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ORIGINAL RESEARCH

A rapid and sensitive method for the quantification of ganciclovir in plasma using liquid chromatography/selected reaction monitoring/mass spectrometry

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ABSTRACT: A method using reversed-phase liquid chromatography coupled with electrospray ionization and selected reaction monitoring mass spectrometry has been developed for the quantitative analysis of ganciclovir in rat plasma. Acyclovir, a structurally related analog of ganciclovir, was used as the internal standard. A small volume of plasma (50 μL) was spiked with the internal standard and plasma proteins were precipitated by methanol. The supernatant was dried under nitrogen, and then reconstituted in water. The use of liquid chromatography/selected reaction monitoring/mass spectrometry effectively eliminated potential interference from endogenous constituents in the plasma. This highly selective and sensitive method made it possible to analyze plasma ganciclovir with a lower limit of quantitation of 10 ng/mL. The assay was reproducible and linear in the range 10–10,000 ng/mL. The precision and accuracy values were in the range 2.0–6.9% and 89.0–109.6%, respectively. The analyte recovery was greater than 88%. This method was successfully used to monitor the pharmacokinetic profile of ganciclovir in normal rats following intraperitoneal administration of the drug. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

Ganciclovir [GCV, Fig. 1(a)], a nucleoside analog of 2′-deoxyguanosine (dGuo) is an effective agent for treating Herpes simplex virus (HSV) infections (Cheng *et al.*, 1983; Martin *et al.*, 1983; Smee *et al.*, 1985). In cells infected with HSV, viral thymidine kinase phosphorylates GCV to the corresponding monophosphate (MP) (Cheng *et al.*, 1983). Cellular kinases then convert GCV-MP into the triphosphate (TP), which competitively inhibits DNA polymerase-catalyzed incorporation of dGuo-TP into DNA (Frank *et al.*, 1984). GCV-TP is incorporated into the end of a growing chain of viral DNA with concomitant loss of pyrophosphate (Hamzeh *et al.*, 1990; Hamzeh and Leitman,1991). In contrast to acyclovir [ACV; Fig. 1(b)], GCV does not appear to serve as a chain terminator but is incorporated internally into DNA strands (Cheng *et al.*, 1983). This causes a decrease in the rate of DNA synthesis and disrupts viral replication (Frank *et al.*, 1984). The specificity of GCV arises because it is an excellent substrate for viral kinases, whereas it is a poor substrate for endogenous mammalian kinases (Crumpacker, 1996; Freeman and Gardiner, 1996).

The ability of GCV-TP to inhibit replication has

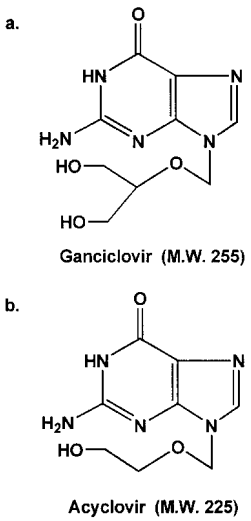

Figure 1. Structures of (a) ganciclovir (GCV) and (b) acyclovir (ACV, internal standard).

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Abbreviations used: ACV, acyclovir; CID, collision-induced dissociation; ESI, electrospray ionization; GCV, ganciclovir; HSV, Herpes simplex virus; LC, Liquid Chromatography; LLQ, lower-limit of quantitation; LOD, Limit of detection; LQC, lower-quality control; MP, Monophosphate; MQC, Middle quality control; QC, quality control; SRM, selected reaction monitoring; TP, triphosphate; UQC, upper quality control.

stimulated a suicide gene therapy approach for the treatment of human tumors (Black *et al.*, 1996; Freeman *et al.*, 1996; Moolten and Wells, 1990; Smythe *et al.*, 1995; Vile and Hart, 1993). Transfection of an HSV*tk* gene into mammalian cells results in the efficient conversion of GCV to GCV-MP. As with virally infected cells, subsequent conversion to GCV-TP then provides a substrate to compete with dGuo-TP for incorporation into elongating DNA. The decreased rate of DNA synthesis and incorporation of GCV-TP into DNA then inhibits host cell DNA replication. A critical component of this approach involves the ability to rapidly and specifically monitor plasma levels of GCV so that appropriate pharmacokinetic models can be constructed and optimal dosing strategies developed.

For quantitative analysis of GCV, assays have been performed primarily by high-performance liquid chromatography (HPLC) (Boulieu *et al.*, 1991a,b; Fletcher *et al.*, 1986; Trang *et al.*, 1993). It was reported by Campanero *et al.* (1998) and Page *et al.* (1996) that the limit of quantitation of GCV was at 50 ng/mL in plasma or serum using HPLC with UV detection. Such HPLC/UV methods were utilized in the pharmacokinetic studies of GCV (Aweeka *et al.*, 1995; Pescovitz *et al.*, 1997; Wolfe *et al.*, 1997). However, these HPLC/UV methods have one or more of the following shortcomings: potential interference from co-eluting matrix constituents, time-consuming extraction procedures, a requirement for large plasma volumes (500 μL), or relatively low sensitivity. In order to overcome these problems, a new method for the analysis of plasma GCV was developed. We report a method for the analysis of GCV, which employs liquid chromatography (LC) coupled with electrospray ionization (ESI) and selected reaction monitoring (SRM) mass spectrometry (MS). The method has been rigorously validated for the measurement of GCV levels in small volumes of rat plasma.

EXPERIMENTAL

Materials. GCV was obtained from Parke-Davis (Morris Plains, NJ). ACV was provided by Faulding Pharmaceuticals Co. (Elizabeth, NJ). HPLC-grade water was obtained from Fisher Scientific Co. (Fair Lawn, NJ); HPLC-grade methanol was purchased from Burdick & Jackson (Muskegon, MI); and ammonium acetate was obtained from J. T. Baker (Phillipsburg, NJ). Blank rat plasma was purchased from Biological Specialty Corporation (Landsdale, PA). Rat plasma samples for the pharamacokinetic studies of GCV were collected in the Thoracic Oncology Research Laboratory (Philadelphia, PA).

Sample preparation. GCV stock solutions were prepared by sequentially diluting a 50 mg/mL aqueous solution of GCV with water to give standard concentrations in the range 0.2–200 μg/mL. Blank rat plasma (9.5 mL) was then spiked with the GCV stock solution (0.5 mL) to make standard plasma samples in the range of 10–10,000 ng/mL. These standard plasma samples were stored at −80°C in aliquots until ready for use. ACV was used as the internal standard at a concentration of 5 ng/μL in water and stored at −80°C until used. The pre-spiked GCV plasma standards and the ACV internal standard were placed in a water/ice mixture and partially thawed by a Whirpool microwave at the 'easy-defrost' level for 2.5 min. An aliquot of the standard ACV solution (25 μL; 125 ng) was added to a 1.5 mL Eppendorf micro-centrifuge tube (VWR Scientific Products, Bridgeport, NJ). A standard GCV plasma sample (50 μL) was then added. The samples were vortex-mixed for 2 min, and methanol (200 μL) was added in order to precipitate plasma proteins. Samples were vortex-mixed again for 2 min, and centrifuged at 14,000 rpm for 2 min. The supernatants were transferred and filtered through a 0.2 μm Costar Spin-X HPLC micro-centrifuge filter (Costar, Cambridge, MA). The filtrates were dried under nitrogen at room temperature. After drying, the residue was reconstituted by adding 250 μL of water, vortex-mixed, and 70 μL was injected onto the LC column. Plasma samples collected from rat models for pharmacokinetic studies were processed in a similar manner to that described above.

HPLC apparatus and conditions. Chromatography was performed using a Waters Alliance 2690 HPLC system (Waters, Milford, MA) with an ODS-AQ column (150 × 4.6 mm, i.d.; 5 μm) obtained from YMC Inc. (Wilmington, NC). A pre-column filter (2 micron; Alltech, Deerfield, IL) was used to prevent particles from clogging the column and to prolong column life. The temperature of the autosampler chamber was held at 15°C. A gradient mobile phase was applied with solvent A containing 5 mM ammonium acetate (NH_4OAc) in water:methanol (95:5), and solvent B containing 5 mM NH_4OAc in pure methanol. The mobile phase flow rate was maintained at 1 mL/min. Immediately following the injection at 90% A, a linear gradient was run to 55% A over 4 min. A second linear gradient was run to 20% A over the next 2 min. The mobile phase was then brought back to the initial condition of 90% A by a linear gradient over 4 min, and the column was re-equilibrated at 90% A for 5 min before the next injection.

Mass spectrometry instrumentation. Mass spectra were acquired on a Finnigan TSQ7000 (Finnigan Corp., San Jose, CA) triple quadrupole mass spectrometer equipped with an ESI source. The TSQ7000 system was set up for unit resolution analysis by performing a standard autotune and calibration procedure. The protonated molecular ion $[M + H]^+$ of GCV at *m/z* 256 was selected in quadrupole 1 (Q1), and collision-induced dissociation (CID) was performed by introducing the collision gas (argon) at a pressure of 2.5 mTorr in Q2. The product ion at *m/z* 152 ($C_5H_6N_5O^+$) was detected in Q3 [Fig. 2(a)]. Similarly, $[M + H]^+$ of the internal standard, ACV at *m/z* 226 was subjected to CID and the major product ion at *m/z* 152 ($C_5H_6N_5O^+$) was detected in Q3 [Fig. 2(b)]. SRM was performed to monitor the specific transitions of *m/z* 256 → *m/z* 152 for GCV and *m/z* 226 → *m/z* 152 for ACV. A collision offset energy was optimized at −17 eV in Q2 for both GCV and ACV transitions. The instrument was operated at a heated capillary temperature of 245°C and a needle potential of 5.50 kV. The pressures for the nitrogen sheath gas and auxiliary gas were maintained at 90 and 30 psi, respectively, to accommodate the high LC flow rate of 1 mL/min.

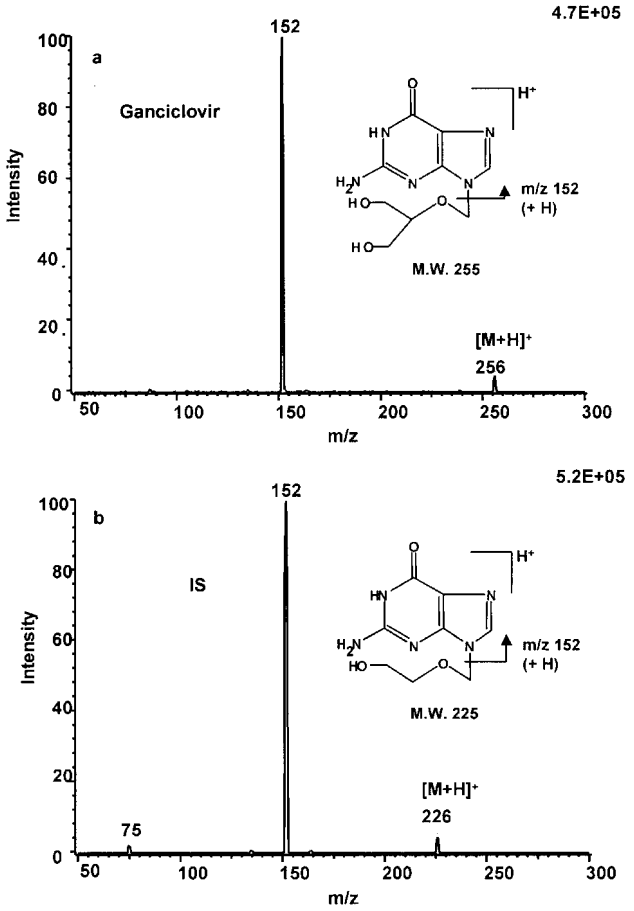

Figure 2. Positive tandem mass spectra for (a) GCV, *m/z* 256 $[M + H]^+$ and (b) ACV, *m/z* 226 $[M + H]^+$. Ionization was performed by ESI/MS at an LC flow rate of 1 mL/min.

Calibration curves, accuracy and precision. Ten-point calibration curves were prepared by analyzing the rat plasma standards at 10, 20, 50, 100, 500, 1000, 2500, 5000, 7500 and 10,000 ng/mL. Areas of the signals derived from both GCV and ACV were measured using Finnigan LCquan version 1.2 software, and their ratio was calculated for each specified GCV concentration. A linear least-squares regression with a weighting index of $1/x^2$ was performed on the peak area ratios of GCV and ACV vs GCV concentrations of the 10 plasma standards to generate a calibration curve. A water blank, rat plasma blank, and control zero (blank rat plasma spiked with ACV) were also processed and analyzed with each calibration curve. A total of three calibration curves was generated during the entire validation process. Six replicates ($n = 6$) of each quality control (QC) sample with GCV concentrations of 10 ng/mL (lower limit of quantitation, LLQ), 50 ng/mL (lower quality control, LQC), 500 ng/mL (middle quality control, MQC), and 5000 ng/mL (upper quality control, UQC) in rat plasma were processed and analyzed in the same manner as the 10 calibration standards. The LQC, MQC and UQC samples were examined on three different validation days. The LLQ samples were analyzed only on the first day. The accuracy of the assay was determined by comparing the means of the measured GCV concentrations with the specified concentrations in the quality control samples. The intra-assay precision (percentage relative standard deviation %RSD) was obtained by calculating the percentage ratio between the RSD of six replicates ($n = 6$) and their mean at each concentration within the same validation day. The inter-assay precision was defined as %RSD of three different validation days ($n = 3$). Plasma samples from pharmacokinetic studies were analyzed together with two of each the LQC, MQC and UQC samples. Assays were acceptable if the accuracy and precision of the QC samples were within ±15%.

Application of the assay. The validated assay was used to measure the GCV pharmacokinetic profile for normal rats dosed with GCV. Fischer 344 rats (~150 g) were appropriately anesthetized to facilitate cannulation of the right internal jugular vein with a soft silastic catheter via a small neck incision. GCV (10 mg/kg) diluted in physiologic saline (1 mL) was administered to the rats by bolus intraperitoneal injection. Blood samples (~300 μL) were collected in Eppendorf tubes containing 20 μL of 1000 units/mL heparin at different time points after GCV administration. The animals were monitored for the remainder of the procedure. Blood samples were vortex-mixed and subsequently centrifuged at 14,000 rpm for 20 s to separate cellular components from plasma. Plasma was then stored at −80°C until analyzed.

RESULTS AND DISCUSSION

Mass spectrometry

The LC/ESI/SRM/MS method has high specificity and sensitivity because only ions derived from the analytes of interest are monitored. Representative CID mass spectra for GCV and the ACV internal standard are shown in Fig. 2. The $[M + H]^+$ ions for GCV and ACV appeared at *m/z* 256 and *m/z* 226, respectively. Under the CID conditions that were employed, $[M + H]^+$ ions for both GCV and ACV were reduced by more than 95%, which resulted in maximum intensity for the product ions at *m/z* 152. GCV and ACV each contain a guanine base, which accounts for the product ions of *m/z* 152 that were generated for both compounds. SRM was performed by monitoring the transition of $[M + H]^+ \rightarrow$ *m/z* 152 for both analytes. The use of atmospheric pressure chemical ionization (APCI) for the analysis of GCV was also explored. Intense $[M + H]^+$ ions were observed for both GCV and ACV which were identical to those obtained using ESI (data not shown). Similar CID spectra were also observed. However, when LC/SRM/MS analysis was performed, the regression lines were nonlinear. Therefore, this ionization technique could not be used for the analysis of GCV. The nonlinearity of the regression line resulted from an increase in sensitivity when increasing amounts of GCV entered the APCI source. There are numerous examples of suppression of ionization occurring with atmospheric pressure ionization techniques (Matuszweski *et al.*,1998). However, it is very unusual to observe an increase in sensitivity.

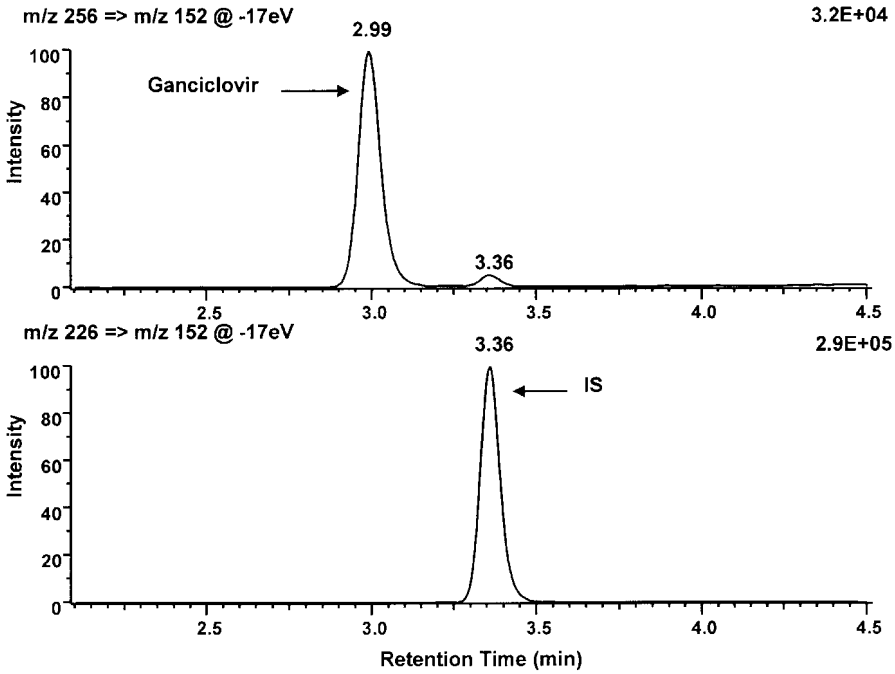

Figure 3. A representative LC/SRM/MS chromatogram for GCV (MQC, 500 ng/mL) and ACV internal standard (125 ng) in rat plasma. The upper chromatogram shows the GCV channel. The lower chromatogram shows the ACV channel. The retention time of GCV was 2.99 min and ACV was 3.36 min. LC was performed with a YMC ODS-AQ column (150 × 4.6 mm, i.d.; 5 μm) using a gradient of water/methanol/5 mM NH_4OAc at a flow rate of 1 mL/min.

Chromatography

Gradient elution of GCV and ACV was performed in order to minimize the potential for interference from metabolites or constituents of the plasma and to prevent non-polar materials from accumulating on the column. A representative LC/SRM chromatogram from a rat plasma GCV sample (MQC) is shown in Fig. 3. GCV had a retention time of 2.99 min as shown in the upper SRM chromatogram. The retention time for ACV, as seen in the lower SRM chromatogram, was 3.36 min. Retention times for both GCV and ACV proved to be highly reproducible under the gradient conditions that were used. It is worth noting that a second peak at 3.36 min was also detected in the upper SRM chromatogram. This signal arose from a methanol adduct of ACV which had an $[M + H]^+$ of *m/z* 258 and formed a product ion of *m/z* 152. A small amount of cross-talk into the *m/z* 256 → *m/z* 152 transition led to the appearance of a signal at 3.36 min that was approximately 1% of the intensity of the signal derived from ACV for the *m/z* 226 → *m/z* 152 transition. The appearance of a signal at 3.36 min in the GCV channel did not affect the high specificity of LC/SRM/MS because of the chromatographic separation of GCV and ACV. LC/SRM chromatograms for the water blank, rat plasma blank, and control zero showed no significant peaks at the retention time corresponding to GCV or ACV, ruling out the possibility of co-elution of interfering endogenous plasma constituents.

Calibration curves

The calibration curve for GCV in rat plasma was a function of peak area ratios (GCV/ACV) vs the GCV concentrations. A linear standard curve was obtained from 10 to 10,000 ng/mL. The variations between the back-calculated values for the plasma standards and the

Table 1. Accuracy for calibration standards during the validation process

Calibration standards (ng/mL)	Accuracy (%)		
	Day 1	Day 2	Day 3
10	100.0	94.0	96.0
20	97.0	112.5	103.1
50	105.0	90.0	106.8
100	105.7	111.5	111.0
500	102.7	109.7	107.9
1000	96.0	102.4	98.1
2500	100.3	97.9	96.0
5000	98.9	95.2	89.8
7500	96.4	95.3	97.6
10,000	98.1	90.9	94.9

Table 2. Precision and accuracy for QC analysis of ganciclovir in rat plasma

Ganciclovir (in rat plasma)	LLQ (10 ng/mL)	LQC (50 ng/mL)	MQC (500 ng/mL)	UQC (5000 ng/mL)
Day 1				
Mean	10.0	51.1	508.9	5055.8
%RSD	6.9	2.2	2.6	4.0
Accuracy (%)	100.0	102.2	101.8	101.1
n	6	6	6	6
Day 2				
Mean		54.8	533.9	4975.5
%RSD		3.0	3.1	2.9
Accuracy (%)		109.6	106.8	99.5
n		6	6	6
Day 3				
Mean		52.7	531.9	4453.8
%RSD		4.0	2.2	2.0
Accuracy (%)		105.4	106.4	89.0
n		6	6	6
Inter-assay				
Mean		53.0	524.9	4828.4
%RSD		3.5	2.6	6.8
Accuracy (%)		106.0	105.0	96.6
n		3	3	3

theoretical concentrations were well within the acceptance criterion of <15%. Detailed information regarding the accuracy for calibration standards is provided in Table 1.

Precision, accuracy and recovery

For the quality control samples of LLQ, LQC, MQC and UQC, the back-calculated results showed a coefficient of variation <15%. The limit of detection (LOD) for GCV in rat plasma was approximately 3 ng/mL, with a signal-to-noise ratio of 3:1. The LLQ for the assay was determined to be 10 ng/mL. The values of means, precision and accuracy for the quality control samples are listed in Table 2. The intra-assay precision for the LLQ sample was 6.9% ($n = 6$), and the intra-assay accuracy was 100.0%. During 3 days of validation, intra-assay precision for the LQC replicates ranged from 2.2 to 4.0%. For the MQC, intra-assay precision ranged from 2.2 to 3.1%. For the UQC, intra-assay precision ranged from 2.0 to 4.0%. The intra-assay accuracy values ranged from 102.2 to 109.6%, 101.8 to 106.8% and 89.0 to 101.1% corresponding to the LQC, MQC and UQC, respectively.

Inter-assay precision and accuracy were evaluated by comparing the data obtained on three validation days. Inter-assay precision ($n = 3$) ranged from 2.6 to 6.8% for the LQC, MQC and UQC. The values of inter-assay accuracy were 106.0% (LQC), 105.0% (MQC), and 96.6% (UQC). In conclusion, the above data met the acceptance criterion of <15% for the same or different validation days, which indicated that the assay was sensitive, accurate and highly reproducible for GCV analysis.

Analyte recovery was determined for the sample preparation procedure by comparing the signal response from a processed 50 μL pre-spiked (500 ng/mL) rat plasma GCV standard with that measured from a processed rat plasma sample (50 μL) to which 25 ng of GCV had been added. The recovery was found to be >88% ($n = 3$). Suppression of ionization for both GCV and ACV due to the plasma matrix was determined to be <16% following protein precipitation ($n = 3$).

Stability

Quality control samples (MQC) were subjected to three freeze/thaw cycles and subsequently measured for the GCV concentrations. Each freeze/thaw cycle consisted of thawing for 2 h at room temperature and subsequently freezing for 22 h at −80°C. The precision and accuracy were 1.7% and 94.5%, respectively ($n = 6$). These results indicated that there was no sample deterioration during the freeze/thaw process. MQC plasma samples ($n = 6$) were analyzed after being kept at −80°C for more than 2 months. Precision and accuracy values were identical with those obtained during the validation procedure. Processed samples which had been analyzed by LC/MS were left in the autosampler compartment at 15°C overnight and then re-analyzed the next day. The LC/MS results met the validation criteria for precision and accuracy. The data were essentially identical. Finally, the

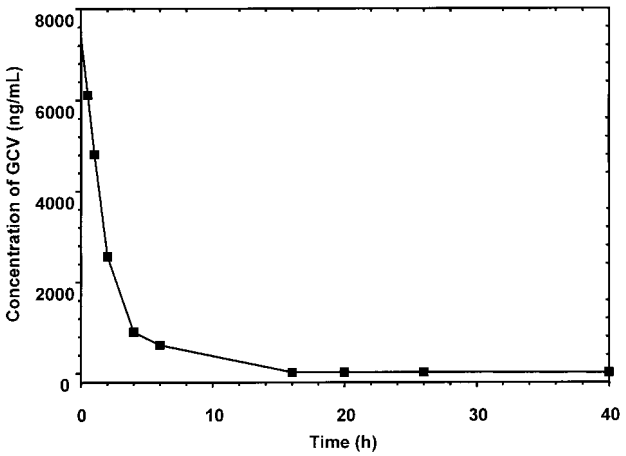

Figure 4. A typical plasma concentration–time profile showing the pharmacokinetics of GCV in normal rats following a bolus dose of GCV (10 mg/kg) in a 1 mL volume by intraperitoneal injection.

stock solutions were shown to be stable when stored at −80°C for 2 months.

Pharmacokinetic profile for normal rats dosed with GCV

The validated assay was used to analyze plasma GCV pharmacokinetics in normal rats following a bolus dose of GCV (10 mg/kg) given by intraperitoneal injection. A typical plasma GCV concentration vs time profile after drug administration is shown in Fig. 4. The experimental data were fitted using a two-compartment model. The maximum plasma concentration (C_{max}) of GCV was determined to be 7389 ng/mL. Plasma GCV concentrations decreased dramatically with time, reaching a plateau of 20 ng/mL after 16 h. Two characteristic half-lives were observed for GCV in rat plasma with $t_{1/2}\alpha$ and $t_{1/2}\beta$ of 1.1 and 4.6 h, respectively. The area under curve (AUC) was 16,143 ng/mL·h, and the volume of distribution was 1353 mL/kg. The plasma clearance was determined to be 619 mL/h/kg.

CONCLUSION

The validated LC/ESI/SRM/MS method showed the capability of selectively and sensitively quantifying GCV in rat plasma using an analog ACV as the internal standard. This method effectively eliminated chemical interference arising from the plasma matrix, and allowed a lower limit of quantitation of 10 ng/mL to be achieved. The plasma volume needed for analysis was also reduced to only 50 μL. Linearity and reproducibility were acceptable in the dynamic range 10–10,000 ng/mL. The assay has provided a reliable means to evaluate the pharmacokinetics of GCV.

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