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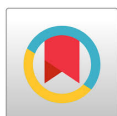
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RESEARCH ARTICLE

Determination of Ganciclovir and Acyclovir in Human Serum using Liquid Chromatography-Tandem Mass Spectrometry



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OBJECTIVES: Currently there is no data about a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay including ganciclovir and acyclovir using stable-isotopically labeled internal standards.

METHODS: A LC-MS/MS assay for measurement of ganciclovir and acyclovir using deuterated standards: ganciclovir-[²H₅] and acyclovir-[²H₄] was developed. The selectivity and sensitivity, linearity, accuracy and precision, recovery, matrix effect, stability, total process efficiency, carry-over and dilution integrity were validated based on EMA and FDA guidelines.

RESULTS: The retention time for ganciclovir was 1.1 min and for acyclovir 1.35 min. Calibration curves were linear over a range of 0.1 to 20 mg/L and the correlation coefficient (R²) was 0.99912 for ganciclovir and 0.99945 for acyclovir. The calculated accuracy was -2.0% to 3.1% for ganciclovir and -1.0% to 6.4% for acyclovir. Within-day precision ranged from 1.8% to 6.6% for ganciclovir and 1.6 % to 6.5% for acyclovir and between-day precision 0% to 9.6% for ganciclovir and 0% to 7.9% for acyclovir.

CONCLUSIONS: A rapid and validated LC-MS/MS method was developed for measurement of ganciclovir and acyclovir in human serum which can be used in routine patient care and clinical research.

KEYWORDS: LC-MS/MS, ganciclovir, acyclovir, cytomegalovirus, therapeutic drug monitoring.

INTRODUCTION

Herpes viruses are a huge burden on the health care system. Herpes simplex virus (HSV) is prevalent in over 60% and cytomegalovirus (CMV) in 40-80% of the population [1,2]. All herpes viruses are not cleared after infection. Most infected individuals suppress replication by their cell-mediated immunity [3]. Transplant re-

ipients, whose immunity is compromised, are more vulnerable to CMV disease (retinitis, encephalitis, hepatitis etc.) which may lead to rejection of the transplanted organ and increase mortality [4,5].

Currently nucleoside analogues ganciclovir (intravenous) and the prodrug valganciclovir (oral), which is in vivo is metabolized to ganciclovir, are used for standard treatment and prophylaxis of CMV [6,7]. Ganciclovir 5 mg/kg or valganciclovir 900 mg twice daily are the dosing regimens suggested by international consensus [6]. Another nucleoside analogue, acyclovir, is used for HSV type 1, 2 and varicella zoster virus (VZV) [8]. Acyclovir is recommended for immunocompetent patients diagnosed with HSV in a dose of 800 mg orally 5 times daily [9]. Unfortunately, both of these medications cause severe side effects and dosages must be adjusted during kidney failure [8,10–12].

The most dangerous side effects that occur during ganciclovir treatment are blood disorders (neutropenia, leucopenia) and graft failure [13]. With extremely high viral loads (>100,000 copies/mL), there is a risk of acquiring resistance to ganciclovir [14]. Adequate exposure of ganciclovir is necessary to provide sufficient treatment response and avoid acquired resistance. Moreover, it is known that acyclovir may cause dose related acute kidney injury [8,10]. These are all factors that suggest measuring serum concentrations of ganciclovir and acyclovir i.e., applying therapeutic drug monitoring (TDM) may be beneficial to guide and individualize therapy. The appropriate dose is estimated based on trough and peak concentrations and area under the curve values (AUC).

The target trough concentrations for ganciclovir in our hospital are 1–2 mg/L and 2–4 mg/L for prophylaxis and therapy, respectively. The target peak concentrations are in the range 5–12.5 mg/L. These concentrations base on inhibitory concentration of CMV and pharmacokinetic parameters from available studies [11,12,15–17]. The target trough concentration range for acyclovir in our hospital is 0.5–1.5 mg/L and target peak concentration range is 5–15 mg/L [18].

Different analytical procedures have been developed to measure ganciclovir and acyclovir drug concentrations. For example high performance liquid chromatography with fluorescence and UV detection [19–22] and liquid chromatography with (tandem) mass spectrometry detection [23–28] have been used to determine ganciclovir in plasma, serum, urine, cell culture media and also intracellularly.

LC-MS/MS has been used before for the assay of ganciclovir and acyclovir, however to our knowledge not analysed with the same assay [25–28]. Several authors used structural analogs as internal standards such as acyclovir for the assay of ganciclovir [26,27]. The drawback of a structural analog is that it does not have as similar properties to the measured and as a stable isotope-labeled standard. Moreover, acyclovir and ganciclovir are both included in antiviral protocol in transplant recipients. Therefore using an active drug for IS can interfere the determination of the drug under analysis [29], especially when both drugs like acyclovir and ganciclovir can be used in the same patient population.

One study used deuterated acyclovir, for the determination of acyclovir together with valacyclovir [25] and in another study fluconazole was used as IS [28]. Stable isotope-labeled IS have the advantage of possessing a similar structure to the original chemical compound with comparable extraction recoveries and chromatographic retention time. Therefore, using these aids to avoids any discrepancies and make the method more stable.

LC-MS/MS was earlier used to analyse valacyclovir together with acyclovir [25,28]. Shi et al. (acyclovir, valacyclovir) reported run time of 9 minutes, Yadav et al. (acyclovir, valacyclovir) 12 minutes and Xu et al. (ganciclovir) 5.5 minutes [25,27,28]. Yadav et al and Billat et al used solid phase extraction to determine acyclovir and ganciclovir, this method

is known to be more time-consuming and costly than direct protein precipitation [26,28]. In another assay, ganciclovir was measured with acyclovir as internal standard (IS) [24].

To our knowledge, no assay, is published where both acyclovir and ganciclovir are measured using LC-MS/MS and stable isotopically labeled IS for rapid throughput, has been published. Moreover, development of individual methods for each drug is more time consuming. Thus, using a single method with a high throughput for multiple drugs is more efficient. Furthermore, we aimed to develop this assay to be used in both for patient care and research purposes, provided that the validated assay meets the requirements of the TDM and study protocol. This study describes a rapid LC-MS/MS assay with deuterated internal standards for both ganciclovir and acyclovir.

MATERIALS AND METHODS

Chemicals and reagents

Ganciclovir, ganciclovir- $^{[2]H_5}$, acyclovir and acyclovir- $^{[2]H_4}$ were purchased from Alschim (Illkirch, France) (in **Figure 1**, chemical structures are presented). Purified water was obtained from a Milli-Q water purifying system (Millipore Corporation, Billerica, MA, USA). Acetic acid and ammonium acetate were received from Merck, NJ, USA and trifluoroacetic acid and acetonitrile were received from Biosolve, the Netherlands. Pooled human serum samples were obtained from local hospital according to the standard operating procedures of the medical center.

Preparation of stock solutions, calibration standard and QC samples

Stock solutions of 500 mg/L for ganciclovir and acyclovir were prepared by dissolving the compounds in methanol. Independent stock solutions were used to prepare calibration and internal quality control (QC) samples. For calibration samples blank human serum was used which was spiked to get 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 mg/L. QC sam-

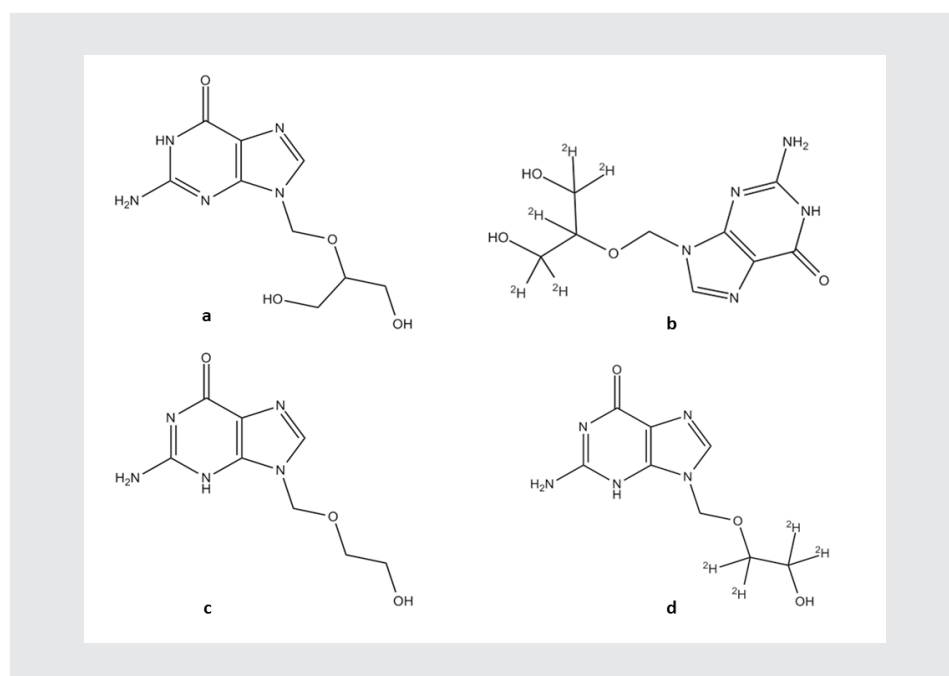


Figure 1. Chemical structures. (a) ganciclovir; (b) ganciclovir- $^{[2]H_5}$; (c) acyclovir; (d) acyclovir- $^{[2]H_4}$

Table 1. Gradient elution

Time (min)	% elution		
	A	B	C
0.00	5	95	0
0.20	5	95	0
1.50	5	85	10
1.51	5	0	95
3.00	5	0	95
3.01	5	95	0
4.5	5	95	0

A = aqueous buffer (acetic acid 35 mL/L, ammonium acetate 5 g/L and trifluoroacetic acid 2 mL/L), B = water, C = acetonitrile

ples were prepared using blank human serum at lower limit of quantification (LLOQ) (0.1 mg/L), low (0.3 mg/L), medium (10 mg/L), high (16 mg/L) and over-curve (OC, 40 mg/L). Samples were stored at -20°C until use. The IS solution was prepared by dilution of 100 mg/L stock solution ganciclovir-[²H₅] and acyclovir-[²H₄] until concentration 0.05 mg/L was obtained for both IS. 10 µL of serum was added to 500 µL of precipitation agents including the IS. As the sample was diluted 50 times with the precipitation reagent the final IS concentration in the diluted sample was 2.5 mg/L. The final concentration, 2.5 mg/L, which is in the middle of the calibration curve. The IS was prepared in 10% trichloroacetic acid and that was used as matrix for the QC, calibration, analysis of patient samples and as precipitation reagent.

Sample preparation

To 10 µL serum of each sample, a volume of 500 µL of precipitation reagent combined with the deuterated IS were added in a vial. The samples were vortexed for 1 min. The vials were centrifuged for 5 min at 11,000 rpm. Five microliters of the upper layer was injected into the LC-MS/MS.

LC MS/MS system

The analysis was carried out on a triple quadrupole Thermo Fisher Scientific TSQ® Quantum Access MAX LC-MS/MS system with a Surveyor® MS pump and a Surveyor plus® autosampler. A Thermo Fisher Scientific 50 mm × 2.1 mm C18, 5-µm HyPURITY Aquastar analytical column was used for the chromatographic separation, with a column temperature of 20°C. The autosampler temperature was set to 10°C. The mobile phase (pH 3.5) contained an aqueous buffer (A) (acetic acid 35 mL/L, ammonium acetate 5 g/L, and trifluoroacetic acid 2 mL/L), water (B) and acetonitrile (C). The flow rate was 0.3 mL/min. The gradient is shown in **Table 1** and run time was 4.5 min.

The mass spectrometer operated in Selected Reaction Monitoring (SRM) mode and positive mode was selected for electrospray ionization. The spray voltage was 3500 V, capillary temperature 350°C and sheath gas pressure and auxiliary gas pressure were 35 and 10 arbitrary units respectively. During the method all the voltages and column were on standard settings. Mass ion transitions for ganciclovir were 256.0 m/z → 152.1 m/z and for ganciclovir-[²H₅] 261.1 m/z → 152.1 m/z and for acyclovir 226.0 m/z → 152.1 m/z and for acyclovir-[²H₄] 230.1 m/z → 152.1 m/z while using a scan width of 0.5 m/z. Collision energies (CE) were 13 V (volts) for acyclovir, 14 V for acyclovir-[²H₄], 13 V for ganciclovir and 16 V for ganciclovir-[²H₅]. Product ion scans are shown in **Figure 2**.

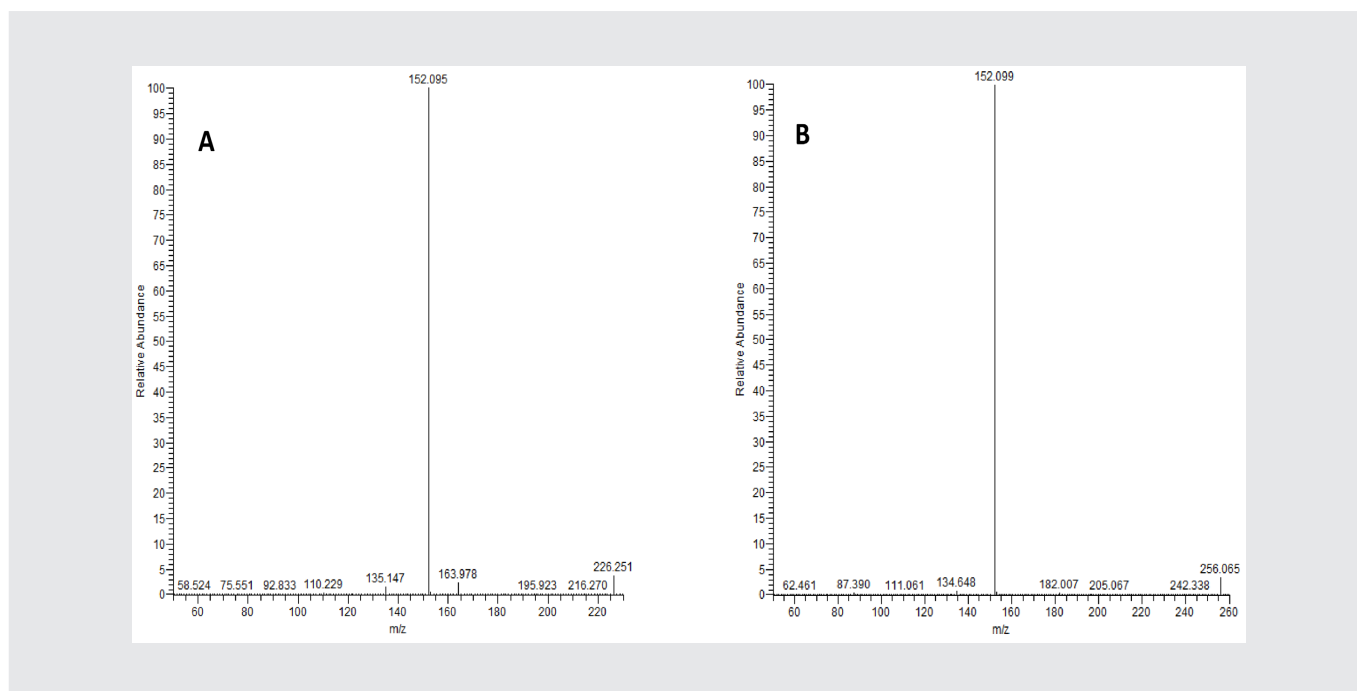


Figure 2. Product ion scans, (A) acyclovir 226.0 → 152.1 m/z (CE 13V) and (B) ganciclovir 256.0 → 152.1 m/z (CE 13V).

Method validation

The analytical method was validated based on European Medicines Agency (EMA) and US Food and Drug Administration (FDA) guidelines and the parameters that were determined for validation were selectivity and sensitivity, linearity, accuracy and precision, recovery, matrix effect, stability, total process efficiency, carry-over and dilution integrity [30,31].

Linearity was tested with different serum concentrations (0.1, 0.5, 1, 2.5, 5, 10, 15, 20 mg/L) for ganciclovir and acyclovir (for regression equations see **Table 2**). The validity of the regression analysis was determined with the calculation of residuals. The plotted residuals showed a random pattern hence the regression was valid.

For selectivity, 6 unpooled human blank serum samples were examined for interference. The responses of blank serum samples were compared to interference of the LLOQ samples. Every day for three consecutive days a single calibration curve was analysed and for accuracy QC samples were analysed in five-fold. Within-run and between-run precision were established using the same method as for accuracy. The accepted deviation for precision was a CV <15% and for accuracy a bias of maximally 15% of the nominal value. The accuracy and precision concentration bias and CV were calculated per run and were expected to be greater than 80% of the nominal value. After a high concentration (20 mg/L) the carry-over was less than 20% of the LLOQ.

The method of Matuszewski et al. was used to analyse matrix effects, recovery and total process efficiency at three concentrations (LOW, MED, HIGH) [32]. For the analysis of matrix effects the relative recovery was measured. This was done by comparing the ratios

of peak height of ganciclovir and acyclovir and the corresponding IS of the QC samples processed with the average peak height of the LOW, MED and HIGH recovery samples. These were post-extraction blank serum samples that were spiked at the concentrations of the QC samples. For dilution integrity OC was diluted ten times with blank serum in five-fold. This was done for three consecutive days with every analyte concentrations above the HIGH.

For storage stability the LOW and HIGH QC samples of ganciclovir and acyclovir were used. The samples were compared to fresh calibration curve for analysis. These samples were tested for stability at room temperature (144 hours 20-25°C), at refrigerator temperature (144 hours 4°C), in the autosampler (after processing of the sample, 120 hours, 10°C) and in four freeze thaw cycles (-20°C). A limit of 15% of loss of the initial drug concentration was accepted.

Clinical application of the method

This method has been used for TDM in routine care of patients receiving acyclovir and ganciclovir. Blood was collected from patients in a Clot Activator tube. For the analysis whole blood was centrifuged at 3,000 g for 5 min and minimum amount of 0.5 mL serum was collected and stored at -20°C. To 10 µL of serum a volume of 500 µL precipitation reagents combined with the deuterated IS were added into a vial. Samples were vortexed for 1 minute and centrifuged for 5 minutes at 11,000 x rpm. Five µL of the upper layer was injected into the LC-MS/MS.

RESULTS

The retention time for ganciclovir was 1.1 min and for acyclovir 1.35 min (**Figure 3**). The chromatograms presented in **Figure 3** were analysed and no interfering peaks at the retention time of ganciclovir, ganciclovir-[²H₅], acyclovir, acyclovir-[²H₄] were observed using blank samples.

The calibration curves were linear over a range of 0.1 to 20 mg/L and the correlation coefficient (R²) was 0.99912 for ganciclovir and 0.99945 for acyclovir. The calibration lines are presented in **Table 2**.

The calculated accuracy was -2.0% to 3.1% for ganciclovir and -1.0% to 6.4% for acyclovir. Within-day precision ranged from 1.8% to 6.6% for ganciclovir and 1.6 % to 6.5% for acyclovir and between-day precision 0% to 9.6% for ganciclovir and 0% to 7.9% for acyclovir (**Table 3**). The results of matrix effects, recovery and total process efficiency are presented in **Table 3**.

Results of stability testing of ganciclovir and acyclovir QC samples LOW and HIGH are shown in **Table 4**. Bench top stability (144 hours) biased between 4.8% and 6.7% for

Table 2. Regression equations

Compound	Y-intercept (± St.dev)	Slope (± St.dev)	Corr. coefficient
Ganciclovir	0.00290±0.00668	0.377±0.00338	0.99912
Acyclovir	0.00335±0.00579	0.413±0.00292	0.99945

St.dev = standard deviation; corr. = correlation.

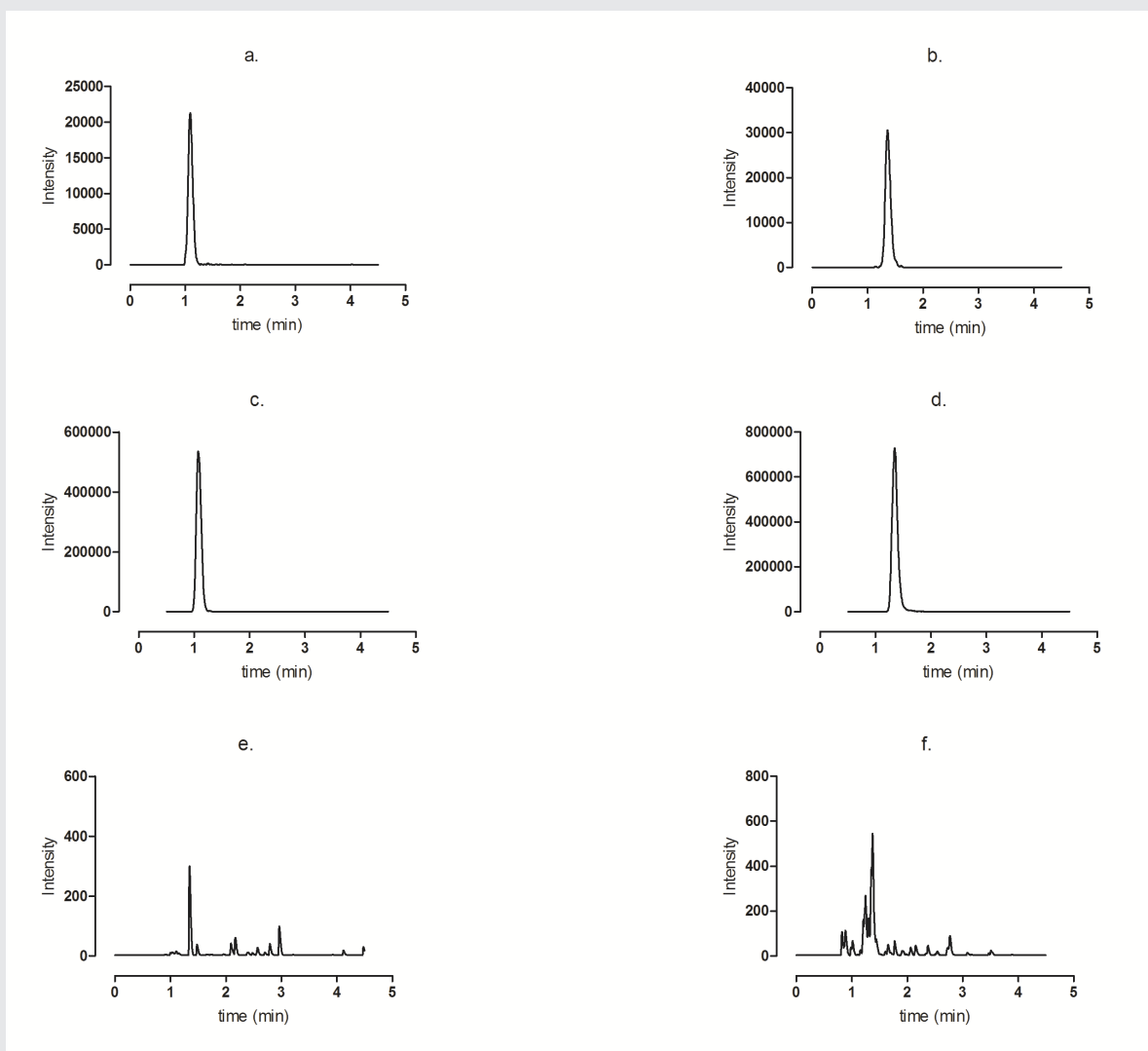


Figure 3. Chromatograms of two analytes at LLOQ of 0.10 mg/L, two internal standards with a concentration of 0.05 mg/L along with blank: (a) ganciclovir; (b) acyclovir; (c) ganciclovir- $^{12}\text{H}_5$; (d) acyclovir- $^{12}\text{H}_4$; (e) ganciclovir blank; (f) acyclovir blank.

Table 3. Validation results

Criteria	QC Concentration Level				
	Nominal Concentration (mg/L)				
	LLOQ	LOW	MED	HIGH	OC
Ganciclovir	0.1	0.3	10	16	40
Acyclovir	0.1	0.3	10	16	40
Accuracy (Bias (%))					
Ganciclovir	0.1	2.8	-0.9	3.1	-2.0
Acyclovir	5.2	5.3	0.9	6.4	-1.0
Within-day precision (CV (%))					
Ganciclovir	6.6	4.9	2.7	2.4	1.8
Acyclovir	6.5	5.0	2.7	1.9	1.6
Between-day precision (CV (%))					
Ganciclovir	5.4	4.0	0.0	3.9	9.6
Acyclovir	3.4	0.0	1.1	2.9	7.9
Matrix effect (bias (%))					
Ganciclovir	n/a	8.8	5.5	1.6	n/a
Acyclovir	n/a	5.2	5.1	1.1	n/a
Recovery (bias (%))					
Ganciclovir	n/a	99.8	95.0	97.0	n/a
Acyclovir	n/a	95.0	95.7	97.1	n/a
Total process efficiency (bias (%))					
Ganciclovir	n/a	108.6	100.2	98.6	n/a
Acyclovir	n/a	100.0	100.5	98.2	n/a

Table 4. Stability results

Criteria	QC Concentration Level	
	LOW	HIGH
	Auto-sampler stability (after 120h) (bias %)	
Ganciclovir	7.0	10.3
Acyclovir	7.0	11.0
	Bench top stability (after 144h) (bias%)	
Ganciclovir	6.7	4.8
Acyclovir	6.2	5.6
	Refrigerator (4°C) stability (after 144h) (bias %)	
Ganciclovir	12.2	9.1
Acyclovir	7.3	7.4
	Freeze-thaw stability (after four freeze thaw cycles) (bias%)	
Ganciclovir	0.3	8.0
Acyclovir	-0.7	10.1

ganciclovir and 5.6% and 6.2% for acyclovir; in a refrigerator 4°C (144 hours) biased between 9.1% and 12.1% for ganciclovir and 7.3% and 7.4% for acyclovir; and in the auto-sampler (120 hours) biased between 7.0% and 10.3% for ganciclovir and 7.0% and 11% for acyclovir. Samples have proven to be stable after 1 year storage in -20°C.

Clinical application results

In the period from April 2016 to September 2018, 65 acyclovir samples with the median of 2 mg/L (IQR 0.9-5.4 mg/L) and 361 ganciclovir samples with the median of 2.2 mg/L (IQR 1-3.9 mg/L) were measured. TDM for acyclovir was used to guide therapy in patients with kidney failure, receiving renal replacement therapy (RRT), virologic failure or to monitor oral absorption of the drug. The population for whom ganciclovir and valganciclovir TDM was used mainly consisted of transplant recipients and patients with human immunodeficiency virus. TDM was indicated in kidney failure, during RRT, for complicated CMV cases and also to analyse oral absorption of valganciclovir. This assay participates in a proficiency program and is being routinely controlled [33]. The testing program in our laboratory is done by the same organization as for antifungal and antiviral medications [34,35].

DISCUSSION

We describe a LC-MS/MS method for the determination of ganciclovir and acyclovir using deuterated ganciclovir- $[\text{?H}_5]$ and acyclovir- $[\text{?H}_4]$ as IS. We present the first LC-MS/MS method where these compounds are measured with the same method using deuterated IS.

Sample clean-up is a simple protein precipitation step. Using protein precipitation makes the method more efficient compared to solid phase extraction which has been used by Yadav et al. [28]. Furthermore, the rapid retention times of 1.1 min and 1.35 min for ganciclovir and acyclovir respectively and a method run time of 4.5 min improve time efficiency.

cy. The shorter run time and sample clean-up method, compared with previous studies, makes this applicable for routine patient care by optimizing and promoting high sample throughput [25,27,28]. Although UPLC can potentially shorten the runtime further, this method requires a higher sample load to benefit from the additional costs made for the method.

The calibration curve was linear within a range of 0.1 to 20 mg/L which the acyclovir and ganciclovir predicted trough and peak concentrations in serum [16–18]. The upper limit is higher compared to Yadav et al. mainly because we also measure peak concentrations which can be higher than 10 mg/L [16,18,36]. These are determined in addition to trough concentrations and applied in pharmacokinetic modelling to determine the correct dose for the specific patient. During the method all the voltages and column remained at standard settings which makes it simple to change between different assays in the laboratory.

There were no interfering peaks from endogenous substances using six pooled serum samples. As mentioned before, using the deuterated IS is a benefit as ganciclovir and acyclovir can be used in the same patient group. Thus in an assay where acyclovir is used as an IS while determining ganciclovir concentration the results might not be valid.

Our method is part of a proficiency testing program [33]. This ensures the safety and quality of this LC-MS/MS assay as it is an important part of QC. An interlaboratory proficiency testing program is important for both clinical care and research. Moreover, an adequate comparison between laboratories is necessary in both clinical care and research to have acceptable results.

Prevention of side effects and achieving better treatment outcomes are among others reasons for performing TDM for acyclovir and ganciclovir. There is currently a gap of knowledge whether routine TDM is indicated for either acyclovir or ganciclovir and the discussion is ongoing [16,35]. Prospective studies are needed to specify the patient population for whom TDM seems most appropriate.

The short run time and small serum volume (10 µL) makes our LC-MS/MS assay a straightforward method, which is easy to apply in routine TDM and prospective pharmacokinetic studies.

CONCLUSION

A rapid and validated LC-MS/MS method was developed for the measurement of ganciclovir and acyclovir in human serum. Ganciclovir-[$^2\text{H}_5$] and acyclovir-[$^2\text{H}_4$] were used as internal standards. The method is suitable for use in routine patient care and in clinical research.

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