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Simultaneous determination of aciclovir, ganciclovir, and penciclovir in human plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A fast, simple and selective HPLC method has been developed for the assay of aciclovir, ganciclovir, and penciclovir in human plasma by coupling HPLC with fluorescence detection. 200 μl plasma, with guanosine 5′-monophosphate as an internal standard, was subjected to protein precipitation with a 7% [v/v] aqueous perchloric acid solution. The 40 μl supernatant was injected into a Diamonsil-5 μm C18 column. Aciclovir, ganciclovir, and penciclovir, with solvents composed of methanol and 0.08% aqueous trifluoroacetic acid solution, were analysed by fluorescence detection at 260 nm (excitation) and 380 nm (emission) using a gradient elution program. The calibration curves of all three analytes were linear between 20 and 2000 ng/ml. The mean absolute recoveries of aciclovir, ganciclovir, and penciclovir were 93.91 ± 1.20%, 97.42 ± 0.75%, and 99.01 ± 3.30%, respectively. The mean inter-day CVs for aciclovir, ganciclovir, and penciclovir, were within 1.29–7.30%, 1.00–5.53%, and 1.19–3.54%, respectively. The intra-day bias for aciclovir, ganciclovir, and penciclovir ranged from −2.01 to 6.33%, 1.81 to 7.37%, and 1.42 to 6.91%, respectively. The method has been validated and applied in pharmacokinetic studies in Chinese adult renal transplant patients.

1. Introduction

Cytomegalovirus (CMV) is a primary cause of disease in immunocompromised subjects, including hematopoietic stem cell transplant (HSCT) patients and solid organ transplant (SOT) recipients [1]. Herpes simplex virus (HSV) types 1 and 2 are ubiquitous pathogens that infect 75% of adults in the United States [2]. Immunocompromised hosts such as transplant recipients and HIV-infected individuals are at a particularly high risk of developing severe complications from HSV infection [3].

Aciclovir (ACV), ganciclovir (GCV), and penciclovir (PCV) are nucleoside analogues with antiviral activity for prophylaxis and treatment against herpes simplex virus or CMV [4–6]. Valaciclovir, valganciclovir, and famciclovir are the prodrugs of ACV, GCV, and PCV, respectively, and are typically used due to their higher bioavailability. The oral dosing regimens of valganciclovir and valaciclovir provide comparable plasma exposure to that of I.V. GCV and ACV [7]. The structures of these antiviral drugs are shown in Fig. 1.

Monitoring ACV and related compounds is useful, particularly in situations where toxic events are experienced at clinically relevant dosages. Given that ACV, GCV, and PCV are predominantly eliminated by kidneys, renal impairment affects their plasma concentrations and rate of elimination [8–14]. Possible neurotoxic effects of ACV in patients with renal disease have been reported [15–17], and GCV has been associated with serious toxic side effects such as haematological toxicity [18] and neurotoxicity [19]. PCV is produced by deacetylation and oxidation of famciclovir in liver and kidney, and poor liver or renal function could impair this conversion process. It is therefore important to study the metabolic process of this drug in patients with hepatic diseases [12]. In cases of multi-organ failure, especially severe hepatic and renal failure that may occur after transplantation, monitoring the concentration of ACV, GCV, and PCV may be useful in transplant patients. Moreover, since these anti-viral agents are often co-administered with other drugs, such analyses demand an assay that is not only simple, rapid, precise, and sensitive, but also capable of determining the pharmacokinetic profile of these aniviral compounds in the presence of other drugs [20].

The quantitation of ACV, GCV, and PCV in biological samples poses an important challenge because these drugs are structurally similar to endogenous substances. Hence, this complicates analysis and requires using a highly selective analytical method. Some analytical methods have been proposed for individual measurement of ACV [21–37], GCV [38–45], and PCV [46–48]. Other techniques have been developed for simultaneous determination of ACV and GCV [49–51], valacyclovir and ACV [52], or valganciclovir, and GCV

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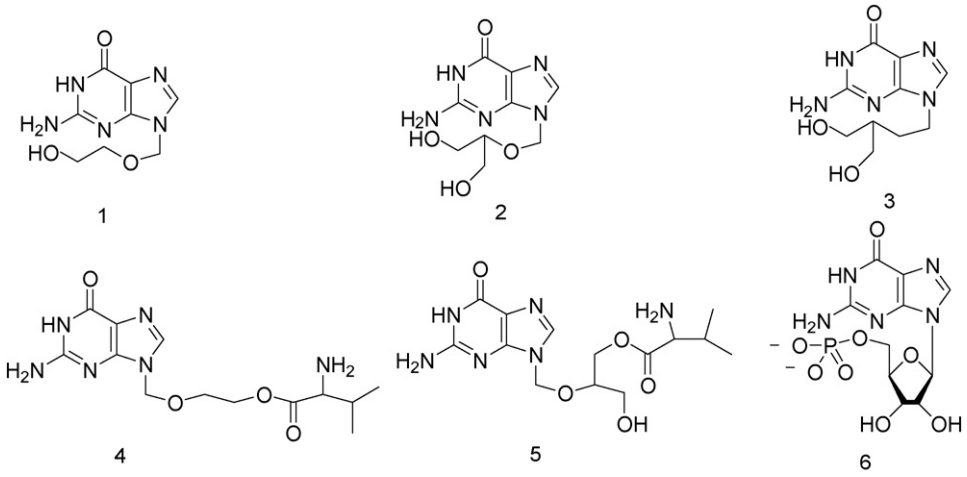

Fig. 1. The chemical structures: aciclovir (1), ganciclovir (2), penciclovir (3), valaciclovir (4), valganciclovir (5), and I.S.-GMP (6).

[53], through high-performance liquid chromatography (HPLC) in biological fluids. However, so far no analytical method for therapeutic drug monitoring allows simultaneous measurement of ACV, GCV, and PCV.

Here we describe a simple but efficient method using HPLC coupled with fluorescence detection for simultaneous monitoring of ACV, GCV, and PCV in human plasma. With small amounts of plasma, this method reaches the levels of selectivity and reproducibility required for pharmacokinetic studies in Chinese adult renal transplant patients.

2. Materials and methods

2.1. Materials

ACV, GCV, and PCV were kindly provided by Livzon Pharm (Guangdong, China), Roche Pharm (Shanghai, China), and Huapont Pharm (Chongqing, China), respectively. Guanosine 5′-monophosphate (Internal Standard, I.S.) was obtained from Sigma–Aldrich (St. Louis, USA). HPLC-grade methanol and perchloric acid (70%) were obtained from Promptar (Elk Grove, CA, USA) and Jinlu Chemical Co. (Shanghai, China), respectively. Trifluoroacetic acid (TFA) was purchased from Sinopharm Group Chemical Reagent (Shanghai, China). Ultrapure water was prepared by using a Milli-Q Academic water-purification system (Millipore, Milford, MA, USA). Drug-free human plasma was supplied by the Blood Bank of Huashan Hospital, Fudan University (Shanghai, China).

2.2. Chromatographic system

The chromatographic system was an Agilent 1100 series (Agilent Technologies, Germany), equipped with a G1311A quaternary pump, a temperature controlled auto-injector and column compartment and a G1321A fluorescence detector set at 260 nm (excitation) and 380 nm (emission). Instrument control, data collection, and processing were performed with Agilent Chemstation version A.10.02 (Agilent Technologies, Germany). The separation was performed at 25 °C on a 4.6 mm × 250 mm, particle size 5 μm, Diamonsil C18 analytical column (Dikma Co. Shanghai, China).

2.3. Mobile phase solutions

Solvent A was prepared from 0.08% [v/v] aqueous TFA solution (pH 2.30–2.35, 25 ± 3 °C) prior to each series of analyses. Solvent B consisted of pure methanol. The mobile phase was delivered at 1.5 ml/min and the gradient elution program is reported in Table 1.

Table 1
Mobile phase compositions for assay of acyclovir, ganciclovir, and penciclovir in human plasma

Time (min)	Solvent A, 0.08%TFA (%)	Solvent B, MeOH (%)
0.00–7.00	96	4
7.01–10.00	40	60
10.01–12.50	96	4

2.4. Preparation of stock solutions, calibration standards and quality control samples

1 mg/ml stock solutions of ACV, GCV, and PCV were prepared by MeOH:H_2O (50:50, v/v). Each stock solution was further diluted with ultrapure water to prepare a series of working solutions. Plasma-based calibration standards were prepared at 2000, 1500, 1000, 500, 100, and 20 ng/ml for ACV, GCV, and PCV. Plasma quality control samples were prepared at 1600, 800, and 40 ng/ml by appropriate dilution of the respective working solution with drug-free plasma. A 200 μg/ml stock solution of guanosine 5′-monophosphate (GMP) was prepared in ultrapure water, and diluted with ultrapure water and perchloric acid. Finally, the 7% aqueous perchloric acid solution (containing 10 μg/ml of the internal standard) was prepared.

All solutions, calibration standards and QC samples were stored at −20 °C.

2.5. Sample preparation

Plasma samples (blank, standard, control, or patient sample; 200 μl) were mixed with 50 μl of 7% [v/v] aqueous perchloric acid solution (containing 10 μg/ml of the internal standard) in 1.5 ml polypropylene tubes. The mixtures were vigorously vortex-mixed for 30 s, then centrifuged at 20,627 × *g* (15,000 rpm) at 4 °C (3K15, Sigma Laborzentrifugen, Germany). The supernatants were transferred to autosampler vials and 40 μl of each solution was injected into the HPLC column.

2.6. Method validation

Assay performance was determined in accordance with FDA guidelines for bioanalytical method validation [54]. The method

Table 2
Drugs potentially co-administered with acyclovir, ganciclovir, and penciclovir were examined for possible interference with this method

Acetaminophen	4-Hydroxy antipyrinum
Adefovir dipivoxil	Hydrochlorothiazide
Almitrine	9-Hydroxy risperidone risperidone
Azathioprine	Ibuprofen
Bepridil hydrochloride	Indometacin
Carbamazepine	Lorcainide
Chlorzoxazone	Metoclopramide
Ciprofloxacin	Metronidazole
Clindamycin sodium phosphate	Naproxen sodium
Clonazepam	Nevirapine
Clozapine	Nifedipine
Cyclosporine A	Nimodipine
Dexamethasone sodium phosphate	Nitrazepam
Dextromethorphan hydrobromide	Ofloxacin
Diazepam	Phenacetin
Digoxin	Phenylpropanolamine
Diltiazem	Phenytoin sodium
Dimethylbiguanide	Propafenone
Doxepin	Pseudoephedrine
Famciclovir	Raubasine
Fenofibrate	Spironolactone
Fenofibrate acid	Tamoxifen
Gentamicin sulphuric acid	Topiramate
Glibenclamide	Valaciclovir
Gliclazide	Valganciclovir
Glipizide	

was validated by determination of selectivity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recovery and stability.

2.6.1. Selectivity

To evaluate potential chromatographic interference by endogenous substances (matrix effects) and other drugs commonly co-administered in Chinese renal transplant patients, pooled blank human plasma samples from different sources and methanol standards containing 10 μg/ml of each drug were tested (Table 2).

2.6.2. Linearity and dilution test

Quantitative analysis of ACV, GCV, and PCV was performed over the range from 20 to 2000 ng/ml. The calibration curves were obtained by linear least-squares regression analysis, plotting of peak to area ratios (ACV, GCV, and PCV/I.S.) versus the ratio of concentrations of ACV, GCV, and PCV using 1/concentration^2 ($1/x^2$) as the weighting factor in each standard sample.

If the concentration was greater than the upper limit of the calibration range, duplicate samples of analyte were analysed after two-fold to four-fold dilution with drug-free plasma. The solutions obtained were assessed as described previously, and the adjusted calculated concentrations were compared with the nominal concentration.

2.6.3. Precision, accuracy and LLOQ

Replicate analysis ($n = 6$) of quality control samples at four concentration levels (LLOQ, low, medium, and high: i.e., 20, 40, 800, and 1600 ng/ml for each drug) was used for determining the precision and accuracy of the assay. Precision was calculated as the coefficient of variation (C.V., %) within a single run (intra-day) and among different runs (inter-day), and the accuracy was calculated as the deviation between nominal and measured concentrations. The LLOQs for ACV, GCV, and PCV were experimentally chosen as the minimal quantitative concentration in plasma samples. The FDA Guidance recommends that the response of the analyte should be at least five times that of the blank and the analyte peak (response), and that the analyte peak should be identifiable, discrete, and reproducible with precision of 20% and accuracy of 80–120%.

2.6.4. Absolute recovery

The absolute recovery was determined by comparing the peak areas of each compound after extraction with those obtained by direct injection of the same amount of analyte in aqueous solution. The absolute recovery of the I.S. was calculated in the same way.

2.6.5. Stability of ACV, GCV, and PCV

The short-term, long-term, and freeze–thaw stability of the analytes was determined in human plasma after each storage period, and the results were compared to the initial concentration (samples that were processed immediately after being freshly prepared). Samples were regarded as stable if the deviation from the initial condition was within ±15%.

2.6.5.1. Short-term stability. Short-term stability was first evaluated using triplicate aliquots of each low- and high-concentration QC sample, which had been kept at RT (25 ± 3 °C) for 0, 4, and 12 h, respectively, before being processed. The stability of stock solutions of ACV, GCV, PCV, and I.S. at RT was also assessed.

Triplicate aliquots of the QC samples were kept at 4 °C for 0, 12, and 60 h before being processed, and were processed and placed in the autosamplers at 10 °C and analysed after 0, 12, and 24 h, respectively. In addition, 12 human blood samples collected from patients (four patients for each analyte) were processed after centrifugation at 2880 × *g* (4000 rpm) for 10 min at 4 °C. The supernatants were divided into duplicate aliquots. One aliquot was immediately analysed and the other was stored in the autosampler at 10 °C and analysed 24 h later.

The stability of the analytes in human blood at room temperature (RT) (25 ± 3 °C) was also investigated. Eight aliquots of quality control blood samples spiked with ACV, GCV, and PCV were prepared. Four aliquots were immediately centrifuged at 2880 × *g* (4000 rpm) for 10 min at 4 °C and the other four aliquots were kept at RT for 2 h prior to centrifugation. These plasma samples were immediately processed and analysed.

2.6.5.2. Long-term stability and freeze–thaw stability. Long-term stability was evaluated using triplicate aliquots of each low- and high-concentration unprocessed QC sample kept at −20 °C for 3 months.

Freeze–thaw stability was determined by using triplicate aliquots of each low- and high-concentration QC sample over three freeze–thaw cycles. For each cycle, the frozen plasma samples were thawed at room temperature for 2 h, and then refrozen for 24 h.

2.7. Applications of the HPLC method

ACV, GCV, and famciclovir are regularly prescribed for transplant patients in our hospital. Therefore, we applied this method to pharmacokinetic studies in Chinese adult renal transplant patients receiving oral ACV (400 mg, thrice daily), GCV (1000 mg, thrice daily), or famciclovir (250 mg, thrice daily) for drug safety and optimization.

Renal transplant patients of Huashan Hospital, aged 18 or above, were invited to participate. Pregnant women or nursing mothers were excluded. Written informed consent was obtained from each subject prior to the beginning of the study. The study protocol was approved by the institutional Ethics Committee and was conducted according to the recommendations described in the Declaration of Helsinki. Serial venous blood samples were collected as a function of time.

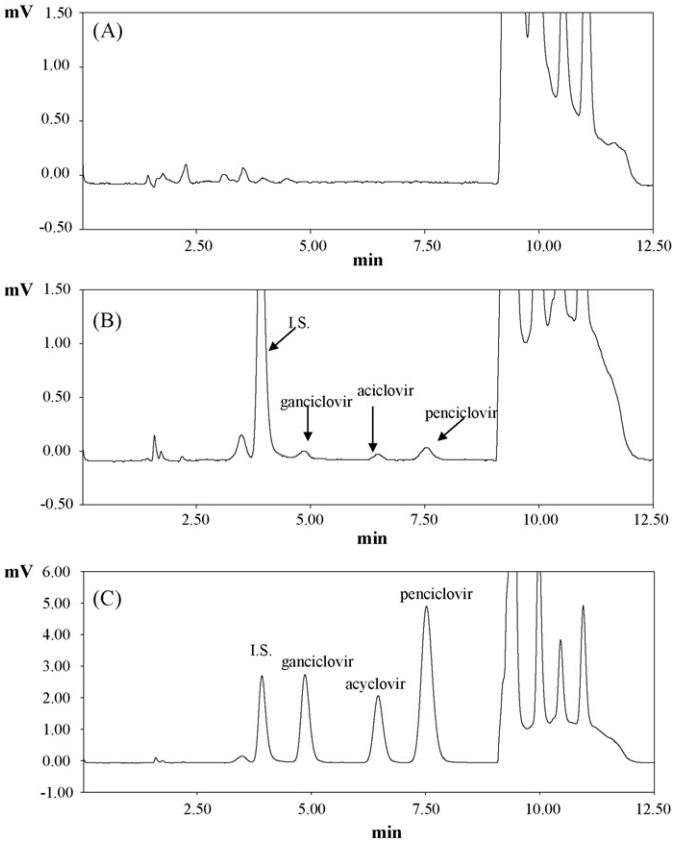

Fig. 2. (A) Chromatogram of a pooled blank plasma. (B) Chromatogram of a plasma control sample of aciclovir (20.00 ng/ml), ganciclovir (20.00 ng/ml), and penciclovir (20.00 ng/ml) spiked with I.S.(GMP). (C) Chromatogram of a plasma control sample of aciclovir (800.00 ng/ml), ganciclovir (800.00 ng/ml), and penciclovir (800.00 ng/ml) spiked with I.S. (GMP).

Blood samples were collected in tubes (BD Vacutainer K2 EDTA 3.6 mg 2 ml, BD, Franklin Lakes, NJ, USA) containing ethylene diamine tetraacetate (EDTA). The samples were rapidly centrifuged at 2880 × *g* (4000 rpm) for 10 min at 4 °C. Then, the plasma of each sample was separated and transferred into 1.5 ml polypropylene tubes and stored at −20 °C until analysis.

3. Results and discussion

3.1. Selectivity

Our proposed HPLC method enables the measurement of ACV, GCV, and PCV in human plasma with fluorescence detection at 260 nm (excitation) and 380 nm (emission). The chromatograms of pooled blank plasma, LLOQ, and a control plasma sample of ACV, GCV, and PCV (I.S. has been added) are shown in Fig. 2.

With the gradient program, the retention times for ACV, GCV, PCV, and I.S. were 6.5, 4.9, 7.7, and 4.0 min, respectively. This gradient elution program yielded sharp peaks without producing any significant drift of the baseline. The assay was found to be free from interference associated with the regularly administrated prescription and over-the-counter drugs shown in Table 2.

3.2. Calibration curves and dilution test

The standard calibration curves were linear between 20 and 2000 ng/ml, with a correlation coefficient (*r*) greater than 0.999. The slope of the calibration curves appeared stable, with average values of 0.00112 ± 0.00004 (*n* = 6), 0.00131 ± 0.00004 (*n* = 6), and 0.00336 ± 0.00011 (*n* = 6) for ACV, GCV, and PCV, respectively.

Different weighting factors were examined for fitting the standard curves, and a weighting factor of $(1/x^2)$ was chosen to compare the plots' linearity and precision.

The dilution test was reproducible (recovery 102.26 ± 0.47% for ACV, 101.46 ± 0.30% for GCV, and 102.02 ± 0.62% for PCV), which will be useful in cases where drug concentrations in study samples exceed the linear range of this method.

3.3. Precision, accuracy and LLOQ

Precision and accuracy results obtained from control samples are given in Table 3. Over these concentration ranges, the mean intra-day precision was better than 8% for each sample. The mean inter-day precisions for ACV, GCV, and PCV were generally good, with mean CVs within 1.29–7.30%, 1.00–5.53%, and 1.19–3.54%, respectively. The intra-day deviations (bias) from the added (nominal) concentrations of ACV, GCV, and PCV were in the ranges of −2.88 to 6.33%, 1.81–7.37%, and 1.42–6.91%, respectively. Therefore, all values lay within the ±15% limit proposed by FDA guidelines [54].

The LLOQ of 10 ng/ml has been reported using fluorescence detection [23], which required 1 ml of human plasma. Notably, the LLOQ is better than the 40 ng/ml LLOQ in previous reports [24,33,39,40,45,46,49]. Our method enabled the LLOQ for ACV, GCV, and PCV to be 20 ng/ml and use only 200 μl plasma, which was more sensitive than the results from the studies described above. The LLOQ of each analyte was given with good accuracy (bias within 10%) and precision (CV% less than 8%), as shown in Table 3.

3.4. Recovery

The mean absolute recoveries of ACV, GCV, PCV, and I.S. measured with QC samples were 93.91 ± 1.20%, 97.42 ± 0.75%, 99.01 ± 3.30%, and 105.26 ± 0.66%, respectively.

3.5. Sample stability

3.5.1. Short-term stability

The stability achieved with control samples at RT, 4 °C, and 10 °C in the autosampler is shown in Table 4. ACV, GCV, and PCV were stable in unprocessed plasma samples for up to 12 h at RT and for 60 h at 4 °C. Stability of processed samples in the autosampler at 10 °C was also good. The processed samples were stable in the autosampler for at least 24 h with acceptable precision and accuracy. For real samples, results showed that they ranged from 300 to 2000 ng/ml and they were also stable in the autosampler for 24 h with a recovery of 99.55 ± 2.80%, 100.92 ± 2.61%, and 97.93 ± 2.25% for ACV, GCV, and PCV, respectively.

The stock solutions were stable at RT for 4 h at 40 ng/ml (recovery 97.80 ± 4.68% for ACV, 94.39 ± 4.94% for GCV, and 101.84 ± 3.22% for PCV) or 1600 ng/ml (recovery 102.89 ± 0.83% for ACV, 102.78 ± 0.64% for GCV, and 102.31 ± 1.25% for PCV), and for 12 h at 40 ng/ml (recovery 99.67 ± 4.89% for ACV, 92.57 ± 1.54% for GCV, and 101.92 ± 1.76% for PCV) or 1600 ng/ml (recovery 102.68 ± 0.67% for ACV, 102.74 ± 1.11% for GCV, and 101.95 ± 0.90% for PCV). The I.S. solutions were also stable at RT for 4 h (recovery 97.22 ± 0.34%) and 12 h (recovery 100.29 ± 0.54%).

For blood sample stability, there was an initial drop in plasma concentration (−8.83%, −28.31%, and −23.14% after 2 h (RT), for ACV, GCV, and PCV, respectively). This phenomenon was similar to a previous report stating that the drug is taken up by erythrocytes until reaching erythrocyte/plasma equilibrium [49]. However, this phenomenon is unlikely to affect the accuracy of drug mea-

Table 3
Precision and accuracy of the assay for acyclovir, ganciclovir, and penciclovir in plasma (20, 40, 800 and 1600 ng/ml)

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Precision CV (%)	Accuracy bias (%)
Aciclovir			
(A) Intra-day (n = 6)			
20	19.60 ± 1.03	5.24	−2.01
40	38.85 ± 1.30	3.34	−2.88
800	850.65 ± 8.61	1.01	6.33
1600	1621.04 ± 23.73	1.46	1.31
(B) Inter-day (n = 6)			
20	19.60 ± 1.43	7.30	−2.00
40	40.01 ± 1.83	4.58	0.02
800	867.39 ± 21.00	2.42	8.42
1600	1644.93 ± 21.28	1.29	2.81
Ganciclovir			
(A) Intra-day (n = 6)			
20	21.32 ± 1.09	5.12	6.62
40	40.72 ± 1.37	3.36	1.81
800	858.93 ± 6.48	0.75	7.37
1600	1638.19 ± 15.20	0.93	2.39
(B) Inter-day (n = 6)			
20	20.34 ± 1.12	5.53	1.69
40	40.78 ± 2.04	5.01	1.94
800	868.80 ± 10.14	1.17	8.60
1600	1655.67 ± 16.52	1.00	3.48
Penciclovir			
(A) Intra-day (n = 6)			
20	20.69 ± 1.65	7.99	3.43
40	41.36 ± 1.07	2.58	3.40
800	855.24 ± 10.47	1.22	6.91
1600	1622.64 ± 20.40	1.26	1.42
(B) Inter-day (n = 6)			
20	21.84 ± 0.77	3.54	9.20
40	42.10 ± 0.50	1.19	5.25
800	862.27 ± 17.69	2.05	7.78
1600	1638.75 ± 28.35	1.73	2.42

Table 4
Stability of acyclovir, ganciclovir, and penciclovir in unprocessed samples left at room temperature (RT) for 4 and 12 h, at 4 °C for 12 and 60 h, and in processed samples left in the autosampler for 12 and 24 h

Conditions	Concentration added (ng/ml)	
	40	1600
Aciclovir[a]		
RT 4 h	102.58 ± 1.60	105.63 ± 0.15
RT 12 h	99.05 ± 4.92	103.18 ± 1.16
4 °C 12 h	98.70 ± 4.77	105.25 ± 0.43
4 °C 60 h	93.73 ± 1.21	104.99 ± 0.71
Autosampler at 10 °C 12 h	95.46 ± 3.62	104.32 ± 0.11
Autosampler at 10 °C 24 h	94.03 ± 0.04	102.80 ± 1.41
Ganciclovir[a]		
RT 4 h	101.69 ± 0.49	105.65 ± 0.36
RT 12 h	95.14 ± 1.97	104.48 ± 1.27
4 °C 12 h	102.83 ± 3.61	105.91 ± 0.49
4 °C 60 h	102.48 ± 4.74	104.97 ± 1.05
Autosampler at 10 °C 12 h	98.40 ± 3.08	104.92 ± 0.63
Autosampler at 10 °C 24 h	98.75 ± 7.14	105.14 ± 0.37
Penciclovir[a]		
RT 4 h	105.86 ± 3.73	104.54 ± 0.66
RT 12 h	103.88 ± 4.68	103.09 ± 1.94
4 °C 12 h	106.56 ± 2.49	104.50 ± 0.74
4 °C 60 h	102.98 ± 3.91	103.58 ± 0.82
Autosampler at 10 °C 12 h	105.73 ± 0.35	103.61 ± 0.14
Autosampler at 10 °C 24 h	103.33 ± 3.01	102.00 ± 2.04

RT, Room temperature.
[a] Recovery ± S.D. (%).

surements in blood from patients treated for few days, as the drug distribution in cell and plasma has already reached an equilibrium in circulating blood at the time of sampling [49].

3.5.2. Long-term stability and freeze–thaw stability

Stability in the freeze–thaw tests is given in Table 5, and it indicates that ACV, GCV, and PCV in human plasma stored at −20 °C and then thawed to RT were stable through three freeze–thaw cycles. If stored at −20 °C, ACV, GCV, and PCV were stable in plasma samples for 3 months. The long-term stability of stock solutions has been validated in published studies [30,41,46].

Overall, the stability of our results was consistent with previously published literature [30,40,41,46,49,51].

3.6. Clinical applications

This assay has been in clinical use for more than half a year, and hundreds of samples from adult renal transplant patients on a wide variety of drug treatments have been assessed. Representative chromatograms of plasma from patients on oral ACV, GCV, or famciclovir are given in Fig. 3.

3.7. Discussion

3.7.1. Fluorescence detection

Various analytical studies have been carried out to determine drug content in pharmaceuticals, pharmacokinetics, and optimal dosing of ACV, GCV, or PCV. These analyses have included HPLC with UV detection [21,22,25–28,30–32,34,35,38,41–44,48,50–52], fluo-

Table 5
Stability of acyclovir, ganciclovir, and penciclovir plasma samples after one, two, and three freeze–thaw cycles for the QC samples at nominal concentrations of 40 and 1600 ng/ml, respectively

Thaw–freeze cycles	Concentration added (ng/ml)					
	Aciclovir[a]		Ganciclovir[a]		Penciclovir[a]	
	40	1600	40	1600	40	1600
1	98.15 ± 3.84	102.05 ± 1.22	102.72 ± 3.58	103.23 ± 0.07	104.40 ± 2.34	102.17 ± 0.62
2	101.03 ± 2.15	100.91 ± 1.49	106.37 ± 1.52	102.49 ± 1.20	106.71 ± 2.16	99.18 ± 1.63
3	105.10 ± 1.64	103.35 ± 2.87	108.16 ± 4.80	103.38 ± 3.64	106.19 ± 2.62	102.69 ± 3.06

[a] Recovery ± S.D. (%).

rescence detection [23,24,29,33,36,37,39,40,45,46,49], and tandem mass spectrometry [47,53]. However, the LLOQs for UV detection were above 50 and 200 ng/ml for GCV, and PCV, respectively, which may not be sensitive enough for pharmacokinetic studies. Although the LLOQ was 10 or 20 ng/ml for ACV, 500–1000 μl of plasma was required for UV detection. Assay by HPLC tandem mass spectrometry could achieve better sensitivity, but the technique it requires is sophisticated and expensive instrumentation. Since ACV, GCV, and PCV (like other nucleoside analogues) are intrinsically fluorescent under acidic conditions, we attempted to overcome the above limitations using fluorescence detection.

3.7.2. Mobile phase composition

Nucleoside analogues are very polar, so the addition of ion-pairing agents or organic modifiers in the mobile phase could increase their retention in reversed phase chromatography [46]. Moreover, as they are both weak acids and weak bases, it is feasible to separate nucleoside analogues as ion-pairs with a strong acid as the counter-ion when the pH is below or near the pK_a [24]. TFA is commonly used as an ion pairing agent [55], and a pH modifier in the mobile phase. To obtain the best chromatographic separation and sensitivity in a short time, different mixtures of methanol and water containing various concentrations of TFA, as well as analytical columns with different packing materials, were systematically investigated. The best separation was achieved with a Diamonsil C18 analytical column (4.6 mm × 250 mm, particle size 5 μm) and a mixture of methanol and 0.08% aqueous TFA as the mobile phase.

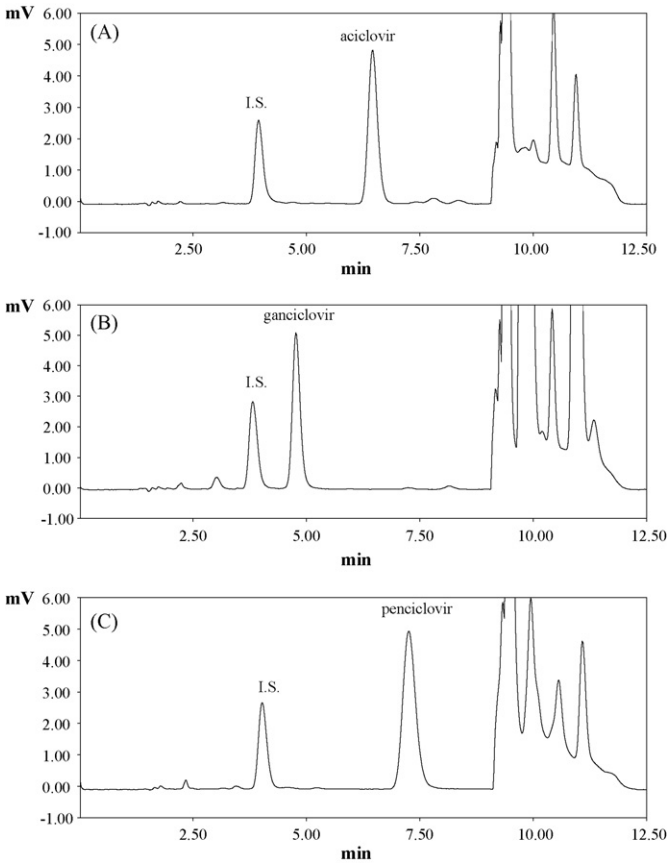

Fig. 3. (A) Chromatogram of plasma from a Chinese adult renal transplant recipient taking aciclovir (400 mg, thrice daily), 2 h after the morning dose (1863.25 ng/ml). (B) Chromatogram of plasma from a Chinese adult renal transplant recipient taking ganciclovir (1000 mg, thrice daily), 2 h after the morning dose (1352.76 ng/ml). (C) Chromatogram of plasma from a Chinese adult renal transplant recipient taking famciclovir (250 mg, thrice daily), 2 h after the morning dose (1133.94 ng/ml).

3.7.3. Pre-processing agent and internal standard

For the analysis of plasma, samples must be purified before chromatography to eliminate interfering compounds. Solid-phase extraction (SPE) [21,24,31,39,45,46], liquid–liquid extraction (LLE) [30], and protein precipitation [23,25,32–34,38,40,41,43,44,47,49–53] have been reported in the preparation of ACV, GCV, or PCV. SPE is expensive, time-consuming, and requires considerable volumes of solvent to elute the drug from the cartridges [30]. Meanwhile, some previous studies [24,33,46] indicated that conventional LLE could not be used since ACV, GCV, and PCV are very polar.

Therefore, we selected protein precipitation methods with direct supernatant injections for preparing samples. We also made a careful choice for the optimization of deproteinized agents. We have tried various agents such as trichloracetic acid (5–20%), acetonitrile, methanol, and perchloric acid (5–60%). Finally, we chose 7% aqueous perchloric acid solution (containing I.S.) for thorough precipitation. Addition of 50 μl of 7% perchloric acid solution to 200 μl of plasma exerted minimal diluting effect on the solution.

There were still late-eluting peaks disturbing the analysis, so we tried to remove these by means of organic solvents [41]. We have tested organic solvents of diverse polarity including acetic ether, butyl acetate, dichlromethane, chloroform, diethyl ether, cyclohexane, and petroleum ether to solve this problem, but were unable to remove the overall late-eluting peaks. Therefore, a simple gradient elution program was selected to assay ACV, GCV, and PCV. The entire HPLC chromatogram run lasted 12.5 min, which permitted the analysis of a large number of samples in a short period.

GMP was selected as the internal standard due to its fluorescence spectrum, retention time, and recovery. 200 μg/ml and 10 μg/ml GMP were found to be stable for at least 1 month in aqueous perchloric acid solution, with a deviation of less than 5%.

4. Conclusion

This work demonstrates the first development of a simple and reliable HPLC method for rapid and simultaneous determination of ACV, GCV, and PCV in human plasma using fluorescence detection. The entire HPLC chromatogram run lasted 12.5 min, thus the process of plasma collection and the duration of one analytical run are

suitable for a great quantity of patient samples. The LLOQ of this method is 20 ng/ml for all analytes using only 200 μl of plasma, hence it is also suitable for pharmacokinetic studies of ACV, GCV, and PCV.

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