$, $r = 0.999$ for ACV) and $Y = 5530.5(\pm 56.7)X - 84.2(\pm 12.9)$ (mean ± SE, $n = 5$, $r = 0.999$ for GCV).

The recoveries of ACV and GCV from human plasma were 99.26–103.07% and the CV were from 2.19 to 4.28% (Table 1). Therefore, it appears that the present HPLC method with no internal standards is useful for simultaneous assay for ACV and GCV.

ACV and GCV in the QC samples were found to be stable when stored either at room temperature (25°C) for 24 h or at –20°C for at least 2 months.

To evaluate the precision of the present method, repeated analyses of ACV and GCV-spiked plasma samples (0.063–2.080 μg/mL) were carried out. The findings in Table 2 show the within- and inter-day variations of the method. The within-run reproducibility of the assay was excellent with relative standard deviations (RSD) of 0.137-8.703%. The averages in the differences between the spiked concentrations of ACV and GCV and their real values (mean relative errors; MRE) were within a range of—8.048–7.662%, showing good accuracy of the present method. The inter-day RSD were 0.148–7.610% and MRE ranged from –5.000 to 6.185%. The detection limits for ACV and GCV, defined as a signal-to-noise ratio of 3, was 0.01 μg/mL for both agents.

The trough plasma concentrations of ACV and GCV in one patient who took oral 200 mg ACV tablets four times daily and injectable 250 mg GCV twice daily for 3 days were 0.42 μg/mL for ACV and 1.06 μg/mL for GCV. Therefore, the present HPLC method for the simultaneous determination of ACV and GCV was simple and specific, thereby being useful for the therapeutic monitoring of antiviral medication.

CONCLUSIONS

We developed a simple HPLC method for the simultaneous determination of ACV and GCV. This method enabled detection of the trough concentrations of both agents in plasma of a patient who took antiviral medications after renal transplantation. The total run time was approximately 15 min, and the detection limit was 0.01 μg/mL for both agents. Therefore, the present method may be useful for the routine monitoring of plasma concentrations of ACV and GCV in immunocompromized hosts.

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Table 2. Precision (RSD) and accuracy (MRE) of the HPLC method for ACV and GCV analyses in plasma samples

ACV				GCV			
Spiked (μg/mL)	Analyzed[a] (μg/mL)	RSD (%)	MRE (%)	Spiked (μg/mL)	Analyzed[a] (μg/mL)	RSD (%)	MRE (%)
Intra-assay							
0.063	0.061 ± 0.003	5.159	–3.746	0.065	0.070 ± 0.004	6.424	7.662
0.126	0.116 ± 0.010	8.703	–8.048	0.130	0.132 ± 0.002	1.717	1.631
0.253	0.261 ± 0.017	6.604	3.501	0.260	0.255 ± 0.002	0.660	–1.908
0.505	0.507 ± 0.025	4.885	0.352	0.520	0.517 ± 0.006	1.112	–0.519
1.010	1.022 ± 0.017	1.673	1.190	1.040	1.039 ± 0.004	0.428	–0.083
2.020	2.013 ± 0.006	0.309	–0.336	2.080	2.081 ± 0.003	0.137	0.069
Inter-assay							
0.063	0.065 ± 0.005	7.610	2.579	0.065	0.069 ± 0.004	5.497	6.185
0.126	0.120 ± 0.006	4.884	–5.000	0.130	0.135 ± 0.005	3.672	3.846
0.253	0.251 ± 0.019	7.472	–0.515	0.260	0.255 ± 0.002	0.907	–2.071
0.505	0.510 ± 0.019	3.640	0.998	0.520	0.514 ± 0.004	0.788	–1.231
1.010	1.013 ± 0.007	0.699	0.297	1.040	1.042 ± 0.006	0.573	0.163
2.020	2.018 ± 0.004	0.186	–0.112	2.080	2.081 ± 0.003	0.148	0.046

[a] Values are means ± SD of five (intra-assay) and five (inter-assay) experiments.
RSD, relative standard deviation; MRE, mean relative error.

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