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A rapid, sensitive HPLC method for the determination of ganciclovir in human plasma and serum ☆

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Abstract

A method for ganciclovir determination in human serum and plasma has been developed and validated. The method has a lower limit of quantification (LLOQ) adequate for sensitive pharmacokinetic studies (≤ 0.05 μg/ml), has run times of ≤ 15 min, and uses aliquot volumes adequate for pediatric studies (0.25 ml). In the method, proteinaceous material in serum or plasma is precipitated by trichloroacetic acid. An aliquot of the supernatant is analyzed by HPLC; automated column switching removes late-eluting materials that might interfere with the analyte peak in subsequent runs. Detection and quantification of ganciclovir is by fluorescence ($\lambda_{ex} = 278$ nm; $\lambda_{em} = 380$ nm). The method has a validated range of 0.0400–4.00 μg/ml and an LLOQ of 0.0400 μg/ml. All intra- and inter-assay % C.V. values were $< 8\%$; all recoveries (accuracy) were within 7% of nominal values. No interference was observed by mycophenolic acid or its glucuronide metabolite, by AZT, salicylic acid, acetaminophen, ibuprofen, naproxen prednisone, acyclovir, or cyclosporine. Ganciclovir is very stable in the samples and the extract during storage and sample processing. Both serum and plasma methods have been validated for use and have been successfully used to analyze samples from clinical studies.

Keywords: Ganciclovir; Column switching; Reverse-phase liquid chromatography; Human serum; Human plasma

1. Introduction

Ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl) guanine or DHPG, see Fig. 1) is a synthetic acyclic nucleoside analog of 2′-deoxyguanosine that has been shown to inhibit the replication of herpesviruses both in vitro and in vivo; sensitive viruses include cytomegalovirus (CMV), Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), Epstein–Barr virus, varicella–zoster virus, human herpes virus 6 (HHV-6), and hepatitis B virus. Ganciclovir has been approved for the treatment of CMV infections in immunocompromised patients, and is available for administration intra-

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venously, orally, and intra-ocularly. Since it is often co-administered with other drugs, there is strong interest in the ability to determine ganciclovir concentrations in plasma and serum samples collected from patients in clinical studies, particularly in drug interaction studies.

Our goal was to develop and validate an assay that had run times of ≤ 15 min, included simple and automatable sample processing procedures, had a lower limit of quantification (LLOQ) that would be adequate for sensitive pharmacokinetic studies (≤ 0.05 μg/ml), and had sample volume requirements that would accommodate the small sample sizes necessary for pediatric studies (0.25 ml). Existing published methods [1–10] had one or more shortcomings: inadequate LLOQ for pharmacokinetic studies, complicated or manual procedures poorly suited for routine automated sample analysis, long HPLC run times (≥ 20 min), or sample volume requirements unsuitable for pediatric studies (≥ 0.5 ml). In many cases validation data were lacking. Of particular concern to us in setting up HPLC methods was the presence of late-eluting endogenous substances that interfered with subsequent chromatograms. To achieve our goal, we therefore chose to develop and validate a method that would use a 0.25-ml sample volume, acid precipitation to deproteinate samples, and HPLC with automated column-switching to minimize potential interferences.

2. Experimental

2.1. Chemicals and supplies

Ganciclovir was obtained from Roche Bioscience, Palo Alto, CA (formerly Syntex Research). Trichloroacetic acid (TCA) and 1-heptanesulfonic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Granular sodium sulfate and concentrated sulfuric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI), and HPLC grade acetonitrile was purchased from VWR Scientific (San Francisco, CA). Samples of serum and heparinized human plasma (individual and pooled) from normal, healthy volunteers were obtained from the American Red Cross (Santa Clara, CA). Water was purified by a Milli Q system (Millipore Corp., Marlborough, MA).

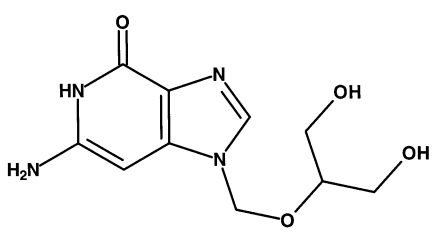

Fig. 1. Chemical structure of ganciclovir.

Aqueous TCA (15%, w/v), the HPLC mobile phase, which was acetonitrile–sodium sulfate/H_2SO_4 (pH 2.4, 0.025 M, containing 0.008 M 1-heptanesulfonic acid) (3:97, v/v), and the HPLC wash solution, which was acetonitrile–H_2O (80:20, v/v), were prepared in house.

2.2. Instrumentation

The HPLC system used for the validation tests consisted of three LC-10AD pumps, an SCL-10A system controller, an SIL-10AXL autoinjector, an RF-10A fluorescence detector (all from Shimadzu Scientific Instruments, Pleasanton, CA); a Model 732 column switching device (Micromeritics, Norcross, GA); and an Eppendorf CH-30 column heater and an Eppendorf TC-50 column-temperature controller (VWR Scientific, San Francisco, CA). The capture column was a 5-cm long, 4.6-mm i.d. Supelcosil LC-8 (5-μm) guard column (Supelco, Bellefonte, PA). The analytical column used for plasma was an HAIsil 120, BD, C18, 5-μm, 250×4.6-mm analytical column (Higgins Analytical, Inc., Mountain View, CA); that used for serum was a Keystone, BDS Hypersil, C18, 5-μm, 150×4.6-mm analytical column (Keystone Scientific, Bellefonte, PA). The centrifuge used was a Jouan CR422 refrigerated centrifuge (Jouan, Inc. Winchester, VA), and the data acquisition system used was a Justice Innovations Chrom Perfect system (Justice Innovations, Mountain View, CA).

2.3. Solution and sample preparation

2.3.1. Stock and spiking solutions

A primary aqueous stock solution of ganciclovir (1.00 mg/ml) was prepared and diluted further with water to prepare secondary stock solutions at 100 and 10.0 μg/ml. These secondary stock solutions were diluted further with water to produce spiking solutions with ganciclovir concentrations of 0.100, 0.200, 0.300, 0.500, 0.750, 1.00, 1.50, 2.00, 5.00, and 10.0 μg/ml. These spiking solutions were used to prepare the calibration standards at the time of the assay. Separate primary and secondary solutions at the above concentrations were used for preparation of QC samples.

2.3.2. QC samples

To prepare the QC samples, appropriate volumes of the secondary stock solutions were diluted to 50.0 ml with blank serum or plasma in a volumetric flask and stirred magnetically at room temperature for 1 h to give final ganciclovir concentrations of 0.0800 (Low QC), 0.450 (Mid QC), and 3.20 μg/ml (High QC). These bulk QC preparations were apportioned into 0.60-ml aliquots in screw-capped polystyrene tubes and stored frozen at −20°C before use. For each batch of study samples analyzed, two vials of each Low, Mid, and High QC were thawed and analyzed. An additional QC sample with a ganciclovir concentration at the lower limit of quantification (LLOQ, 0.0400 μg/ml) was similarly prepared, but analyzed only during the validation process in order to determine the precision and accuracy at the LLOQ.

2.3.3. Preparation of calibration standards

For preparation of the calibration standards used for generation of the standard curve and for validation of the method, 0.100-ml aliquots of the spiking solutions were added to 11 × 92-mm polypropylene tubes that each contained a 0.250-ml aliquot of pooled blank human serum, or plasma, previously thawed and centrifuged at 2000 × *g* for 10 min. The tubes were vortexed 15 s to mix the contents. This procedure generated a set of ten calibration standards with concentrations of equivalent to 0.0400, 0.0800, 0.120, 0.200, 0.300, 0.400, 0.600, 0.800, 2.00, and 4.00 μg/ml ganciclovir of serum or plasma. These ten calibration standards were used to generate a standard curve; additional calibration standards were prepared and analyzed for validation runs to collect precision and accuracy data.

2.3.4. Extraction of calibration standards

To each calibration standard tube was added 0.125 ml of 15% aqueous TCA. The tubes were then capped, vortexed for 15 s, and centrifuged (2000 × *g*) for 10 min. Aliquots (300 μl) of the supernatants were transferred to HPLC injection vial inserts, which were placed in injection vials. The vials were capped and placed on the HPLC autoinjector. For analysis, 40 μl of supernatant was injected into the HPLC system.

2.3.5. Preparation of and extraction of QC samples and unknown samples

Frozen study samples and QC samples were thawed at room temperature and then centrifuged at 2000 × *g* for 10 min. The sample matrix (serum or plasma) matched the matrix used for preparation of calibration standards. Aliquots (250 μl) of the samples were transferred by pipette to 11 × 92-mm polypropylene tubes. Water (100 μl) was added to each tube, and the tubes were vortexed for 15 s. The same procedures described above for the extraction (addition of 0.125 ml of 15% TCA) and HPLC injection of calibration standards were also applied to the diluted samples.

2.3.6. Chromatography with column switching

The HPLC system used is depicted in Fig. 2. It consisted of three HPLC pumps, an autoinjector, and a switching valve, all operated by a system controller. Pumps A, B, and C were connected to reservoirs containing HPLC mobile phase, water, and HPLC wash phase, respectively. The system also included a capture column, an analytical column, and a fluorescence detector ($\lambda_{ex}/\lambda_{em}$: 278/380 nm). A column heater maintained at 30°C (slightly above ambient temperature) maintained a constant analytical column temperature. The cycle begins with the switching valve in Position I (see Fig. 2). The sample is injected and eluted by

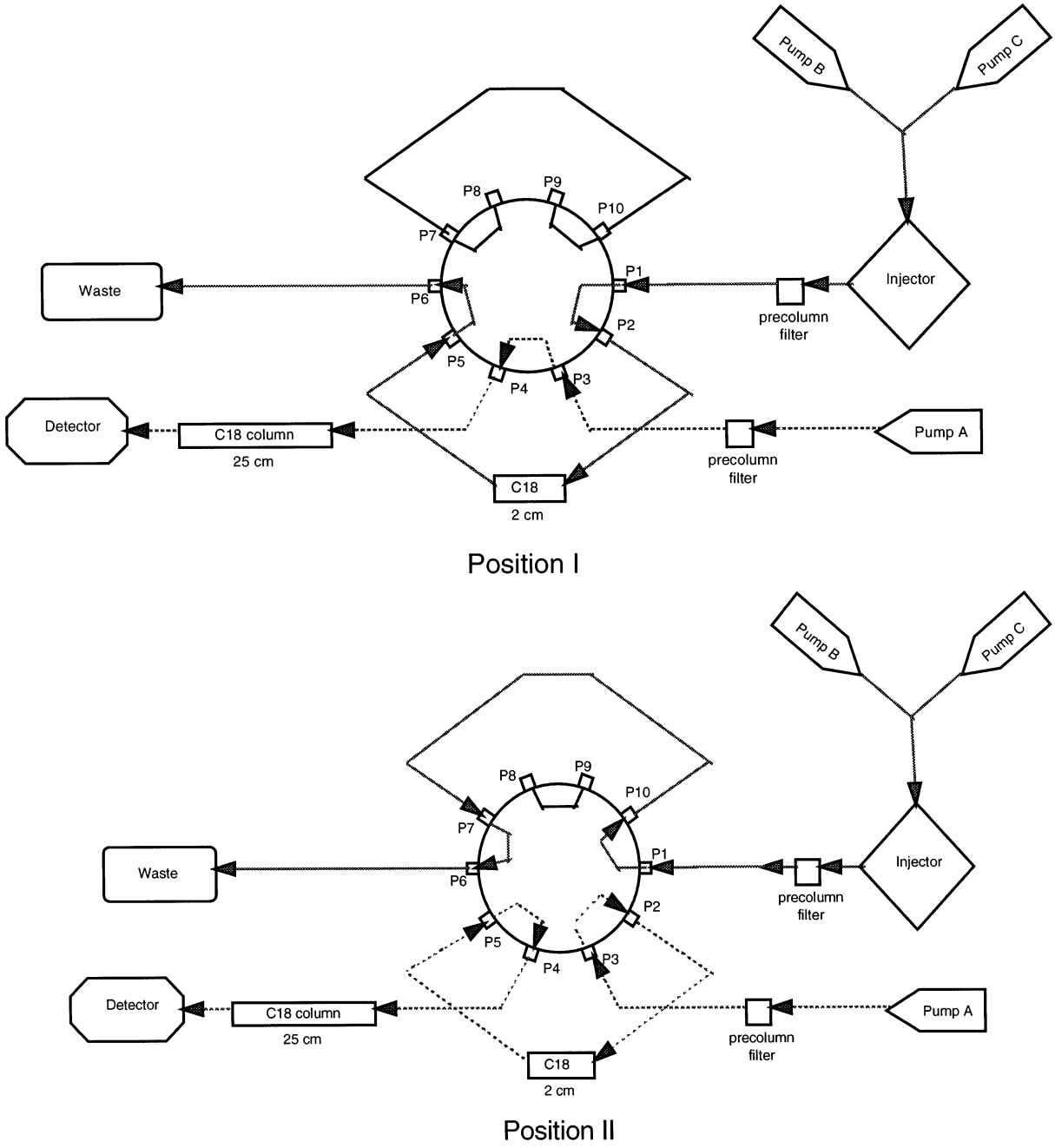

Fig. 2. Diagram of column-switching HPLC System for ganciclovir determination. Upper diagram is Position I; lower diagram is Position II.

H_2O from Pump B onto the capture column. The valve then switches to Position II, whereupon ganciclovir in the sample is eluted by HPLC mobile phase (Pump A) from the capture column onto the analytical column. The valve then switches back to Position I, and while ganciclovir

from the sample is eluted through the analytical column by HPLC mobile phase (Pump A); Pump C is activated, Pump B is shut off, and slower-moving materials are eluted from the capture column by HPLC wash solution and sent to waste. Finally, as Pump A elutes the ganciclovir off the analytical column and through the detector, Pump C is shut off and Pump B is reactivated to re-equilibrate the capture column with H_2O. This sequence of events is shown in Table 1.

2.4. Calculations

The peak area vs. concentration data for the calibration standards were fit by linear regression with weighting factor $W = 1/\text{concentration}$ to generate a calibration curve equation of the form peak area $= m(\text{concentration}) + b$.

2.5. Validation

2.5.1. Precision and accuracy

To assess the precision and accuracy of the method, five aliquots of each of the QC samples were analyzed in each of three assay runs. Also, two calibration standards at each concentration, in addition to those used to generate the standard calibration curve, were prepared and analyzed in each of four runs. The data were analyzed by EXCEL5.0 ANOVA: Single Factor to generate intra-assay and interassay coefficients of variation expressed as percentages (% C.V.). Intermediate precision, also expressed as a % C.V., was calculated as the square root of the sum of the intra-assay and interassay variances. Accuracy was assessed by evaluation of mean recovery, defined as the ratio of mean found concentration to nominal concentration, expressed as a percentage.

2.5.2. Procedural recovery and stability

Procedural recovery of the extraction procedure in serum and plasma was assessed by replicate analysis ($n = 5$) of the Low QC and the High QC; results were compared to those obtained by injection of aqueous solutions of ganciclovir prepared at the same ganciclovir concentrations as the nominal concentrations of corresponding QC samples. Both extracted QC samples and aqueous standards were injected onto the capture column by the standard injection procedure described for the method. Stability of ganciclovir in frozen serum was determined by periodic analysis of QC samples stored at -20°C. Stability of ganciclovir in thawed serum or in serum extracts was evaluated by replicate analysis of the Low QC and High QC after storage refrigerated at 4°C for 0, 24, and 48 h, after storage at room temperature (≈ 22°C) for 0, 24, and 48 h, after storage of the

Table 1
Sequence of HPLC actions and switching events used for ganciclovir determination

Time (min)	Valve position	Flow rate (ml/min)			Action
		Pump A[a]	Pump B[b]	Pump C[c]	
0.00	I	1.00	0.25	0	Load sample with H_2O onto capture column
1.20	II	1.00	0.25	0	Switch to Position II to connect capture column with analytical column.
2.60	I	1.00	0.25	0	Switch to Position I to divert late-eluting peaks to waste.
3.00		1.00	0	1.00	Begin wash of capture column
6.10		1.00	1.50	0	End wash, begin re-equilibration of capture column.
11.90		1.00	0.25	0	Reduce Pump B flow rate
12.50		1.00	0.25	0	End cycle, start new injection

[a] HPLC mobile phase (acetonitrile–sodium sulfate/H_2SO_4 (pH 2.4, 0.025 M, containing 0.008 M 1-heptanesulfonic acid) [3:97, v/v]).
[b] H_2O.
[c] HPLC wash phase (acetonitrile–H_2O [80:20, v/v]).

corresponding extracts at 4°C for 0, 24, 48, 96, and 168 h, after storage of the extracts on instrument for 0 and 17 h, after storage of the extracts at 22–24°C (ambient temperature) for 0 72, and 144 h, and after subjecting the QC samples to one or three cycles of freeze and thaw. Stability of ganciclovir in plasma and plasma extracts was evaluated similarly with QC samples, except that stability of ganciclovir in plasma extracts was assessed at 4°C up to 48 h and at room temperature ($\approx$ 22°C) up to 72 h.

3. Results and discussion

3.1. Sample preparation and chromatography

The method that was developed and validated used TCA to precipitate proteins from a 0.250-ml sample of serum or plasma. To avoid deleterious effects of overloading the column with TCA, the injection volume was held at 40 μl. For ganciclovir, which is administered at relatively high doses and is present in plasma at relatively high concentrations, the injection volume was adequate, since achievement of very low quantification limits is not critical. Detection and quantification of ganciclovir was by fluorescence. The method used external standards, and no internal standard was used. The use of automated column switching techniques removed late-eluting materials that otherwise would have lengthened the sample run time or interfered with subsequent sample analyses. 1-Heptanesulfonic acid has often been used as a mobile phase ion-pairing agent at approximately 8 mM for HPLC bioanalytical methods in our lab and at commercial contract labs. This concentration gave adequate retention of ganciclovir on the HPLC column, and no additional concentrations were investigated.

The method was validated for both serum and plasma by use of the appropriate calibration standards and QC samples matched to the matrix of the samples. The method for plasma was validated first, and with the 250-mm column used, the ganciclovir retention time was approximately 10.8 min, which corresponded to a capacity factor, K', of 1.7. K' is defined as $(t_r - t_0)/t_0$, where t_0 and t_r are respective retention times for an unretained peak and the peak of interest. When the method was later validated for serum, a shorter (150-mm) column was used and resulted in a correspondingly shorter ganciclovir retention time (approximately 7.2 min) and K' of 0.80. The original timing of the automated programming steps was retained, however, so that both serum and plasma methods could be run on the same equipment without the need to alter the programming. However, those who set up the method in other labs may potentially reduce the run time further by using the shorter column and beginning the capture wash and reequilibration steps earlier. A reduction of run time from 11 to 7.3 min corresponds to a 50% increase in analyses per time unit. It also represents a reduction in almost 1 h in the time needed to analyze a single sample in a complete run containing ten standards and six QCs.

3.2. Range and linearity

The method was validated for the range of ganciclovir concentrations from 0.0400 to 4.00 μg/ml for both serum and plasma. For both matrices, the signal-to-noise ratio at the LLOQ (0.0400 μg/ml), was >10. To test the validity of the linear regression model, we performed a statistical lack-of-fit test on the data. The linear model adequately describes the calibration curve, as evidenced by the lack of departures from linearity observed in the quantitations (lack-of-fit $P = 0.7889$ for serum, and $P = 0.3568$ for plasma). The weighting factors were chosen to be proportional to the reciprocal of the SD, which is optimal under least square estimation. The coefficient of variation is constant over the range of the data, which implies that the SD is proportional to the nominal concentration, or equivalently, to the assay response (peak area). The slopes (m), intercepts (b), and coefficients of determination (r^2) obtained for the linear regression equations determined during the validation for serum and plasma are presented in Table 2. All values of r^2 were ≥ 0.997.

Table 2
Summary of parameters for the regression equations, peak area = m(concentration) + b, determined for runs analyzed during the validation of serum and plasma ganciclovir methods

Serum				Plasma			
Run	m	b	r^2	Run	m	b	r^2
1	3.01×10^6	−12900	0.9999	1	2.52×10^6	10400	0.9982
2	3.02×10^6	−28500	1.0000	2	2.57×10^6	−17400	0.9996
3	3.01×10^6	−21600	0.9999	3	2.56×10^6	4000	0.9986
4	3.04×10^6	−7900	0.9998	4	2.63×10^6	8100	0.9989
5	2.93×10^6	4460	0.9995	5	2.72×10^6	21800	1.0000
6	3.22×10^6	−14900	0.9999				
7	3.26×10^6	11300	0.9968				

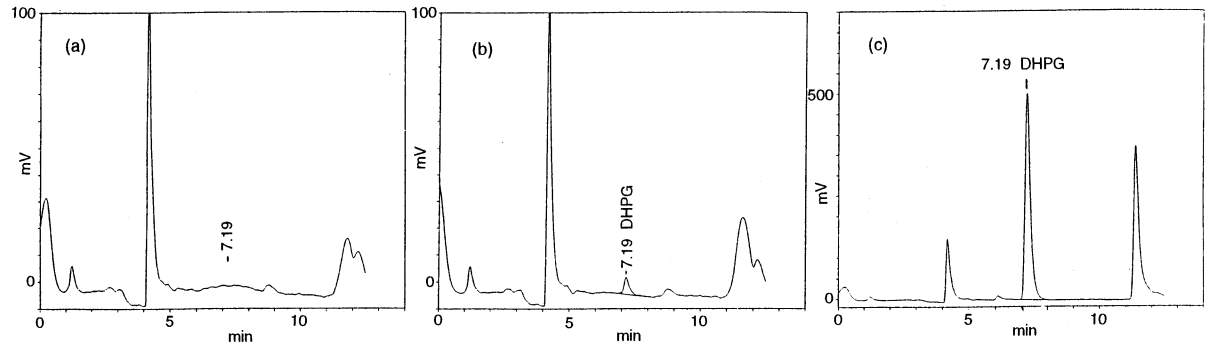

Fig. 3. Chromatograms from analysis of ganciclovir (labeled as DHPG) in human serum. (a) blank human serum, (b) serum LLOQ QC (0.0400 μg/ml), (c) serum High QC (3.20 μg/ml).

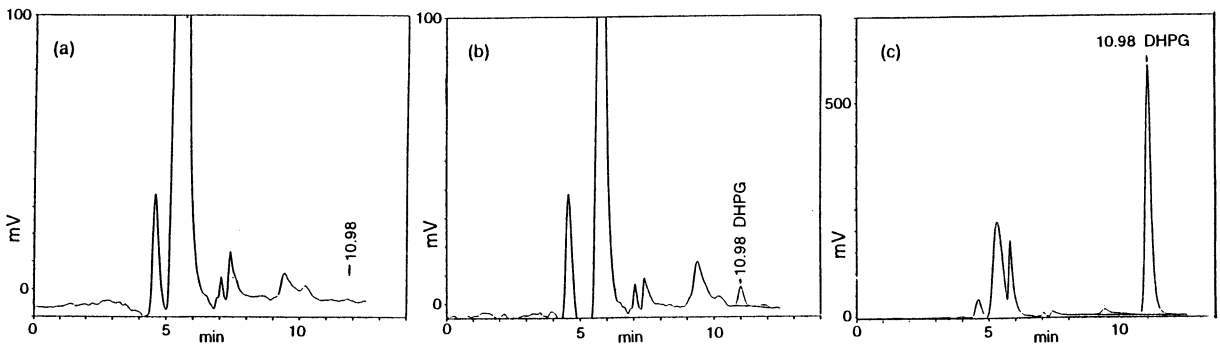

Fig. 4. Chromatograms from analysis of ganciclovir (labeled as DHPG) in human plasma. (a) blank human plasma, (b) plasma LLOQ QC (0.0400 μg/ml), (c) plasma High QC (3.20 μg/ml).

3.3. Specificity

The analysis of blank human serum from six individuals and plasma from nine individuals showed no interfering peaks at the retention time of ganciclovir. Representative chromatograms for serum and for plasma are shown in Figs. 3 and 4. In addition, no chromatographic interference was

observed from the following potentially co-administered drugs or their metabolites: aspirin, salicylic acid, acetaminophen, ibuprofen, naproxen, AZT, acyclovir, cyclosporine, prednisone, mycophenolic acid, or mycophenolic acid glucuronide.

3.4. Precision and accuracy

The precision of the method for both serum and plasma was assessed by determination of the intra-assay and inter-assay coefficients of variation (% C.V.) of the method from five replicates of each QC sample in each of three runs, and from two replicates of each calibration standard in each of four runs. These calibration standards were separate from the set used to generate a standard curve. The accuracy of the method for both serum and plasma was evaluated for both QC samples and calibration standards by the mean recovery, defined as the ratio of the mean found concentration to the nominal concentration, expressed as a percentage. Summaries of precision and accuracy data for calibration standards and QCs are presented in Table 3 for serum and Table 4 for plasma. For both serum and plasma, all % C.V. values were $\leq 9.38\%$ for QC samples and $\leq 9.52\%$ for calibration standards. All mean recoveries ranged from 97.4 to 105% for QCs and from 94.0 to 110% for calibration standards.

3.5. Absolute recovery

Absolute recovery of ganciclovir for both serum and plasma by the extraction process was performed by comparison of peak areas from extracted Low and High QC samples to those from aqueous standards. Absolute recoveries for serum and plasma ranged from 97.9 to 103%. These results indicated essentially complete recovery of ganciclovir during the extraction.

3.6. Stability

Stability studies of ganciclovir in serum indicated no changes in ganciclovir concentrations upon storage of the samples for 48 h at either 4°C or room temperature (≈ 22°C); upon storage of serum samples frozen for 20 months at -20°C; upon storage of serum sample extracts for 7 days at 4°C, for 6 days at room temperature (≈ 22°C), or for 17 h at ambient temperature on-instrument; or upon subjecting the samples to up to three cycles of freeze and thaw. Stability studies of ganciclovir in plasma indicated no changes in ganciclovir concentrations upon storage of the samples for 48 h at either 4°C or room temperature (≈ 22°C); upon storage of samples frozen at -20°C for at least 2 months; upon storage of plasma sample extracts for 48 h at 4°C or for 72 h on instrument at room temperature (≈ 22°C); or upon subjecting the samples to up to three cycles of freeze and thaw.

4. Conclusions

A simple HPLC method with automated column switching has been developed and validated for determination of ganciclovir in human serum and plasma. Run times are 12.5 min and could potentially be shortened further. The validated range and LLOQ (0.0400 μg/ml) of the method is suitable for pharmacokinetic and drug interaction studies. Endogenous substances and a wide range of potentially co-administered substances and their metabolites do not interfere with ganciclovir quantification. The use of a small sample volume (0.250 ml) makes the method suitable for pediatric studies. The method has been set at both at our research site (Roche Bioscience) and at a commercial contract laboratory (PHARMout Laboratories). Several thousand study samples have been analyzed, and assay performance has been comparable at both locations.

Acknowledgements

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Table 3
Human serum: Intra- and inter-assay precision and accuracy data for determination of ganciclovir

	Nominal conc. (μg/ml)	Number of runs	Replicates per run	Mean conc. found (μg/ml)	C.V. (%)			Mean recovery (%)	LCL (%)	UCL (%)
					Intra-assay	Inter-assay	Intermediate precision			
Calibration Standards[a]										
STD 1	0.0400	4	2	0.0440	5.76	NS	5.76	110	104.7	115.3
STD 2	0.0800	4	2	0.0821	2.05	2.32	3.10	103	99.7	106.3
STD 3	0.120	4	2	0.124	3.18	5.42	6.28	103	95.8	110.2
STD 4	0.200	4	2	0.204	9.52	NS	9.52	102	93.9	110.1
STD 5	0.300	4	2	0.299	6.80	NS	6.80	99.6	93.9	105.3
STD 6	0.400	4	2	0.397	7.90	NS	7.90	99.1	92.5	105.7
STD 7	0.600	4	2	0.599	7.34	NS	7.34	99.8	91.7	107.9
STD 8	0.800	4	2	0.781	5.17	NS	5.17	97.7	93.5	101.9
STD 9	2.00	4	2	1.93	3.83	NS	3.83	96.6	93.5	99.7
STD 10	4.00	4	2	3.76	3.54	NS	3.54	94.0	91.2	96.8
QC samples										
LLOQ QC	0.0400	3	5	0.0403	7.32	4.05	8.37	101	94.5	107.5
Low QC	0.0800	3	5	0.0813	3.17	6.55	7.28	102	93.6	110.4
Mid QC	0.450	3	5	0.444	4.87	3.70	6.12	98.6	93.4	103.8
High QC	3.20	3	5	3.12	5.10	2.43	5.65	97.4	93.4	101.4

[a] These calibration standards were separate from the set used to generate the calibration curve. NS: Not significant compared to intra-assay value. LCL and UCL are lower and upper 95% confidence limits of the mean.

Table 4
Human plasma: Intra- and inter-assay precision and accuracy data for determination of ganciclovir

	Nominal conc. (μg/ml)	Number of runs	Replicates per run	Mean conc. found (μg/ml)	C.V. (%)			Mean recovery (%)	LCL (%)	UCL (%)
					Intra-assay	Inter-assay	Intermediate precision			
Calibration Standards[a]										
STD 1	0.0400	4	2	0.0415	3.77	5.26	6.47	104	96.8	111.2
STD 2	0.0800	4	2	0.0859	3.18	2.57	4.09	107	102.7	111.3
STD 3	0.120	4	2	0.121	5.72	NS	5.72	101	96.2	105.8
STD 4	0.200	4	2	0.200	3.54	NS	3.54	100	97.0	103.0
STD 5	0.300	4	2	0.297	2.87	2.10	3.56	99.0	95.6	102.4
STD 6	0.400	4	2	0.393	4.86	NS	4.86	98.3	94.3	102.3
STD 7	0.600	4	2	0.593	3.10	NS	3.10	98.8	96.2	101.4
STD 8	0.800	4	2	0.793	1.14	1.35	1.77	99.2	97.4	101.0
STD 9	2.00	4	2	1.99	1.15	NS	1.15	99.3	98.3	100.3
STD 10	4.00	4	2	3.90	1.78	0.972	2.03	97.5	95.7	99.3
QC samples										
LLOQ QC	0.0400	3	5	0.0418	7.08	6.15	9.38	105	96.0	114.0
Low QC	0.0800	3	5	0.0817	4.94	2.81	5.68	102	97.5	106.5
Mid QC	0.450	3	5	0.440	2.32	NS	2.32	97.8	96.5	99.1
High QC	3.20	3	5	3.15	3.04	0.938	3.18	98.3	96.3	100.3

[a] These calibration standards were separate from the set used to generate the calibration curve. NS: Not significant compared to intra-assay value. LCL and UCL are lower and upper 95% confidence limits of the mean.

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