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RESEARCH ARTICLE

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Rapid determination of acyclovir, its main metabolite 9-carboxymethoxymethylguanine, ganciclovir, and penciclovir in human serum using LC–MS/MS

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Abstract

A novel MS-based analytical method for simultaneous analysis of the antiviral drugs acyclovir, its metabolite 9-carboxymethoxymethylguanine, ganciclovir, and penciclovir in human serum is described. These antiviral drugs are active against herpes virus infections. Acyclovir and penciclovir are regarded as safe and effective medicines with mild side effects such as headache and gastrointestinal discomfort, and ganciclovir is regarded as more toxic and is known to cause, for example, bone marrow suppression. Acyclovir's main metabolite 9-carboxymethoxymethylguanine is a presumptive neurotoxin and should be monitored in patients with impaired renal function or in cases with neurotoxic symptoms. A sample was prepared using protein precipitation with 1% formic acid in methanol containing isotopically labeled internal standard. Chromatographic separation on a biphenyl column and mass spectrometric detection were performed in multiple reaction monitoring (MRM) mode on a Xevo TQ-S micro with ESI in positive ion mode, within 3 min. Inter-day assay accuracies for the quality controls varied between 95 and 104% and intra-day assay between 93 and 105%. Inter-day and intra-day assay imprecision for the quality controls ranged between 1.4 and 4.2% and 1.7 and 6.5% respectively. The lower limit of quantification for all four substances was 0.156 μmol/L. It is an accurate and reproducible method for therapeutic drug monitoring.

KEYWORDS
antiviral agents, LC–MS/MS, serum analysis, therapeutic drug monitoring, validation

1 | INTRODUCTION

Acyclovir (ACV), ganciclovir (GCV), and penciclovir (PCV) are nucleoside analogues that share structural similarities with guanosine and are widely used for the treatment of herpes simplex, herpes zoster, and cytomegalovirus (CMV) infections. ACV has been life-saving in patients with herpes encephalitis and GCV in patients with serious CMV infections. All three drugs can be administered intravenously (IV). The oral bioavailability is low (ACV, GCV) or nearly absent (PCV), and they are improved as the prodrugs valacyclovir, valganciclovir, or famciclovir. In infected cells, administrated drugs are converted to a monophosphate by the viral thymidine kinase and thereafter by cellular enzymes to the di- and triphosphorylated forms. The latter competitively

inhibits viral DNA polymerase and terminates the viral DNA chain.

GCV and PCV as well as the major part of ACV are excreted unchanged by the kidneys. Approximately 5–15% of ACV is metabolized in the liver by alcohol dehydrogenase to an ACV aldehyde and further on via aldehyde dehydrogenase to the main metabolite 9-carboxymethoxymethylguanine (CMMG) (de Miranda et al., 1982; Hellden et al., 2006).

Despite these antiviral drugs being available on the market for several years, no therapeutic levels nor a reliable concentration-toxicity relationship has been clearly defined for either of the substances. The use of therapeutic drug monitoring (TDM), for example, for GCV, has therefore been questioned (Galar et al., 2021; Jager et al., 2016; Scott et al., 2004). Too high a dose in renal-compromised patients increases the probability of toxic drug levels, which is why dose adjustment is necessary for patients with impaired renal function to avoid the risk of accumulation resulting in toxic drug and metabolite levels. The incidence of ACV-induced nephrotoxicity is approximately 10–15% (Bean & Aeppli, 1985; Lee et al., 2018; Ryan et al., 2018) with frequent case reports every year since the early 1980s (Bach, 1987). For GCV, cases of neurotoxicity, hepatitis, and bone marrow suppression have been reported, but relatively few cases compared to the number of cases reported for ACV. It can be hypothesized that toxicity symptoms in these cases can be difficult to diagnose without TDM due to the similarity between side effects and the disease.

We have previously shown that CMMG is consistently increased in patients with ACV-induced neurotoxicity, mainly in patients with acute or chronic kidney disease (Hellden et al., 2003). Recent publications have shown the utility of TDM in CMMG in bone-marrow transplanted patients (Berry & Venkatesan, 2014; Brandariz-Nunez et al., 2021; Lindstrom et al., 2019). In Sweden, measurements of CMMG levels have been used as a marker of ACV-induced toxicity since 1994 and helped many Swedish physicians to distinguish between ACV toxicity and symptoms of viral encephalitis infections.

Even though TDM studies of GCV have provided contradictory results, (Vezina et al., 2014; Wiltshire et al., 2005) promising results have been shown in the prevention of drug toxicity as well as cases of subtherapeutic drug concentrations not only in Sweden (Martson et al., 2019; Martson et al., 2021; Peredo et al., 2015).

Previously published analytical methods for determination of ACV and its metabolite CMMG have used HPLC in reversed-phase mode for the separation followed by UV, UV-diode array, fluorescence detection, or MS (Dao et al., 2008; Darville et al., 2007; Svensson et al., 1997; Urinovska et al., 2021; Weller et al., 2009). In these publications, the most common reversed phases used for chromatographic separation have been various C8 and C18 phases with, for example, HSS T3 tri-functionally bonded C18 ligands that promote polar compound retention and are compatible with aqueous mobile phases. As these antiviral compounds are fairly polar and include an aromatic moiety, other reversed phases such as phenyl or biphenyl could produce variations in retention and selectivity compared to the traditional C8 and C18 phases. Previously, a phenyl-hexyl phase has been used for the separation of seven antiretroviral agents (Zheng et al., 2020). Because the polar nature of these compounds may result in poor retention and peak shape when analyzed in reversed-phase mode, chromatographic separation using hydrophilic interaction liquid chromatography (HILIC) has also been attempted (Brown et al., 2002). With the introduction of UHPLC and the use of sub-2 μm particle columns, a reduction in separation time can be obtained while maintaining separation efficiency. Combining the peak capacity of the UHPLC column with the selectivity of the mass spectrometer generally results in increased sensitivity and reduced analysis time, particularly for multicompound methods. Several methods exist for the determination of ACV, GCV, and PCV in human plasma or serum, with or without simultaneous determination of their corresponding prodrugs (Heinig et al., 2011; Kasiari et al., 2008; Schimek et al., 2018; Shi et al., 2018; Xu et al., 2007; Yadav et al., 2009) but only a few methods include the measurement of CMMG (Darville et al., 2007; Svensson et al., 1997; Urinovska et al., 2021; Yang et al., 2007).

The aim of this work was to develop and validate a rapid, simple, sensitive, and robust LC–MS/MS method for the quantification of ACV, its metabolite CMMG, as well as GCV, and PCV in human serum for the purpose of monitoring antiviral drug levels and diagnose toxicity in critically ill patients.

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

Analytical standards of ACV and PCV were purchased from Sigma-Aldrich (St. Louis, MO, USA), CMMG was purchased from Toronto Research Chemicals (Ontario Canada), and GCV was purchased from Alschim (Illkirch, France). The internal standards 9-carboxymethoxymethylguanine-$^{13}C_2$-^{15}N (CMMG-IS) was purchased from Toronto Research Chemicals and acyclovir-D_4 (ACV-IS), GCV-D_5 (GCV-IS), and PCV-D_4 (PCV-IS), from Alschim. Water was purified with a MilliQ-gradient water purifying system from Merck (Darmstadt, Germany). Methanol (LC–MS grade), ammonium acetate (EMSURE®), formic acid (EMSURE®), and hydrochloric acid (EMSURE®) were purchased from Merck and dimethyl sulfoxide (DMSO) (BioUltra) from Sigma-Aldrich.

2.2 | Standards and quality control samples

Stock solutions of 1 mg/mL of ACV, GCV, and PCV, ACV-IS, GCV-IS, and PCV-IS, respectively, were prepared in methanol with 1% (v/v) of hydrochloric acid. Stock solutions of 1 mg/mL CMMG and CMMG-IS were prepared in DMSO. Calibrators and quality controls (QCs) were made by spiking ACV, CMMG, GCV, and PCV into drug-free serum from healthy volunteers (blood donors from Linköping University Hospital). A six-point calibration curve was prepared by spiking drug-free serum with each of the analytes to a concentration of 160 μmol/L, followed by a serial dilution to obtain the concentration of 40, 10, 2.5,

0.625, and 0.156 μmol/L. QC samples were prepared by using separate stock solutions by adding the four analytes to drug-free serum to make the high-level QC at 120 μmol/L and was further diluted to obtain the mid-level QC (OC-II, 20 μmol/L) and the low-level (QC-I, 0.5 μmol/L) respectively. An internal standard working solution (5 μmol/L of ACV-IS, CMMG-IS, GCV-IS, and PCV-IS) was prepared in methanol with 1% (v/v) formic acid.

2.3 | Sample collection

Samples from patients were collected in serum tubes containing no anticoagulants and centrifuged for 10 minutes at 2,000*g*, after which serum was transferred to a new tube prior to analysis or storage at −20°C.

2.4 | Sample preparation

About 150 μL cold internal standard solution was added to 50 μL of the calibration standard, QC or serum sample, vortexed briefly followed by centrifugation at 15,000*g* for 5 min at 10°C. An amount of 100 μL of the supernatant was transferred to a 96-well plate and diluted with 100 μL of 1% formic acid in Milli-Q water followed by mixing for 10 min.

2.5 | Chromatography and instrumentation

LC–MS/MS analysis was performed on an Acquity (Waters, Milford, MA, USA) system equipped with a binary solvent manager, flow-through needle (FTN) autosampler, and a mass spectrometer with an electrospray interface (ESI) operating in the positive ion mode, XEVO TQ-S micro. Chromatographic separation was performed at 40°C using a Kinetex biphenyl column (100 × 2.1 mm, 100 Å pore size, 2.6 μm particle size) preceded by a pre-column, SecurityGuard™ Ultra biphenyl 2*2.1 mm, all from Phenomenex (Copenhagen, Denmark). The autosampler purge solution was 10:90 methanol:water, the needle wash solution was 80:20 methanol:water, and the injection volume was 2 μL. The four analytes and their internal standards were separated in a gradient chromatographic run using a mobile phase consisting of 10 mmol/L ammonium acetate pH 6.8 (A) and methanol (B) at a flow rate of 0.5 mL/min over the total run time of 3 min. The following mobile phase gradient was used: phase B was started at 2% for 0.3 min, then increased to 25% from 0.3 to 2.0 min, further increased to 75% from 2.0 to 2.5 min, after which it was returned to the initial composition and equilibrated until 3.0 min.

The mass spectrometer was operated in positive ion mode. The instrument conditions were as follows: capillary voltage, 1.3 kV; desolvation gas flow and temperature, 1000 L/h and 500°C respectively; the source temperature, 150°C; and the sample cone gas flow, 50 L/h.

All analytes and internal standards were detected using multiple reaction monitoring (MRM) recording one quantifier ion, one qualifier ion for each of the analytes, and a quantifier ion for the internal standards. The specific mass spectrometric settings for each compound are presented in Table 1. Six-level calibration was performed before every analysis, and concentrations were calculated using TargetLynx software (MassLynx 4.1, Waters).

2.6 | Method validation

Validation of selectivity, linearity, sensitivity, accuracy, and precision was performed according to the European Medicines Agency (EMA) guidelines (EMA, 2012).

2.7 | Selectivity

Selectivity was assessed by analyzing serum samples from six different drug-free individuals without the addition of internal standard or analytes for evaluation of interference from endogenous components. In addition, the drug-free serum sample was prepared with the internal standard working solution to evaluate purity and potential interferences affecting the analytes. Signal interference of less than 20% of the LLOQ and 5% of the internal standard was considered acceptable as per the EMA guidelines.

2.8 | Matrix effect, extraction recovery, and process efficiency

Two methods were used to evaluate matrix effects. Qualitative matrix effects were investigated by post-column infusion. The analytes at a concentration of 1 μmol/L in methanol were infused at a flow rate of 10 μL/min postcolumn. Simultaneously with the post-column infusion, serum extracts ($n = 6$) were injected without the internal standard or analyte. An increase or decrease in the continuous signal at the expected retention time for each analyte or internal standard indicates ion enhancement or suppression. Matrix effects were also evaluated quantitatively together with recovery and process efficiency according to Matuszewski et al. (Matuszewski et al., 2003). ACV, CMMG, PCV, and GCV in concentration levels corresponding to the six calibration levels (0.156, 0.625, 2.5, 10, 40, and 160 μmol/L) and the internal standards, ACV-IS, CMMG-IS, GCV-IS, and PCV-IS (5 μmol/L of each) were either dissolved directly in water (set one) or added to serum samples from six drug-free individuals after (set two) or before (set three) the extraction procedure. The matrix effects were defined as the ratio between the mean peak areas of sets one and two, expressed as percentages. Consequently, a calculated value of 100% indicates no measurable matrix effects. Recovery and process efficiency were determined in a similar way by the division of set three by set two and set three by set one, respectively. The calculated matrix effect should be within the interval 85–115%, and the extraction recovery should be more than 50% (Rudzki et al., 2018).

TABLE 1 The specific mass spectrometric settings for each substance and their corresponding internal standard

Substance	ESI mode	Retention time (min)	MRM transitions (*m/z*) quantifier/qualifier	Cone voltage (V)	Collision energy (eV)
ACV	+	1.40	226.10 > 152.00	12	10
			226.10 > 134.95	12	28
CMMG	+	0.74	240.03 > 152.00	4	12
			240.03 > 134.95	4	30
PCV	+	1.53	254.10 > 151.99	48	16
			254.10 > 135.01	48	32
GCV	+	1.08	256.10 > 152.01	12	12
			256.10 > 134.95	12	32
ACV-IS	+	1.38	230.13 > 152.00	2	10
CMMG-IS	+	0.75	243.10 > 155.00	2	12
PCV-IS	+	1.52	259.22 > 152.07	48	14
GCV-IS	+	1.07	261.13 > 151.99	2	12

MRM transitions with protonated molecule and monitored fragments in positive mode. Retention time, cone voltage, and collision energy for each analyte.
Note: ACV, acyclovir; CMMG, 9-carboxymethoxymethylguanine; GAN, ganciclovir; PCV, penciclovir; and their analogous internal standards ACV-IS, CMMG-IS, PCV-IS and GCV-IS; MRM, multiple reaction monitoring.

2.9 | Calibration model

Calibration curves were constructed by plotting the analyte/internal standard peak area ratio using the residual plot to compare linear and quadratic curve fit, with or without weighting of 1/*x*, for each compound resulting in a calibration curve with a coefficient of determination (r^2) > 0.995.

2.10 | Lower limit of quantification

Six replicates of the lowest calibration point 0.156 μmol/L followed by further dilution 1:1 with drug-free serum to 0.078 μmol/L ($n = 6$) were analyzed to determine the lower limit of quantification (LLOQ). Acceptable precision and accuracy were considered to be ≤ 20% and 80–120%, respectively (EMA, 2012).

2.11 | Accuracy and precision

The accuracy and precision of the method were determined through repeated measurements of QC samples at three levels ($n = 6$). Intra-day and inter-day assay precision and accuracy were performed in one run and multiple runs, respectively. Accuracy was calculated as a bias, mean measured concentration divided by spiked concentration * 100 and precision as the coefficient of variation (CV%). Mean inaccuracy should be within 15% (20% for LLOQ) of nominal values for quality control samples. The imprecision should not exceed 15% (20% for LLOQ).

2.12 | Dilution integrity

To evaluate the dilution process performed on samples exceeding the highest calibrator, drug-free serum ($n = 5$) was spiked to 250 μmol/L and diluted fivefold with drug-free serum. According to EMA guidelines, accuracy should be within 85–115%, and precision should be within ± 15%.

2.13 | Carry-over

Carry-over was assessed by injecting 10 sample extracts of the lowest calibration standard, followed by a series of injections alternating between the highest and lowest calibrators ($n = 10$ per level). A *t*-test was used to compare the mean peak areas of the two sets of low calibration standards. Carry-over was considered insignificant if $p > 0.05$ (Honour, 2011).

2.14 | Stability

A system suitability check was used regularly before every batch confirming retention time, peak area, height, width, and the signal-to-noise ratio (S/N).

The stock solution and working solutions of antiviral drugs and internal standards' stability were evaluated after 6 months' storage at −80°C. The long-term storage stability of the patient samples over a time period of 6 months at −20°C was evaluated. Storage stability was claimed if the bias between the freshly prepared samples and samples stored for 6 months was ≤ 15%.

3 | RESULTS AND DISCUSSION

3.1 | Chromatography and sample preparation

Several C_{18}, phenyl, and biphenyl stationary phases were tested in combination with methanol or acetonitrile with additives such as

ammonium acetate, ammonium formate, acetic acid, or formic acid. Our experiment showed that the biphenyl phase provided changes in selectivity relative to C_{18} with a methanolic phase, but with acetonitrile-based phase the biphenyl behaved more similarly to a C_{18}, which has been shown by Appulage et al. (Appulage et al., 2016). For these rather polar substances analyzed, in addition to interaction driven by the hydrophobic effect a biphenyl phase can give additional retention by π-π- and cation-π interactions compared to a C_{18} phase. A mobile phase consisting of 10 mmol/L ammonium acetate and methanol with a linear gradient on a biphenyl stationary phase gave the optimum separation and peak shape symmetry of the analytes with a chromatographic run time of 3 min. Based on the pKa values of ACV, GCV, and PCV published on PubChem (https://pubchem.ncbi.nlm.nih.gov/), and the pH of mobile phase A (6.8) it can be concluded that these analytes are present in a neutral state during the chromatographic run and that hydrophobic and π-π-interactions are responsible for the selectivity/difference in retention times. Log *P* values (Pubchem) of ACV, GCV, and PCV show an increasing order following the retention order. The pKa values reported for CMMG indicate that it will be present in a charged state (−1) at pH 6.8 resulting in limited retention. The precipitation solvents acetonitrile and methanol in different combinations with formic acid and zinc sulfate were used to investigate which combination gave the best extraction recovery. Acetonitrile and zinc sulfate were excluded due to low extraction efficacy and broad and asymmetric peaks for all the analytes when the extract was injected into the biphenyl column. The combination of 1% formic acid with methanol as precipitation solvent and dilution of the supernatant with 1% formic acid in ultrapure water before injection was chosen as it displayed significantly better extraction recovery. Figure 1 shows a representative MRM chromatogram from the serum extract of the calibration standard spiked with 0.156 μmol/L of ACV, CMMG, PCV, and GCV.

3.2 | Selectivity

Analyzing serum from six drug-free individuals showed an absence of endogenous interferences at the mass transitions at the retention times for ACV, CMMG, GCV, and PCV. Drug-free serum spiked with internal standards, ACV-IS, CMMG-IS, GCV-IS, and PCV-IS, showed no interfering peaks for ACV, CMMG, GCV, and PCV.

3.3 | Matrix effect, extraction recovery, and process efficiency

The qualitative matrix effect experiment showed no ionization suppression regions that would interfere with ACV, CMMG, GCV, or PCV. For CMMG, an ionization suppression region between 0.4 and 0.6 was found but with a retention time of 0.7 for CMMG and the use of CMMG-IS ensured a valid measurement of the analyte (Figure 2). For the quantitative experiment the mean extraction recovery, matrix effect, and process efficiency are summarized in Table 2, in the standard concentration range 0.156–160 μmol/L and their corresponding internal standards at 5 μmol/L. The extraction recovery within the standard curve range was greater than 83.3%, for all compounds. For the matrix effect, a variation between 76.3 and 93.6% was obtained with a process efficiency of 67.6–87.7%. The extraction recovery for the internal standards was greater than 86.2%. A variation between 81.8 and 93.4% was obtained for the matrix effect with a process efficiency of 78.2–90.2%.

To further evaluate the quantitative matrix effect, recovery, matrix, and internal standard normalized matrix factors were calculated. Absolute and compensated values for matrix effect and process efficiency as well as variability of matrix effect at the six different calibration levels are all summarized in Table 3. For uncompensated matrix effects, the CV ranged from 0.7 to 3.5% and for IS normalized matrix effects, the CV ranged from 0.5 to 3.3% for the six calibration levels.

3.4 | Calibration model and LLOQ

A quadratic regression model with $1/x$ weighting was used for all analytes for the concentration range 0.156–160 μmol/L, resulting in correlation coefficients between 0.99962 and 0.99999. The recalculated concentrations of each calibration standard were all within ± 5 (4.3)% of nominal values for all the analytes. LLOQ was determined to a concentration 0.156 μmol/L equal to the lowest standard point for the four substances.

3.5 | Accuracy and precision

Accuracy and precision of the analysis of QC samples at three concentration levels for each compound are presented in Table 4. The mean intra-day assay accuracy was 93 to 105% ($n = 6$), and inter-day assay accuracy ranged from 95 to 103% ($n = 6$) for ACV, CMMG, GCV, and PCV. CV of intra-day assay imprecision was 1.7–6.5%, and the inter-day assay imprecision was 1.4–4.2%.

3.6 | Dilution integrity

Drug-free serum was spiked with a concentration of 250 μmol/L for each of ACV, CMMG, GCV, and PCV and diluted to 62.5 μmol/L. Accuracy and precision of the analysis were not affected as the five-fold diluted samples were measured with an inaccuracy of < 9.6% and imprecision < 7.1%.

3.7 | Carry-over

Application of the optimal washing procedure to prevent carry-over effects and ensure the precision and accuracy of the method was

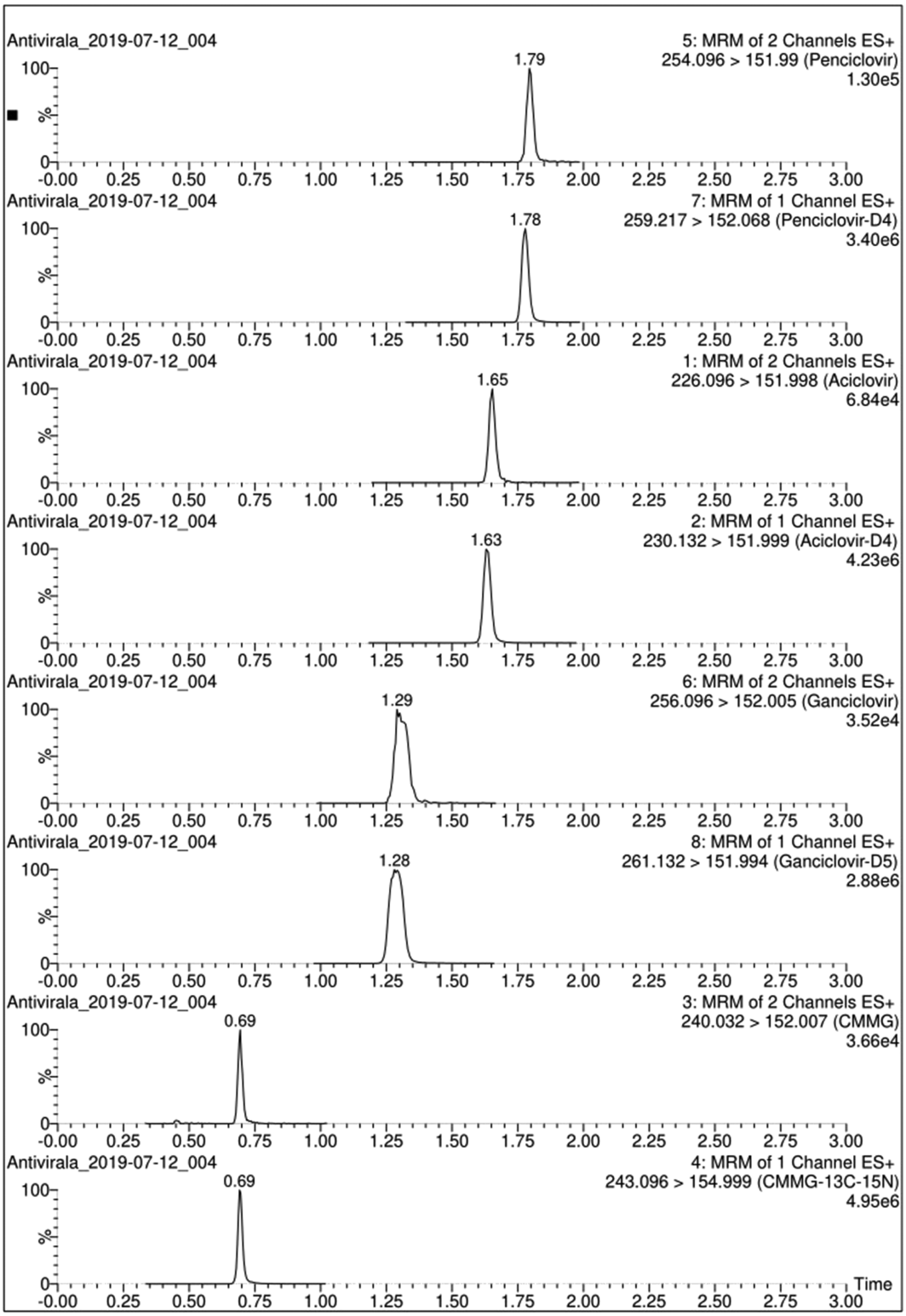

FIGURE 1 Representative multiple reaction monitoring (MRM) chromatograms from serum extract of calibration standard spiked with 0.156 μmol/L of each substance

tested. The alternate injections of highest calibration and lowest calibration standard compared to 10 repeated injections of lowest calibration standard were performed. Using the independent sample *t*-test to compare the mean of the two sets of the lowest calibration standards, the result in *p*-values was above 0.05 for all analytes, indicating no significant carry-over, after extending the time for the post-inject wash time from 6 to 12 s in the FTN injection system.

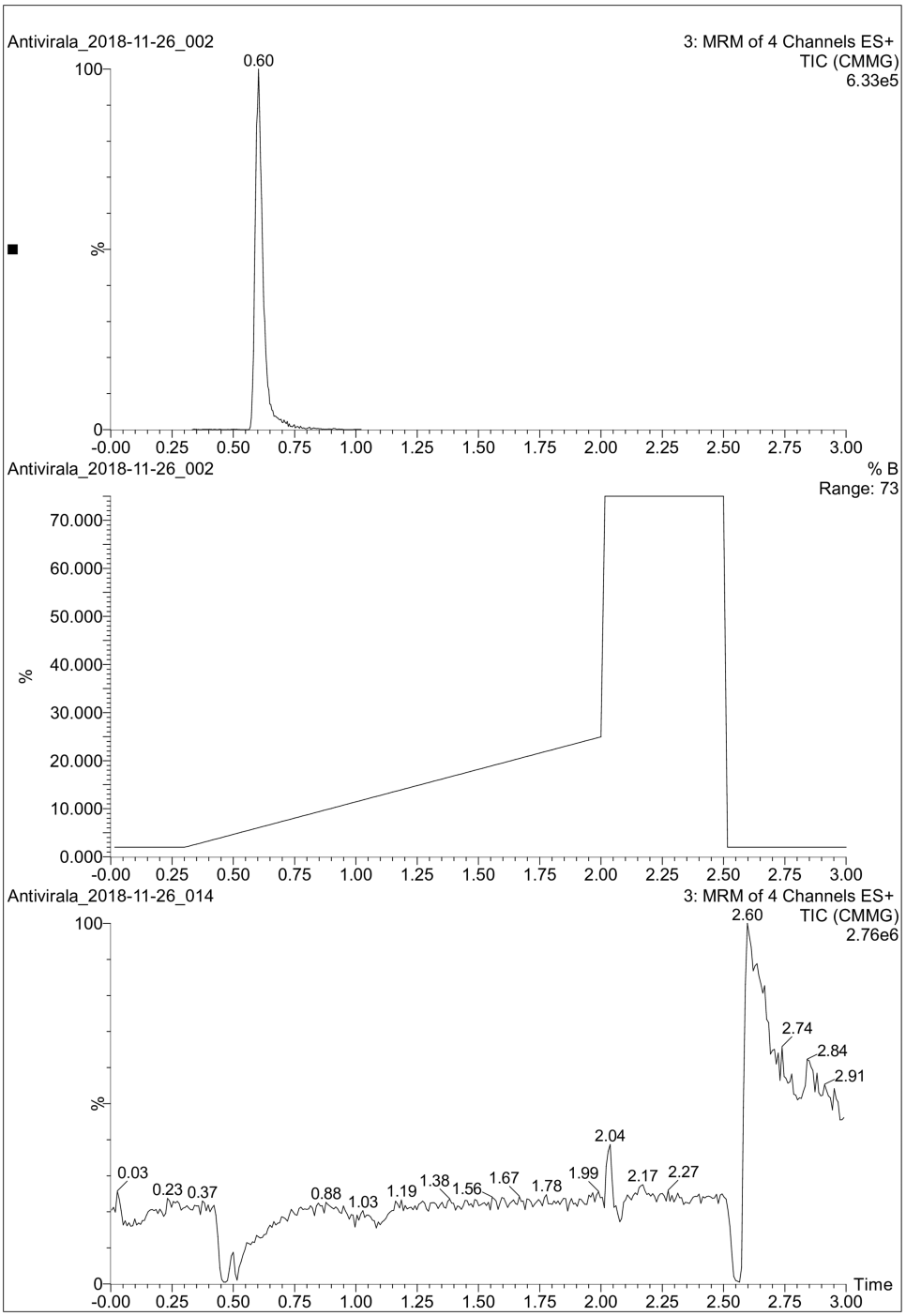

FIGURE 2 Qualitative matrix effect for 9-carboxymethoxymethylguanine (CMMG)

3.8 | Stability

The analytes and internal standards were found to be stable for 24 h in autosampler at 5°C. Stock and working solutions of analytes and internal standards were stable for 6 months at −80°C. Patient samples stored at −20°C were found to be stable for 6 months, after being reanalyzed with freshly prepared calibration standards. Unpaired two-sided *t*-test gave *p*-values of 0.827, 0.932, 0.999, and 0.929 for ACV, CMMG, GAN, and PNV, respectively, showing a good stability for up to 6 months' storage.

3.9 | Application of method

To evaluate method applicability, serum samples from patients undergoing treatment for herpes virus infection with ACV ($n = 10$) or GCV ($n = 10$) were analyzed.

GCV results ranged between 1.9 and 16.9 μmol/L (mean 12.6 μmol/L), ACV ranged between 2.2 and 31.0 μmol/L (mean 12.6 μmol/L), and CMMG 0.5–18.2 μmol/L (mean 3.4 μmol/L). Figure 3 shows serum samples collected during 24 h from a subject who was administered a 200 mg tablet of ACV to study the formation of CMMG.

TABLE 2 Results of matrix effect (ME), extraction recovery (ER), and process efficacy (PE) for each substance and their corresponding internal standard, as mean values in six different serum samples

	Calibrator I			Calibrator II			Calibrator III			Calibrator IV			Calibrator V			Calibrator VI		
µmol/L	0.156			0.625			2.5			10			40			160		
Compound	ME%	ER%	PE%	ME%	ER%	PE%	ME%	ER%	PE%	ME%	ER%	PE%	ME%	ER%	PE%	ME%	ER%	PE%
ACV	86.1	91.0	78.3	86.7	87.6	75.9	80.2	89.3	71.6	88.1	91.1	80.3	91.6	92.0	84.2	93.6	93.8	87.7
CMMG	86.3	83.3	71.9	86.2	84.4	72.7	78.4	86.2	67.6	85.3	86.0	73.3	87.4	87.8	76.8	88.1	91.1	80.2
GCV	83.6	84.1	70.3	82.9	88.1	73.0	76.3	89.3	68.1	84.0	90.2	75.8	87.5	90.9	79.5	91.2	91.9	83.8
PCV	82.4	87.6	72.2	83.0	89.1	74.0	76.7	90.7	69.5	84.0	91.7	77.0	88.9	92.8	82.5	91.4	94.3	86.2
	Internal standard 5 µmol/L																	
ACV-IS	91.4	95.1	87.0	90.5	94.2	85.2	91.4	95.0	86.9	91.9	94.9	87.2	93.2	94.5	88.0	93.4	96.6	90.2
CMMG-IS	93.2	86.8	80.2	90.7	86.2	78.2	90.8	86.4	78.5	90.4	87.0	78.7	90.1	88.3	79.5	89.8	91.0	81.7
GCV-IS	82.1	98.6	81.0	81.8	98.2	80.3	82.5	98.4	81.1	82.3	98.9	81.4	83.7	99.2	83.1	85.3	100.3	85.6
PCV-IS	84.5	96.8	81.8	85.5	95.4	81.6	86.1	95.7	82.5	87.0	96.3	83.8	88.5	98.1	86.8	89.7	99.4	89.2

Note: ACV, acyclovir; CMMG, 9-carboxymethoxymethylguanine; GAN ganciclovir; PCV, penciclovir; and their analogous internal standards ACV-IS, CMMG-IS, PCV-IS and GCV-IS.

TABLE 3 Absolute and compensated (IS normalized) values for matrix effect (ME) and process efficiency (PE) for each substance, as mean values in six different serum samples

		Calibrator I				Calibrator II				Calibrator III				Calibrator IV				Calibrator V				Calibrator VI			
		0.156				0.625				2.5				10				40				160			
Compound	$n = 6$ µmol/L	ME (%) + CV		PE (%) + CV		ME (%) + CV		PE (%) + CV		ME (%) + CV		PE (%) + CV		ME (%) + CV		PE (%) + CV		ME (%) + CV		PE (%) + CV		ME (%) + CV		PE (%) + CV	
ACV	Absolute	86.1	2.1	78.3	6.7	86.7	2.6	75.9	2.0	87.5	1.4	78.1	2.7	88.1	2.3	80.3	3.1	91.6	1.7	84.2	1.1	93.6	1.0	87.7	1.1
	Compensated	94.2	2.1	90.1	6.7	95.7	3.1	89.1	2.2	95.7	0.7	90.0	2.6	95.9	1.2	92.1	3.0	98.3	0.6	95.7	1.8	100.2	1.8	97.2	1.3
CMMG	Absolute	86.3	3.5	71.9	3.1	86.2	2.6	72.7	1.7	86.1	1.7	74.2	1.7	85.3	2.7	73.3	1.3	87.4	2.5	76.8	1.1	88.1	1.6	80.2	1.8
	Compensated	93.5	3.3	89.7	4.3	94.9	2.1	93.0	1.4	94.8	0.9	94.6	1.6	94.3	0.7	93.2	2.4	97.1	0.7	96.7	1.8	98.1	1.6	98.2	0.8
GCV	Absolute	83.6	1.6	70.3	7.3	82.9	1.7	73.0	4.6	83.8	1.6	74.8	3.2	84.0	2.4	75.8	3.1	87.5	2.0	79.5	1.3	91.2	1.2	83.8	1.3
	Compensated	101.8	1.7	86.8	6.8	101.3	1.2	90.9	3.7	101.6	1.0	92.2	2.7	102.0	1.2	93.1	3.0	104.5	0.6	95.8	1.6	106.9	1.3	97.9	1.3
PCV	Absolute	82.4	1.1	72.2	3.5	83.0	0.9	74.0	1.6	83.5	1.7	75.7	2.4	84.0	2.4	77.0	1.8	88.9	2.6	82.5	0.7	91.4	2.1	86.2	1.7
	Compensated	97.5	1.2	88.2	2.9	97.1	0.8	90.7	1.4	97.0	0.8	91.8	1.9	96.5	1.3	91.8	1.0	90.5	0.5	95.0	0.7	101.9	1.3	96.7	2.0

Note: ACV, acyclovir; CMMG, 9-carboxymethoxymethylguanine; GAN ganciclovir; PCV, penciclovir; and their analogous internal standards ACV-IS, CMMG-IS, PCV-IS, and GCV-IS.

TABLE 4 Accuracy and precision for the quality controls for all included substances. From a single run on six replicates at each quality control level (inter-day assay) and from six different days (intra-day assay)

		Intra-day assay ($n = 6$)			Inter-day assay ($n = 6$)		
		QC I	QC II	QC III	QC I	QC II	QC III
Substance		0.5 µmol/L	20 µmol/L	120 µmol/L	0.5 µmol/L	20 µmol/L	120 µmol/L
ACV	Mean	0.46	20.4	119.0	0.48	20.6	119.8
	SD	0.021	0.59	2.0	0.007	0.50	2.7
	Imprecision (CV%)	4.4	2.9	1.7	1.4	2.4	2.3
	Accuracy (%)	93	102	99	95	103	100
CMMG*	Mean	0.50	19.7	118.3	0.52	20.5	120.7
	SD	0.026	0.55	2.8	0.022	0.58	2.8
	Imprecision (CV%)	5.5	2.8	2.4	4.2	2.9	2.3
	Accuracy (%)	100	98	99	105	102	101
PCV	Mean	0.48	20.1	116.5	0.52	20.9	118.6
	SD	0.028	0.60	2.8	0.011	0.63	2.4
	Imprecision (CV%)	5.8	2.9	2.4	2.1	3.0	2.1
	Accuracy (%)	97	105	97	104	104	99
GAN	Mean	0.47	19.9	118.8	0.48	20.4	120.8
	SD	0.030	0.57	2.3	0.008	0.52	3.0
	Imprecision (CV%)	6.5	2.9	1.9	1.6	2.5	2.5
	Accuracy (%)	94	100	99	96	102	101

Note: ACV, acyclovir; CMMG, 9-carboxymethoxymethylguanine; CV,coefficient of variation; GAN ganciclovir; Mean and SD in µmol/L; PCV, penciclovir; QC, quality control.

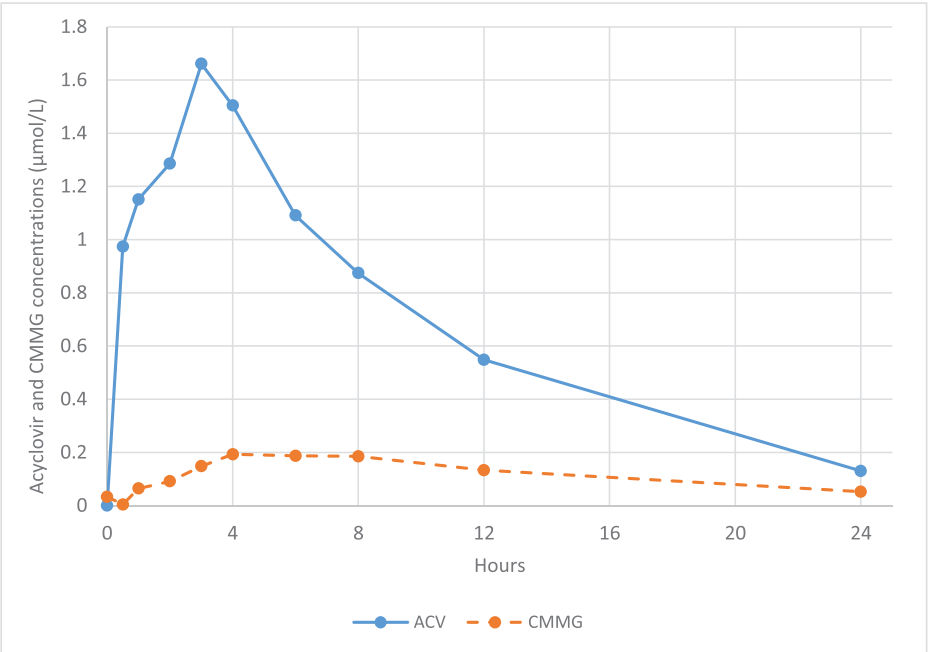

FIGURE 3 Acyclovir (ACV) and 9-carboxymethoxymethylguanine (CMMG) concentrations during a 24-h sampling period collected from one subject given a single 200-mg tablet acyclovir

4 | CONCLUSIONS

This work describes a fully developed and validated method for rapid quantification of ACV, its metabolite CMMG, GCV, and PCV, with the advantage of a short preprocessing, using a small amount of serum and protein precipitation with 1% formic acid in methanol containing corresponding isotopically labeled forms of the four substances analyzed as internal standards that result in high extraction recovery,

minimized assay variation, and matrix effects. Further on, this study shows an improvement compared to previous methods by the use of a XEVO TQ-S micro for detection of the analytes with an LLOQ of 0.156 μmol/L for all four analytes. Optimal chromatographic performance, including selectivity and peak shape, was achieved using a column with a biphenyl stationary phase, resulting in a chromatographic runtime of 3 min. The novelty of using an alternative chromatographic selectivity by the choice of a core-shell biphenyl stationary phase demonstrated that this stationary phase could be an alternative even for these rather polar antiviral drugs.

In all, the validated LC–MS/MS method presented for the determination of the total concentration of ACV and its metabolite CMMG as well as GCV and PCV in human serum proved to be sensitive, specific, and accurate, making it suitable for therapeutic drug monitoring of these substances to improve patient treatment.

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