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An HPLC method for the determination of diastereomeric prodrug RS-79070-004 in human plasma☆

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Abstract

Ganciclovir is an antiviral nucleoside analogue approved for treatment and prevention of cytomegalovirus infections in immunocompromised subjects. RS-79070-194, a diastereomeric monovalyl ester of ganciclovir (hydrochloride salt), is under evaluation as a prodrug to increase the bioavailability of ganciclovir. An HPLC method with column switching has been developed and validated for quantification of the corresponding free base RS-79070-004 in human plasma. In the method, proteinaceous material in 0.25 ml of plasma is precipitated by trichloroacetic acid. An aliquot of the supernatant is analyzed by HPLC, with automated column switching to remove late-eluting materials that might interfere with the analyte peaks in subsequent runs. Detection of RS-79070-004 is by UV ($\lambda = 254$ nm). The peak areas for each isomer are summed to generate a value for total RS-79070-004. The method has a validated range of 0.0400–4.00 μg/ml and a lower limit of quantification of 0.0400 μg/ml. All intra- and inter-assay %CVs were $< 7.5\%$, and all recoveries (accuracy) were within 6% of nominal values. No interference was observed by ganciclovir, caffeine, acetaminophen, or ibuprofen. Analyte stability in plasma and in the sample extracts is adequate for the specified collection, storage, and analysis conditions. The validated method has been successfully used to analyze clinical study samples.

Keywords: Ganciclovir; Column switching; Prodrug; Reverse-phase liquid chromatography; Diastereomer

1. Introduction

RS-79070-194, the hydrochloride salt of the mono-L-valylester of ganciclovir (free base: RS-79070-004, see Fig. 1), is under evaluation as a prodrug to increase the limited oral bioavailability (6–10%) [1,2] of ganciclovir. 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 herpes viruses both in vitro and in vivo, 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 RS-79070-194 exists as a mixture of two diastereomers (see Fig. 1), and is administered as an approximately 1:1 mixture of these diastereoisomers, a method for quantification of the total (sum of both diastereomers) prodrug was desired for analysis of samples from clinical pharmacokinetic and drug interaction studies. We also needed to determine sample storage and assay conditions that would preserve the sensitive ester function of the ganciclovir prodrug. Our goal was to develop and validate a method for quantification of total prodrug as free base (RS-79070-004) in human plasma by HPLC with column switching. The method that was developed and validated is reported here.

2. Experimental

2.1. Chemicals and supplies

The following chemicals, reagents and disposables were used (US source in parentheses, unless otherwise indicated): RS-79070-194 and ganciclovir (Roche Bioscience, Palo Alto, CA); caffeine, ibuprofen, acetaminophen, and 100% w/v, 6.1 N trichloroacetic acid (TCA) (Sigma, St Louis, MO); UV high-purity grade acetonitrile and methanol (Burdick and Jackson, Muskegon, MI); 85% aqueous phosphoric acid and concentrated (38%, 12 M) hydrochloric acid (Mallinckrodt, St. Louis, MO); blank plasma and heparinized whole human blood collected from healthy subjects (Roche Bioscience blood collection center, Palo Alto, CA); 1.5-ml disposable conical, screw-cap micro centrifuge tubes (Fisher Scientific, Pittsburgh, PA); 16 × 100-mm disposable screw-cap test tubes (Baxter Healthcare Corp., McGaw Park, IL).

Water was purified in-house by a Milli-Q system (Millipore Corp., Bedford, MA).

15% (w/v) aqueous trichloroacetic acid, 0.0425% (w/v) aqueous phosphoric acid, 0.001 M HCl, the HPLC mobile phase, which was acetonitrile–0.0425% aqueous phosphoric acid (5:95, v/v), and HPLC wash solution, which was methanol–H_2O (80:20, v/v) were prepared in-house.

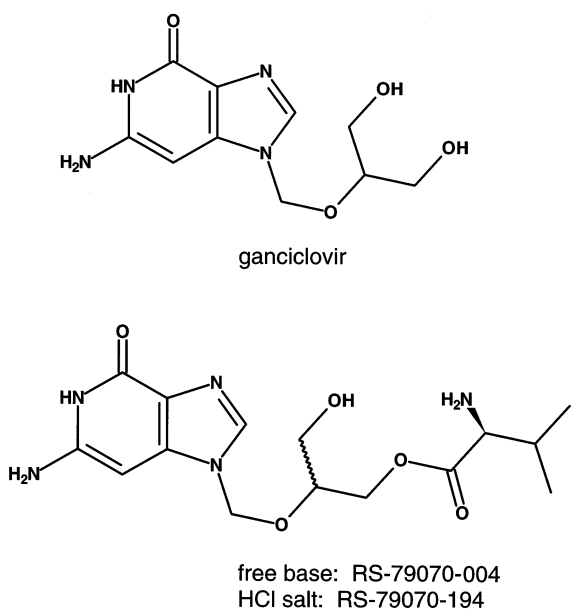


Fig. 1. Structures of RS-79070-194, RS-79070-004, and ganciclovir.

2.2. Instrumentation

A 250-μl Gilson Microman digital positive displacement micropipet and disposable 250-μl tips (Rainin Instruments, Woburn, MA) were used for pipetting plasma and methanolic solutions. An Eppendorf Repeater Pipetter (Baxter Healthcare Corp., McGaw Park, IL) and disposable 5.0- and 12.5-ml combitips (Baxter Healthcare Corp., McGaw Park, IL) were used for pipetting aqueous solutions. A refrigerated ModelTJ-6 centrifuge and a Microfuge E, (Beckman Instruments, Palo Alto, CA) were used for centrifugation. The HPLC system consisted of a Rainin HPLX ternary HPLC (Rainin Instruments Co., Woburn, MA); a Model 4000 autosampler and a Model L-4000 UV detector (Hitachi LTD, Tokyo, Japan) set at 254 nm; an electrically actuated, high-pressure 10-port switching valve (Valco, Houston, TX); a precolumn solvent filter with a 2.0-μm frit (Upchurch Scientific, Oak Harbor, WA), and a 20 × 4.6-mm i.d., 5-μm C18 BDS Hypersil column (capture column) and a 250 ×

4.6-mm ID, 5-μm C18 BDS Hypersil column (analytical column) (Keystone, Bellefonte, PA).

Data collection and calculations were performed with the Nelson 6000 Laboratory Data System (Nelson Analytical, Cupertino, CA) on an HP-1000 computer (Hewlett Packard, Palo Alto, CA).

2.3. Solution and sample preparation

2.3.1. Stock and spiking solutions

A standard Stock Solution A of RS-79070-194 was prepared by dissolving a weighed amount of RS-79070-194 in the appropriate volume of 0.001 M HCl to give a 0.500 mg/ml solution. The concentration was verified by UV ($E_{1\ cm}^{1\%} = 337.3$ at 254 nm) by dilution of stock solution A to 0.0500 mg/ml with methanol and determining the absorbance at 254 nm. Solutions were acceptable for use if they gave absorbances within 5% of theoretical. For Stock Solution B, 2.208 ml of Stock Solution A was diluted with 0.001 M HCl to 10.0 ml in a volumetric flask to give a solution with a concentration of 100 μg/ml in terms of free base RS-79070-004. Stock Solution C was prepared by dilution of Stock Solution B with 0.001 M HCl to give a 10.0 μg/ml solution of free base RS-79070-004. A set of 10 spiking solutions for preparation of calibration standards was prepared by dilution of Stock Solutions B and C with 0.001 M HCl. to give RS-79070-004 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. A separate set of Stock Solutions at the same concentrations were prepared from a separate initial weighing for preparation of Quality Control (QC) samples. The Stock Solutions and spiking solutions were stored at 4°C for up to 4 months in 16 × 100-mm screw-top test tubes (Baxter Healthcare Corp., McGaw Park, IL).

2.3.2. QC Samples

Appropriate volumes (0.08–0.64 ml) of Stock Solutions B or C were diluted to 20.0 ml with blank plasma in an ice bath followed by 10 min of magnetic stirring to give LLOQ QC, Low QC, Mid QC, and High QC samples with RS-79070-004 concentrations of 0.0400, 0.0800, 1.60, and 3.20 μg/ml, respectively. The bulk QC samples were apportioned at once into approximately 1-ml aliquots in screw-top polypropylene tubes, which were immediately frozen at −80°C. For each batch of study samples analyzed, two vials of each Low, Mid, and High QC were thawed and analyzed. The LLOQ QC was analyzed only during the validation process in order to determine the precision and accuracy at the LLOQ.

2.3.3. Preparation and extraction of calibration standards

For preparation of the calibration standards used for generation of the standard curve and for validation of the method, all reagents were chilled in an ice bath before use and calibration standard tubes were set up in an ice bath as well. Aliquots (0.100-ml) of cold 15% TCA solution were added by pipette to into 1.5-ml conical microfuge tubes set in an ice bath. Aliquots (100 μl) of the cold spiking solutions were added to the tubes, followed by 250-μl aliquots of cold blank plasma, to generate a set of calibration standards with nominal concentrations of RS-79070-004 equivalent to 0.040, 0.0800, 0.120, 0.200, 0.300, 0.400, 0.600, 0.800, 2.00, and 4.00 μg/ml of plasma. For validation purposes, additional calibration standards were prepared and analyzed in addition to those used to generate the standard curve. The tubes were capped, vortexed for 15 s to mix the contents, and centrifuged at 4°C in a microfuge at 15,000 × *g* for 5 min. Supernatants were transferred to autosampler vials, and an 80-μl aliquot of each was injected into the HPLC system.

2.3.4. Preparation and extraction of QC samples and unknown samples

Reagents and equipment were set up and chilled as for calibration standards (see above). Plasma samples and QC samples were thawed at room temperature, and immediately vortexed (5–10 s) and centrifuged (2000 g) at 4°C after thawing. To a 100-μl aliquot of cold 15% TCA solution in a 1.5-ml conical microfuge tube set in an ice bath was added 100 μl of 0.001 M HCl solution followed by 250 μl of the plasma or QC sample. The tubes were capped, vortexed for 15 s to mix the contents, and centrifuged at 4°C in a microfuge at 13,000 rpm for 5 min. Supernatants

Table 1
Sequence of HPLC actions and switching events used for RS-79070-004 determination

Time (min:s)	Valve position	Pump Flow Rate (ml/min)			Action
		A[a]	B[b]	C[c]	
0.00	I	1.3	0	1.3	Sample injected and delivered with H_2O onto capture column, faster-eluting materials eluted to waste, RS-79070-004 retained.
2:16	II	0.5	0	0	Valve switches to Position II; RS-79070-004 is eluted in reverse direction from capture column onto analytical column by HPLC mobile phase.
2:47	I	1.3	1.3	0	Valve switches back to Position I; HPLC mobile phase elutes RS-79070-004 through analytical column to detector while HPLC wash solution washes capture column.
5:31	I	1.3	0	1.3	As elution of RS-79070-004 on analytical column continues, reequilibration of capture column in initial (forward) direction with H_2O begins.
9.45	I	1.3	0	1.3	End cycle, start next sample injection.

[a] HPLC mobile phase (acetonitrile–0.0425% aqueous phosphoric acid [5:95, v/v]).
[b] HPLC wash solution (methanol–H_2O [80:20, v/v]).
[c] H_2O.

were transferred to autosampler vials, and an 80-μl aliquot of each was injected into the HPLC system.

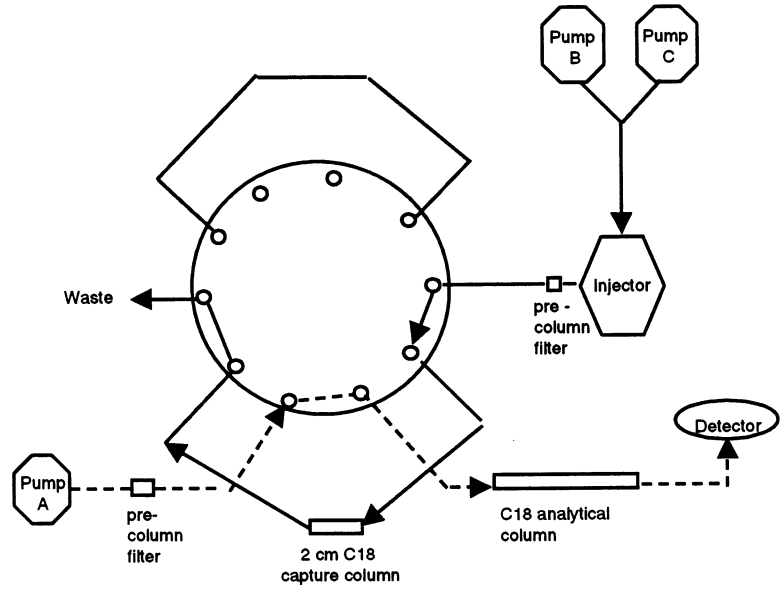
2.4. Chromatography with column switching

The HPLC system used is depicted in Fig. 2. Pumps A, B, and C were connected to reservoirs containing HPLC mobile phase, HPLC wash solution, and water, respectively. The system also included a guard column, a capture column, an analytical column, and a UV detector ($\lambda = 254$ nm). The run cycle begins with the switching valve in Position I (see Fig. 2). Upon injection, the sample extract is delivered onto the capture column by water from Pump C, while Pump B is inactive and HPLC mobile phase delivered by Pump A flows through the analytical column at 1.3 ml/min. Fast-eluting materials exit the capture column and are sent to waste. At 2:16 min the valve switches to Position II, Pump C is shut off, and HPLC mobile phase is delivered by Pump A at a reduced rate of 0.5 ml/min to elute RS-79070-004 (in the reverse direction from which it was captured) onto the analytical column. At 2:47 min the valve switches to position I, HPLC mobile phase flow from Pump A increases to 1.3 ml/min, and Pump B is activated to deliver HPLC wash solution at 1.3 ml/min. RS-79070-004 is eluted through the analytical column by HPLC mobile phase, and late-eluting materials are washed in the initial direction from the capture column to waste by HPLC wash solution. At 5:30 min, while HPLC mobile phase continues to elute RS-79070-004 at 1.3 ml/min through the analytical column to the detector, Pump B is shut off and Pump C is reactivated to reequilibrate the capture column with water at 1.3 ml/min. At 9:45 min the cycle begins again with the next injection. These steps are summarized in Table 1.

The integration window for RS-79070 was approximately 7.6–9.1 min, and was checked and reset if needed at the beginning of a run.

2.5. Calculations

The peak area vs. concentration data for the eight calibration standards from 0.040 to 0.800 μg/ml were fit by unweighted linear regression with the Nelson 6000 Data System to generate a calibration curve equation of the form peak area $= m$(concentration) $+ b$. The peak area was the total integrated area for the time interval that contained the two peak areas that corresponded



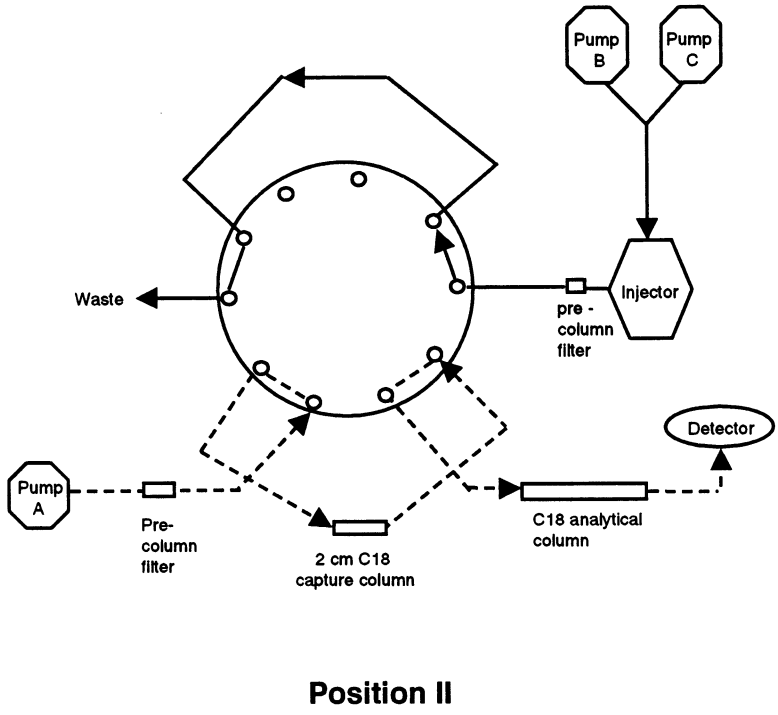


Fig. 2. Diagram of the column-switching HPLC system used for RS-79070-004 determination. Upper diagram shows switching valve in Position I; lower diagram shows the valve in Position II.

to the two RS-79070-004 diastereomers. The Nelson 6000 Data System was incapable of weighted linear regression; to avoid the dominating effect of upper points on the lower end of a curve generated by unweighted linear regression, and to minimize the need to for sample dilution to achieve concentrations within a narrow curve range, the calibration standards at 2.00 and 4.00 μg/ml were used as verification standards in each run to verify extrapolations of the curve up to 4.00 μg/ml. A verification standard had to be within 10% of its nominal value in order to quantify sample concentrations for that run in the extrapolation range.

2.6. Validation

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 and verification 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 within-run and between run 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 recovery, defined as the ratio of mean found concentration to nominal concentration, expressed as a percentage.

2.6.1. Procedural recovery and stability

Procedural recovery of the extraction procedure was determined in triplicate by spiking known amounts of RS-79070-004 into blank plasma to give samples with RS-79070-004 concentrations of 0.0400, 0.400, and 4.00 μg/ml and then comparing the peak areas generated by analysis with a calibration curve prepared by injection of aliquots of the RS-79070-004 spiking solutions directly into the HPLC system by the same column switching method described in this report. Appropriate correction was made for the TCA supernatant volume.

Stability of RS-79070 in whole human blood (21–24°C) and in human plasma (21–24°C and 4°C) was determined by spiking RS-79070-194 into freshly drawn whole blood samples or plasma samples from three individuals to give a RS-79070-004 concentration of 1.53 μg/ml, and then monitoring the samples over time, after storage at the indicated temperatures, for the appearance of ganciclovir (the degradation product of RS-79070-004) [3]. The results (log of RS-79070-004 concentration vs. time) for each individual blood or plasma specimen were fit by linear regression to generate an equation of the form $\log(\text{concentration}) = m(\text{time}) = b$; this equation was then used to calculate half life ($t_{1/2}$) and also the time required to drop to 90% of the initial value ($t_{90\%}$) for each blood or plasma specimen. The stability of RS-79070-004 in plasma samples stored frozen at −80°C was determined by periodic determination of RS-79070-004 in QC samples stored at −80°C. The effects of freeze and thaw on RS-79070-004 concentrations were assessed by comparison of RS-79070-004 concentrations determined for QC samples subjected to 1, 2, and 3 cycles of freeze and thaw. Stability of RS-79070-004 in the TCA extracts was determined with extracts from QC samples before and after storage on-instrument at ambient temperature for 31 h, and at 4°C for 96 h.

3. Results

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 plasma. All processing of plasma was performed at ice-bath temperatures to avoid loss of RS-79070-004 by ester hydrolysis. The method used external standards, and no internal standard was used. Automated column switching was used to remove interferences, especially those late-eluting materials that would cause interference in subsequent chromatograms. Detection and quantification of RS-79070-004 was by UV. Concentrations were reported in terms of the total free base, RS-79070-004, to accommodate the possibility of dosing with either the free base or a salt.

Table 2
Summary of parameters for the regression equations, peak area = m(concentration) + b, determined for runs analyzed during the validation of the RS-79070-004 method

Run	m	b	r^2
1	4.538×10^4	137.2	0.9998
2	4.579×10^4	−214.7	0.9997
3	4.580×10^4	−966.0	0.9999
4	4.676×10^4	−443.1	0.9999
5	4.360×10^4	−577.5	0.9993

3.2. Range and linearity

The method was validated for the range of RS-79070-004 concentrations from 0.0400 to 4.00 μg/ml in plasma. The calibration curve range is 0.0400–0.800 μg/ml with verification of extrapolation to 4.00 μg/ml by use of verification standards in each run. The signal-to-noise ratio at the LLOQ (0.0400 μg/ml) was 10. The slopes (m), intercepts (b), and determination coefficients (r^2) obtained for the linear regression equations determined during the validation of the method are presented in Table 2. All values of r^2 were $>$ 0.999.

3.3. Specificity

The specificity of the method was determined by analysis of blank plasma collected from six untreated human subjects. No endogenous interfering peaks were observed in the chromatograms from these samples. A representative chromatogram from blank plasma is shown in Fig. 3, along with chromatograms from an LLOQ QC, and a High QC. In addition, no chromatographic interference was observed in plasma samples spiked with physiologically relevant concentrations of caffeine, acetaminophen, ibuprofen, or ganciclovir.

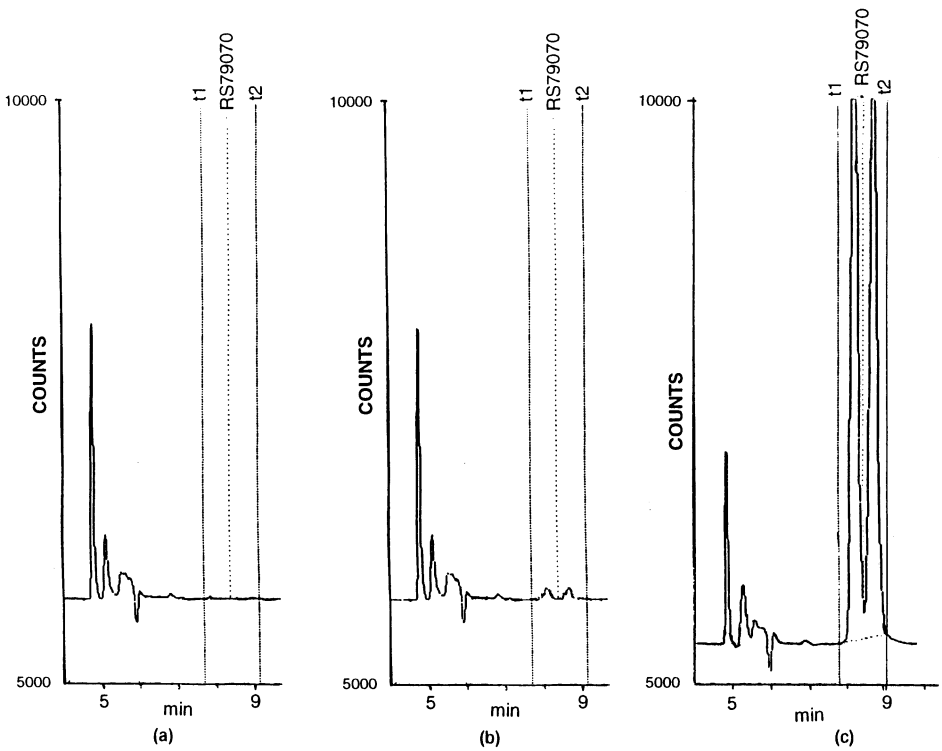


Fig. 3. Representative chromatograms from analysis of RS-79070-004: (a) blank plasma, (b) LLOQ QC sample (0.0400 μg/ml), (c) High QC sample (3.20 μg/ml). Vertical lines bracket the integration window.

Table 3
Intra- and inter-assay precision and accuracy data for determination of RS-79070-004 in human plasma

	Nominal conc. (μg/ml)	Number of runs	Replicates per run	Mean Conc. found (μg/ml)	C.V. (%)		Mean (%)	recovery
					Intra-assay	Inter-assay	Intermed precision	
Calibration Standards[a]								
STD 1	0.0400	4	2	0.0398	10.9	NS	10.9	99.5
STD 2	0.0800	4	2	0.0797	0.753	2.15	2.27	100
STD 3	0.120	4	2	0.127	9.64	7.49	12.2	106
STD 4	0.200	4	2	0.203	1.97	1.83	2.69	101
STD 5	0.300	4	2	0.300	0.859	NS	0.859	100
STD 6	0.400	4	2	0.397	0.660	0.984	1.18	99.3
STD 7	0.600	4	2	0.597	0.701	0.317	0.769	99.5
STD 8	0.800	4	2	0.796	0.679	NS	0.679	100
STD 9	2.00	4	2	2.03	1.17	0.427	1.24	101
STD 10	4.00	4	2	4.09	1.15	0.847	1.43	102
QC samples								
LLOQ QC	0.0400	3	5	0.0409	6.60	NS	6.60	102
Low QC	0.0800	3	5	0.0848	2.99	1.77	3.48	106
Mid QC	1.60	3	5	1.65	1.68	NS	1.68	103
High QC	3.20	3	5	3.32	1.45	1.69	2.23	104

[a] These calibration standards were separate from the set used to generate the calibration curve. NS: Not significant compared to intra-assay value.

Table 4
Stability data ($t_{1/2}$ and $t_{90\%}$) for RS-79070-004 in human blood (21–24°C) and plasma (4 and 21–24°C)[a]

	Blood				Plasma			
	Subject 1A	Subject 1B	Subject 1C	Mean	Subject 2A	Subject 2B	Subject 2C	Mean
4°C								
$t_{1/2}$ (h)	ND	ND	ND	ND	97.1	122	76.6	98.5
$t_{90\%}$ (h)	ND	ND	ND	ND	16.5	19.6	13.0	16.4
21–24°C								
$t_{1/2}$ (h)	18.3	19.5	18.5	18.8	24.9	27.6	18.4	23.6
$t_{90\%}$ (h)	3.00	2.79	3.32	3.04	4.09	4.44	3.09	3.87

[a] ND: Not determined (whole blood not studied at 4°C).

3.4. Precision and Accuracy

Summary results calculated by Excel 5.0 ANOVA: Single Factor from the analysis of quintuplicate analysis of QC samples on three different days and of duplicate analysis of calibration standards (separate from those used to generate a standard curve) on four different days are presented in Table 3. All intra- and inter-assay% C.V. values were $<10\%$, except for the calibration standard at the LLOQ, which had an intra-assay% C.V. of 10.9%. Mean recoveries (found concentration/nominal concentration) ranged from 99.3 to 106%. These results demonstrate good precision and accuracy for the method.

3.5. Absolute recovery

Absolute recoveries of the extraction process, determined in triplicate at nominal plasma concentrations of 0.0400, 0.400, and 4.00 μg/ml, averaged 75.1, 76.9, and 76.1%. The % C.V. values for the triplicate determinations were $<2.1\%$ at the LLOQ and $<0.70\%$ at the higher concentrations.

3.6. Stability

The results of the stability studies of RS-79070-004 in whole blood and plasma specimens from three individuals produced linear plots ($r^2 < -0.994$) of log(remaining RS-79070-004 concentration) vs. time for individual plasma and blood specimens. This indicated first-order kinetics, and the results were used to calculate the $t_{1/2}$ and $t_{90\%}$ values shown in Table 4, as well as mean $t_{1/2}$ and $t_{90\%}$values for blood and plasma at the designated storage temperatures. Analytically significant losses, defined as the loss of 10% of the initial concentration, (i.e. $t_{90\%}$), occurred after storage of whole blood for 2.8–3.3 h at 21–24°C and after storage of plasma for 3.1–4.4 h at 21–24°C or for 13–19 h at 4°C.

No loss in RS-79070-004 concentrations was observed in QC samples stored at −80°C for 90 days or subjected to three cycles of freeze and thaw, or in sample extracts stored on-instrument at ambient temperature (20–24°C) for 31 h or stored at 4°C for 96 h.

4. Discussion

Quantitative determination of RS-79070-004 in plasma presented a challenge because of the hydrolytic sensitivity of its ester function, and because of the presence of two diastereomeric components of the drug. As was verified by stability studies of RS-79070-004 in whole blood and plasma, immediate chilling of freshly collected blood samples, followed by 4°C separation of plasma and subsequent immediate freezing and storage of the sample at −80°C, were adequate procedures for the prevention decomposition of RS-79070-004 during sample collection and storage. It was also determined that sample processing at ice bath temperatures was adequate to prevent degradation of RS-79070-004 during sample analysis. No degradation of RS-79070-004 was

observed in the TCA sample extracts stored for extended time periods at ambient or refrigerated temperatures. Therefore, refrigeration of samples awaiting injection into the HPLC system was unnecessary.

The presence of two stereoisomeric components of RS-79070-004 resulted in two adjacent but distinct peaks (e.g. Fig. 3c) under most chromatographic conditions that also separated other peaks. Determination of total RS-79070-004 was achieved by integration of the chromatographic window that contained both peaks. Other alternatives involving peak heights instead of areas or involving separate quantification of individual peaks were not feasible with our data collection system. By use of the column switching technique to isolate the analyte of interest, chromatographic conditions were readily found that generated the required interference-free chromatographic window.

In the method we validated, we used verification of an upper extrapolation region by two control samples in every run to extend the calibration region as explained in the Calculations section. Although this practice has been found acceptable by the various regulatory agencies to which bioanalytical methods have been submitted for many recently approved drugs, we recommend that those who wish to implement this method consider the use of the data from all standards, with weighting by 1/nominal concentration, to generate a regression equation.

The method has been successfully used for the analysis of clinical study samples from HIV-positive subjects.

5. Conclusions

An HPLC method with column switching and UV detection has been developed for quantification of ganciclovir prodrug RS-79070-004 in human plasma. The method is applicable to analysis of clinical study samples, provided that blood collected during studies is chilled and processed at refrigerated temperatures to avoid degradation of the analyte by ester hydrolysis. Sample processing for the analysis should also be performed at refrigerated temperatures. RS-79070-004 is stable in the sample extracts, and no refrigeration is necessary after sample processing. Integration of the chromatographic window containing the two peaks for the diastereomeric analyte to give total peak areas is adequate for quantification with good precision and accuracy.

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