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Determination of ganciclovir in different matrices from solid organ transplanted patients treated with a wide range of concomitant drugs

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Abstract

The aim of the present study was to develop a time-efficient chromatographic method for the analysis of therapeutic concentrations of ganciclovir (GCV) in plasma, urine as well as dialysate (from continuous renal replacement therapy) from solid organ transplant recipient treated with either GCV or its prodrug valganciclovir (VGCV) in combination with a wide variety of other concomitant drugs. Sample preparation was performed by reversed phase solid phase extraction and was followed by separation of the analytes on a reversed phase column using isocratic elution with a mobile phase consisting of acetonitrile—a counter ion (50 mM 1-heptanesulfonic acid) in an aqueous sodium dihydrogen phosphate buffer (pH 2.1; 10 mM) (10:90 v/v) and a fluorescence detector. Validation of the method showed linearity within the concentration range of 0.1–40 μg/mL for plasma and 0.1–120 μg/mL for urine and dialysate ($R^2 > 0.99$, $n \geq 5$). Accuracy and precision (evaluated at 0.1, 5 and 40 μg/mL) were both satisfactory. The LLOQ was determined to be 0.1 μg/mL. The method was successfully applied on clinical samples from renal transplant recipients treated with VGCV in combination with a variety of usually used concomitant drugs for solid organ transplant recipients.

Keywords: Counter ion; Fluorescence detection; Ganciclovir; Reversed phase chromatography; Solid organ transplanted patients; Valganciclovir

1. Introduction

Cytomegalovirus (CMV) infections are the most common infections following solid organ transplantation and is the direct cause of a variety of clinical disorders (gastritis, colitis, hepatitis, retinitis and pneumonia) associated with substantial morbidity and mortality in solid organ transplanted patients [1]. The incidence of symptomatic disease related to CMV infection varies between different transplant settings, but is in general between 5 and 50% [2–8]. CMV infections are also important in acquired immunodeficiency syndrome (AIDS) patients due to their immunosuppressed state, even though the present incidence is lower as compared to the pre-HAART (highly active antiretroviral therapy) era, and is still strongly associated with death in AIDS patients [9].

The nucleoside analog ganciclovir (GCV), 9-(1,3-dihydroxy-2-propoxymethyl) guanine (Fig. 1), is the gold standard for treatment of CMV infections [10,11]. The valyl ester prodrug of GCV, valganciclovir (VGCV, Fig. 1) was recently introduces in the market and with its considerably higher oral bioavailability of GCV it seems to be an alternative to intravenous administration of GCV [12–15]. The valyl ester is rapidly, extensively and irreversible converted to GCV drug in the absorption phase and more than 97% of absorbed VGCV appear in the systemic circulation as GCV [12,13]. Therapeutic drug monitoring of ganciclovir during treatment of CMV infections is not generally applied but the course of the infection is rather followed by viremia measurements [16]. However, therapeutic drug monitoring is extensively used in solid organ transplant recipients and could possible also be relevant for GCV, and its new prodrug VGCV. To further elucidate on the relevance of GCV drug monitoring in this populations, especially with regard to efficacy and side-effects further investigations on its pharmacokinetics is needed. This requires a fast and sensitive bio-analytical method

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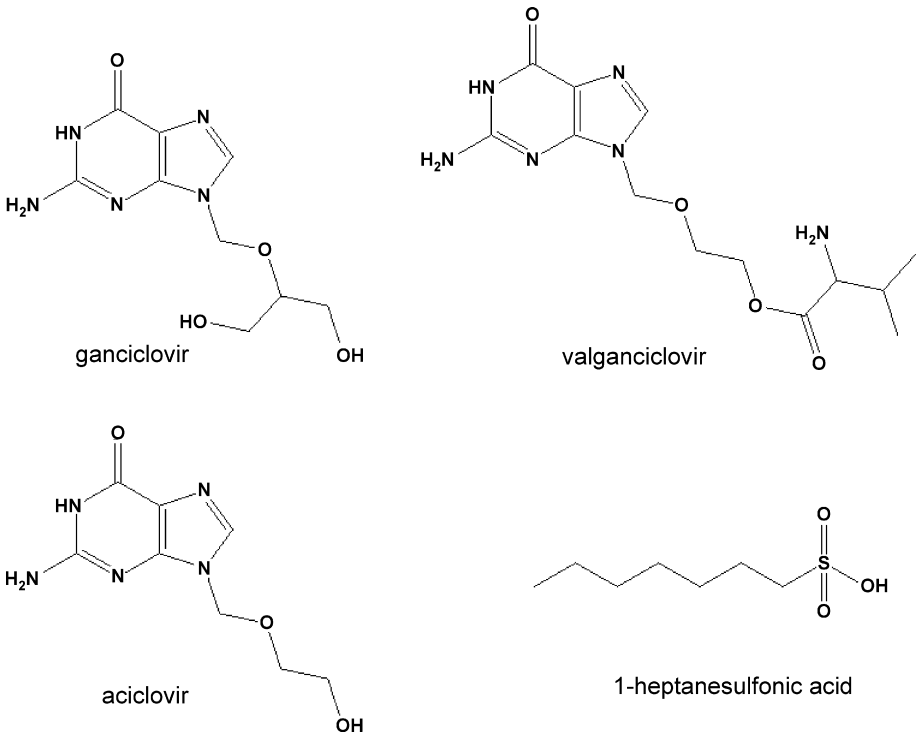

Fig. 1. Structural formulas of ganciclovir, valganciclovir, acyclovir (IS) and 1-heptanesulfonic acid.

that can be applied in several matrices, such as plasma, urine and renal replacement dialysate. In addition, it is also important that the method is selective with regard to co-administered drugs since solid organ transplant recipients are treated with a wide variety of drugs. Previously published methods have not been shown to have all these characteristics [17–24]. In this paper we describe a reversed phase HPLC method fulfilling these criteria by combining 1-heptanesulfonic acid (Fig. 1) as a counter ion and applying fluorescence detection for selective and sensitive determination of GCV in plasma, urine and dialysate.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical-reagent grade. Distilled water was used for all aqueous solutions. Ganciclovir, acyclovir (internal standard (IS), Fig. 1) and 1-heptanesulfonic acid sodium salt were purchased from Sigma (Germany), sodium dihydrogen phosphate from Prolabo (France), acetonitrile and methanol from Merck (Germany) and ortho-phosphoric acid 85% from Fluka (Switzerland).

Aqueous stock solutions of both GCV (1 mg/mL) and IS (250 μg/mL) were prepared and stored at −20 °C.

2.2. Sample preparation

A previously described solid phase extraction [19] was slightly modified for our purpose. In brief the following procedure was applied: sample (200 μL plasma, urine or dialysate) spiked with 10 μL IS was applied to a 30 mg (1 mL) Oasis HLB cartridge (Water Oasis, Ireland) which had been preconditioned with 1 mL methanol and 1 mL water. After sample application the cartridge was washed with 250 μL water (for plasma samples) or 1 mL of water (for urine and dialysate). GCV and IS were in the next step eluted with 750 μL of 10% methanol. Finally the eluate was evaporated to dryness in a SpeedVac concentrator (ThermoSavant, US) making sure that the temperature was kept below 50 °C. The residue was dissolved in 200 μL of 5 mM 1-heptanesulfonic acid and 10 mM sodium dihydrophosphate buffer (pH 2.1), vortexed for 10 s, centrifuged at 10,000 × *g* for 10 min at room temperature and the clear supernatant was transferred to sample vials of glass (Agilent Technologies, Germany). An aliquot of 30 μL was injected in the high-performance liquid-chromatography (HPLC) system.

2.3. Instrumentation and chromatographic conditions

An Agilent series 1100 HPLC equipped with a quaternary pump, temperature controlled auto injector and column compartment and fluorescence detector fitted with an 8 μL flow cell were used (Agilent Technologies, Germany). Since retention times are kept short, there are, especially in the case of urine, high signals near the injection front, which interfere with the GCV determination using UV detection. Fluorescence detection was chosen instead to make the detection of ganciclovir more specific. The detector was operated at an excitation wavelength of 265 nm and an emission wavelength of 378 nm. The separation was performed on an Agilent Zorbax® SB-Aq reversed-phase column (150 mm × 4.6 mm, 5 μm) with a corresponding guard column, by isocratic elution with a mixture of acetonitrile–50 mM 1-heptanesulfonic acid in 10 mM sodium dihydrophosphate buffer

(pH 2.1) (10:90 v/v) at a flow-rate of 1.0 mL/min. Each run took no longer than 6 min. The auto sampler tray was kept at 10 °C while the column temperature was 25 °C.

HPLC ChemStation® LC 3D (Agilent Technologies, Germany) software was used to control the machine and to analyze the chromatographic data.

2.4. Method validation

The validation of the method was performed based on FDA guidelines for Bio-analytical Method Validation [25]. Blood was drawn into EDTA vacutainer tubes on ice, centrifuged at 800 × *g* (4 °C) for 20 min from which plasma was decanted and stored at −20 °C until analysis was used for the full validation of the method. Both drug free plasma from healthy volunteers at the laboratory as well as plasma with and without ganciclovir from renal transplant recipients on different commonly occurring drug combinations for solid organ transplant recipients was used. Plasmas were not pooled but analyzed individually. Urine from renal transplant recipients on GCV treatment and one healthy, drug free, volunteer and GCV free dialysate from one patient treated with continuous renal replacement therapy was used for cross validation of the method for these matrices. The Regional Committee for Medical Research Ethics, health region south approved the collection of these samples and patients donating blood gave their informed consent.

The concentration of the compounds in the different matrices was determined from their peak heights, using acyclovir as IS. Peak height is chosen above peak area since small peaks, eluting near the internal standard will have larger influence on the result when peak area is used. Linearity was tested both for the plasma standard curve (ranging 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 20 and 40 μg/mL) as well as for the urine and dialysate standard curves (ranging 0.05, 0.1, 1, 5, 10, 20, 40 and 120 μg/mL). Plasma from three drug-free individuals were spiked with 0.1, 0.3, 6, 24 and 40 μg/mL to determine the lower limit of quantification (LLOQ), accuracy and precision of within as well as between-day runs. Absolute recovery was determined at 0.3, 6 and 24 μg/mL by comparing the response of extracted samples spiked before extraction with the response of extracted blank samples spiked after extraction. Selectivity was determined in plasma from nine renal transplant recipients on commonly occurring drug combinations for solid organ transplant recipients. One plasma sample spiked to a concentration of 60 μg/mL was diluted with different plasma to investigate the applicability of diluting samples outside of the standard curve. The GCV concentration in plasma from six renal transplanted patients treated with GCV/VGCV were also determined. Long-term −20 °C storage and freeze-thaw stability was not tested as this has been performed by others previously [23]. Stability of GCV at 4 °C was tested in periods from one night in the auto sampler to 7 weeks in the refrigerator.

3. Results and discussion

3.1. Sample preparation and chromatography

A previously described SPE extraction procedure [19] for the preparation of human plasma was modified in the present study and showed also to be applicable for urine and dialysate matrices. Samples were applied directly on the reversed phase SPE cartridge and washed with water to elute highly hydrophilic compounds. Washing with acidified water did not affect the extraction procedure. GCV and IS was eluted with 10% methanol and 750 μL was needed to achieve an appropriate recovery (Table 1). The eluate was evaporated to dryness in a vacuum concentrator at <50 °C. Samples were dissolved in 5 mM 1-heptanesulfonic acid in 10 mM sodium dihydrophosphate buffer (pH 2.1), allowing complex formation before application on the HPLC system.

Table 1
Absolute recovery for GCV at three concentrations levels ($n = 4$ at each level) as well as for IS (acyclovir, $n = 12$)

	Recovery (%)	S.D. (%)
GCV, 0.3 μg/mL	58	4.5
GCV, 6 μg/mL	68	2.8
GCV, 24 μg/mL	56	0.6
IS (ACV)	60	5.0

Representative chromatograms of the analysis of both plasma and urine samples from two patients is shown in Fig. 2. Therapeutic GCV plasma concentrations in solid organ transplant recipients receiving oral VGCV therapy typically range from 0.2 to 20 μg/mL [13]. At these levels the peaks of GCV and IS were baseline separated. At higher levels there was no baseline separation, however, this did not interfere with the peak height estimations and linearity was obtained in the full standard curve range. The retention times of GCV and IS were 3.4 and 3.8 min, respectively. A total run time of 6 min was appropriate as long as a washing run was included every 12th injection to minimize carry-over. During the washing run the ACN concentration was gradually increased to 50% over a period of 4 min, kept at 50% for 12 min and reversed back to 10% over a period of 4 min (total wash time was 20 min).

3.2. Validation of the method

Plasma samples from nine renal transplant recipients not treated with GCV, VGCV, acyclovir or valaciclovir showed no interfering peaks in the present method. A summary of drugs these patients were treated with are given in Table 2. This makes the method suitable for application in these patients as long as

Table 2
Selectivity of the method

Allopurinol	Felodipine	Nifedipine
Amilorid	Fluvastatin	Pantoprazol
Amlodipine	Frusemid	Prednisolone
Aspirine	Hydrochlorthiazide	Ranitidin
Atorvastatin	Irbesartan	Sirolimus
Calcitriol	Isosorbide	Spironolactone
Cyclosporine A	Lisinopril	Sulphamethoxazol
Darbepoetin alpha	Metoprolol	Tacrolimus
Esomeprazol	Mycophenolate mofetil	Trimethoprim
Everolimus	Nateglinide	

No interference was shown during analysis of plasma samples from patients treated with the following drugs.

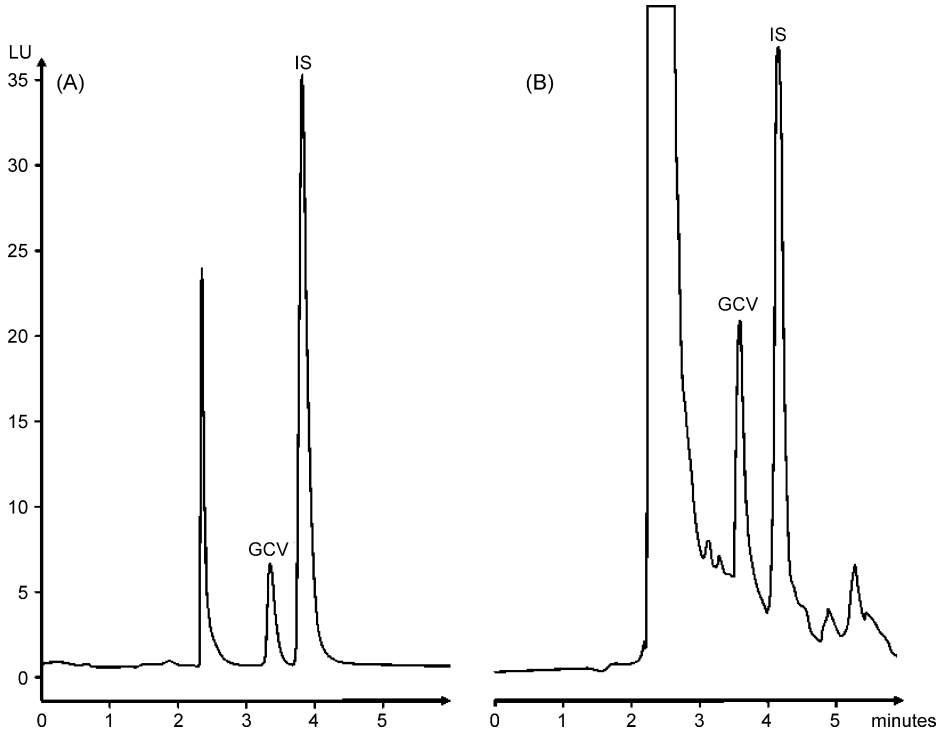

Fig. 2. Representative chromatogram of ganciclovir (GCV) with acyclovir as internal standard (IS) from a renal transplant recipient treated with valganciclovir. Panel A for plasma and panel B for urine.

Table 3
Standard curve (mean ± S.E.M. peak height ratios) characteristics according to the general formula $y = ax + b$

	Low concentration range[a] (μg/mL)			High concentration range[b] (μg/mL)			
	a	*b*	R^2	*a*	*b*	R^2	*n*
Plasma	0.1013 ± 0.0019	0.0002 ± 0.0012	0.9996	0.0936 ± 0.0048	−0.0279 ± 0.024	0.9989	5
Urine	0.0454 ± 0.0027	−0.0061 ± 0.0014	0.991	0.0480 ± 0.0017	−0.0287 ± 0.029	0.9997	4
Dialysate	0.0713 ± 0.0030	−0.0233 ± 0.0011	0.9997	0.0455 ± 0.0025	−0.2893 ± 0.050	0.994	4

[a] Plasma: 0.1–2.5; urine/dialysate: 0.1–5.
[b] Plasma: 2.5–40; urine/dialysate: 5–120.

they are not treated with acyclovir or its derivatives. The method showed appropriate linearity within the selected concentrations ranges of 0.05–40 μg/mL for plasma and 0.1–120 μg/mL for urine and dialysate as long as the standard curves were split in two. Characteristics for the plasma, urine and dialysate standard curves are shown in Table 3. The method also allowed dilution of samples showing concentrations above the given standard curve range. The average deviation from the nominal value of 1:2 and 1:4 dilutions was closer to zero than −7%, with a linear regression $R^2 > 0.99$. The coefficient of variation (CV) for the standard curves for all three matrices were below 17.5% at LLOQ (0.1 μg/mL) and below 12.0% at the other concentrations, which is within acceptable limits (Table 4) [25].

The accuracy was determined in multiple parallels of plasma and deviated less than ±10% within run and less than ±7.3% between-run from the true values within the range of the standard curve (Tables 4 and 5). Corresponding within run data for urine and dialysate is also shown in Table 4.

The within-run precision measured both in five parallels of spiked plasma (0.1, 5 and 40 μg/mL) and in four parallels of

Table 4
Accuracy and precision for plasma, urine and dialysate samples spiked with known amounts of GCV

Nominal concentration (μg/mL)	Measured concentration (μg/mL)	Bias (%)	CV (%)
Plasma			
0.1 (*n* = 5)	0.12	17.5	10.3
0.3 (*n* = 8)	0.33	8.8	5.6
2.5 (*n* = 8)	2.40	−4.0	7.3
6 (*n* = 8)	5.5	−9.1	8.0
24 (*n* = 8)	21.5	−10.4	8.2
40 (*n* = 5)	39.6	−1.0	12.0
Urine			
0.1 (*n* = 4)	0.12	23.1	17.5
5 (*n* = 4)	5.06	1.3	10.3
40 (*n* = 4)	40.8	2.0	6.8
Dialysate			
0.1 (*n* = 4)	0.11	7.8	14.7
5 (*n* = 4)	4.99	−0.3	8.2
40 (*n* = 4)	46.3	15.6	3.5

CV, coefficient of variation.

Table 5
Between-run variance (R.S.D., $n = 3$), between-run accuracy and average difference in concentration from immediate analysis and following storage one night in autosampler to 7 weeks at 4 °C

Concentration	R.S.D. (%)	Accuracy (%)	Relative concentration difference storage (%)
0.1	20.4	−7.3	5.5
5	10.2	3.5	0.2
40	9.8	2.7	−0.1

spiked urine and dialysate (0.1, 5 and 40 μg/mL) ranged from 3% to 17%. The between-run precision in spiked plasma (0.1, 5 and 40 μg/mL) ranged from 9.8% to 14.6%.

The recovery for all analytes ranged from 56% to 68% (Table 1).

Based on repeated measurements was LLOD determined to be 0.05 μg/mL and LLOQ to be 0.1 μg/mL for GCV (Table 4).

GCV has previously been shown to be stable during storage at −20 °C for at least 20 months [23]. In addition showed samples good stability at 4 °C and could be reanalyzed following a night in the auto injector. The analytes were actually stable enough so that reanalyzes was feasible following storage (Table 5) for up to 7 weeks in a refrigerator (4 °C).

3.3. Application of the method

The method was developed to determine GCV in human plasma, urine and in dialysate from continuous renal replacement therapy in pharmacokinetic investigations. Special emphasis on selectivity towards commonly co-administrated drugs in solid organ transplant recipients resulted in an applicable method for pharmacokinetic investigations in this patient population. The LLOQ is appropriate for measurement of trough concentrations in a clinical setting for these patients and the upper concentration of the standard curve should cover extraordinary high levels that may be found during acute renal failure. The plasma concentration of GCV was 0.2, 1.0 and 2.7 μg/mL in three solid organ transplant recipients treated with oral VGCV at standard doses once daily (QD) for CMV viremia. In Fig. 3 is the mean ± S.D. of three solid organ transplant recipients treated with oral VGCV twice daily (BID) for CMV disease shown, both on the third as well as the 21st day of treatment. Urine concentration of GCV in three other transplanted patients was 7.5, 47.4 and 115.9 μg/mL following oral QD VGCV therapy. In case of extraordinary high concentrations of GCV it has been shown that dilution of the samples before reanalysis is feasible.

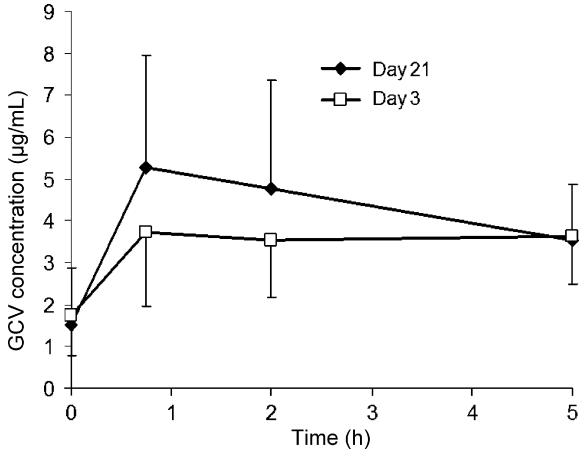

Fig. 3. Mean ± S.D. ganciclovir (GCV) concentrations in three solid organ transplant recipients during valganciclovir (VGCV) treatment BID for CMV disease. Open squares are at the third day and closed diamonds are at the 21st day of treatment.

4. Conclusion

In the present work we presented a simple, sensitive and time-efficient method for analysis of GCV in human plasma, urine and dialysate from continuous renal replacement therapy. Sample preparation was performed by SPE, followed by HPLC separation and detection using a fluorescence detector without prior conjugation reactions. The LLOQ was 0.1 μg/mL and the method was linear up to concentrations well above therapeutic concentrations. The method was successfully applied on samples from renal transplant recipients treated with a wide variety of concomitant drugs, which would make it feasible for use in pharmacokinetic investigations as well as in therapeutic drug monitoring in this patient population.

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