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Simple Green RP-UPLC.MethodForThe Analysis Of Ganciclovir In Its Bulk Form And Pharmaceutical Preparations

Semirna Daniel,[a] Fatma Gamal,[a] Nada Saad,[a] Hala Hamdy,[a] Ghada Adel,[a] Mahmoud Elnahas,[a] Fady Wael,[a] Sohair Aboelghar,[b] Nada Hesham,[b] Mohamed Tarek,[b] Hebatallah A. Wagdy[b*]

[a]*The Center for Drug Research and Development (CDRD), Faculty of Pharmacy, The British University in Egypt, Suez Desert Road, P.O. Box 43, El-Sherouk City, Cairo 11837, Egypt.*

[b]*Pharmaceutical Chemistry Department, Faculty of Pharmacy, The British University in Egypt, P.O. Box 43, Sherouk City,Cairo11837,Egypt.*

Abstract

A green, simple, validated reversed-phase ultra -. performance liquid chromatographic method was developed for the analysis of ganciclovir in both bulk and two different dosage forms. The optimum separation was reached using Acclaim™ RSLC.120 C18…column..(2.1.x.100.mm,.2.2.µm) at 30°C using the mobile phase composed of methanol (60%) and 20 mM phosphate buffer,. pH (4.00) 40%; at 0.5 mL/min. flow rate. The measurement took place at 253 nm using a photodiode array detector. The method was linear at concentrations ranging from 20.00 to 300.00 µg/mL. The limit of detection and the limit of quantification were 7.00.µg/mL and 20.00 µg/mL, respectively. The range for the percentage relative standard deviation of intra.-. day was 0.38 to 1.10 %, while for inter-day precisions was 0.56 to 2.08 %, respectively. The method was accurate, with percentage recovery ranging from 98.71 to 101.02 % and percentage relative standard…deviation ranging from 0.38 to 1.10 %. The method was favorably applied for determining of ganciclovir in bulk, pharmaceutical tablet, and gel preparation. The greenness of the method was assessed by the analytical. eco-scale system, and it was found to be eco-friendly.

Keywords: Ganciclovir; anti-viral; Ultra-performance liquid chromatography; validation; green chemistry; Valcyte® tablets; Ganvir® eye gel; dosage form analysis

1. Introduction

Ganciclovir **Figure 1**, is an antiviral drug belongs to guanosine analogues which are used for herpes virus treatment, Varicella..Zoster virus infections and human..Cytomegalovirus (HCMV) [1]. It is used as a prophylactic treatment from cytomegalovirus retinitis in immune-compromised patients or patients who have organ transplantation. HCMV spread through the fluid of the body from a person to another. It can also affect lungs, liver, esophagus, stomach, intestine and brain [2]. The physical and chemical properties of ganciclovir were reported in **Table 1**.

Ganciclovir exerts its pharmacological action by inhibiting the replication of viral..DNA by the ganciclovir-..5'-..triphosphate, including selective. inhibition of viral DNA..polymerase. After, it will be metabolized to its triphosphate form, ganciclovir-5'-triphosphate, by cellular enzymes: guanylate kinase, phosphoglycerate kinase and deoxyguanosine kinase

*Corresponding author e-mail: Hebatallah.wagdy@bue.edu.eg (Hebatallah A. Wagdy)
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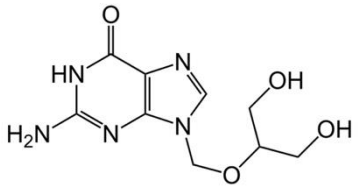

Figure 1: Chemical structure of ganciclovir [4].

Table 1: Chemical and physical properties of ganciclovir [3, 4].

IUPAC name	9-[(1,3-dihydroxy-2-propoxy) methyl] guanine
Chemical formula	$C_9H_{13}N_5O_4$
Solubility	Soluble in water about 4300 µg/mL at (pH 7.00)
Color	White to off white solid
Melting point	250°C
Molecular weight	255.23 g/mol
pKa	2.2

inside the infected cells. Ganciclovir-5'-triphosphate was found to inhibit both HCMV DNA polymerase and cellular DNA polymerase. However, it was proven to have a selective antiviral action owing to the fact that weaker effect on the inhibition of cellular DNA than HCMV DNA polymerase. Moreover, its activity and selectivity is improved by ganciclovir-5'-triphosphate's ability to accumulate in the infected cells. The mechanism of action is shown in **Figure 2**. Ganciclovir's metabolism is very low as most of the drug is eliminated in urine unchanged. Ganciclovir is eliminated through kidney; unchanged drug is excreted by glomerular filtration and the major route of elimination is active tubular secretion [5,6]. The most commonly reported adverse effects of ganciclovir are abdominal pain, neutropenia, anemia, fatigue, bone marrow suppression and leukopenia [5].

There were different methods reported in literature for ganciclovir analysis such as electrochemical method [8-14], spectrophotometric method [15-25], Raman spectroscopic method [26-28] and chromatographic methods [29-38].

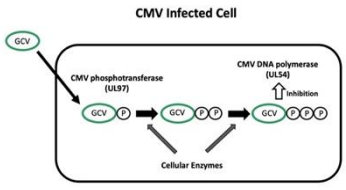

Figure 2: Mechanism of Ganciclovir [7].

Chemical products can result in harmful hazards to human and environment as, flammability, mutagenicity, carcinogenicity, and atmospheric damage. In analytical chemistry, this can be minimized by using green analytical methods. Not all analytical techniques can be categorized as green and it could be improved by getting rid of toxic chemicals, lowering energy, and improving the analytical process from sampling to waste management. Recently, evaluation of how green the analytical method is has been an important parameter in assessing analytical methods. The method is evaluated according to environmental impact starting from sampling till the detection. Therefore, the green analytical chemistry (GAC) aims to develop a green and eco-friendly analytical method [39-41].

Finally, the aim of this work is to develop and validate a simple green RP-UPLC method for the determination of ganciclovir in bulk and pharmaceutical preparations. To the best to our knowledge that this the first green analytical method for the determination of Ganciclovir.

2. Experimental

2.1. .Instruments and software

Thermo Fisher UHPLC .Dionex Ultimate.3000..(.Germering, Germany.) was the UPLC system used. It was equipped with an ISO-3100SD pump, an autosampler model WPS 3000 SL and a TCC-3000 SD column thermostat. The UPLC system was coupled to a photo diode array..detector model 3000..RS (.Germering, Germany.). Data acquisition and analysis were performed using Chromeleon software of version 6.8 (.Germering, Germany.). The pH meter used for adjusting the buffer pH..was..regulated by Jenway..pH-meter 3310 (.Dunmow, Essex, .United Kingdom.). The water used was purified using Milli-Q ultrapure water purification system (vThermo Scientific Barnstead.Smart2Pure 3.UV, Hungary.). An ultra-sonicator was used in to extract ganciclovir from its dosage form (Elmasonic S.60 (H), Germany).The analytical column used for separation was an Acclaim^{TM}RSLC.120.C18.column (2.1.x.100 mm, 2.2 µm).

2.2. Chemicals and reagents

High-grademonobasic.sodium..phosphate..and..phosphoric

acid were obtained from Sigma-Aldrich, …Germany.
HPLC..grade..acetonitrile..and..methanol were provided by Fisher Scientific (..Loughborough,..Leicestershire,..United..Kingdom.). Standard of ganciclovir was purchased from Sigma Pharmaceutical Company (Cairo, Egypt). Ganciclovir pharmaceutical preparation (Valcyte® tablets, Ganvir® eye gel) were purchased from the local market of Egypt.

2.3. Standard stock solution preparation

The stock. solution was constituted by accurately weighing 25.00 mg. of ganciclovir in 25.00 mL.volumetric flask and the volume was completed to the mark using ultrapure water to prepare a solution of concentration 1.00 mg/mL. A working solution of concentration 60.00 µg/mL was then prepared by adding 0.60 mL from the stock solution into 10.00 mL volumetric. flask and complete to the mark using ultrapure water. The serial dilution was prepared by dilution of 0.20, 0.40, 0.60, 0.80, 1.00, 1.50 and 3.00 mL from the stock. solution in 10.00 . mL volumetric flask using ultrapure water to prepare concentrations of 20.00, 40.00, 60.00, 80.00, 100.00, 150.00, 300.00 µg/mL.

2.4. Preparation of pharmaceutical preparation

One tablet of Valcyte® 450 mg was accurately weighed before and after removing the coat using methanol, then the tablet was crushed into fine powder using mortar and pestle. An amount of the powder equivalent to 10.00 mg ganciclovir was accurately .weighed and dissolved in 10.00 mL volumetric. flask using ultrapure water to prepare a stock solution of concentration 1.00 mg/mL. The solution was first sonicated and then it was filtered using a nylon syringe filter. Then 0.60 mL from the stock solution was carried it into 10.00 mL volumetric flask and completed to the mark using ultrapure water to prepare a concentration of 60.00 µg/mL. The other pharmaceutical preparation was Ganvir® 0.15 % eye gel was analyzed by accurately weighting amount of the eye gel equivalent to 10.00 mg and dissolving the weighed amount in 10.00 mL volumetric flask using ultrapure water to prepare a stock solution of concentration 1.00 mg/mL. The solution was first sonicated and then it was filtered using a nylon syringe filter. Then 0.60 mL from the stock solution was transferred into 10.00 mL volumetric flask and completed using ultrapure water to prepare a concentration of 60.00 µg/mL.

2.5. Chromatographic conditions

Chromatographic separation was accomplished on Acclaim™ RSLC.120. C18 column 2.2.µm (2.1.× .100 mm) using isocratic mobile phase composed of methanol: phosphate buffer pH (4.00) (60:40; *.v/v*). The flow rate was 0.5 mL/min using PDA detector adjusted at 253 nm. The injection volume was 10 µL and the temperature of the column oven was 30˚C. The chromatographic run time was 5 min.

2.6. Method validation

Method validation was accomplished according to the ICH guidelines to the respect of the mentioned parameters hereunder.

2.6.1. Linearity

Linearity is the capability of a method to get a. response that is directly proportional to the concentration of the sample over certain range of concentrations. The linearity was determined by preparing seven concentrations in range from 20.00 to 300.00 µg/mL. Each and every concentration was injected three. times into .UPLC. The average peak area was calculated for each concentration. Afterward, concentrations against the average peak area were.layed. Analyzing the linear regression showed that the regression equation and the square of the regression coefficient (R^2) were obtained.

2.6.2. Limit of detection and limit of quantification

The limit..of..quantification..(.LOQ.) is the least amount of the analyte in sample that can be quantitatively determined with acceptable accuracy..and..precision, it is equal to the analyte concentration in which signal to noise ratio is 10. While, limit of detection (LOD) is defined as the least amount that can be detected, it is equal to the analyte concentration in which signal to noise ratio is 3.

2.6.3. Precision

Precision measures the degree of closeness of the method readings to each other. It is determined by intra-day and inter-day precision to be then evaluated using % relative standard deviation...(..% RSD). Intra-

day precision was performed by injecting the seven serial concentrations into the..UPLC on the same day, each concentration was injected three times. The average peak area of the three repeated injections was then determined to calculate the % RSD. While inter..-..day precision was evaluated by injecting the seven concentrations into the.. system and each concentration was injected three times on three different days. The average peak area of the three days was used to calculate the % RSD.

2.6.4. Accuracy

Accuracy is the measure for the proximity of experimental values to a reference (true) value. Seven different concentrations of standard solution were prepared, chromatographed, the peak area for each concentration was determined and the concentrations were calculated then, compared to the theoretical concentrations by calculating the percentage recovery (% R) and % RSD.

2.6.5. System Suitability parameters

System suitability parameters of the proposed method was tested by calculating the tailing factor (T_f), capacity factor (K), number of theoretical plates (N) and height equivalent to theoretical plate (HETP).

3. Results and discussion

3.1. Method development

In the sake of a high-quality separation, the most important thing that should be maintained is the optimum conditions. Thus, temperature, composition of the mobile phase,. flow rate and the.wavelength were optimized. At first, a comparison was done between methanol and acetonitrile to choose which would be the organic solvent of the mobile phase. Methanol was chosen as the peak of the eluent was of a better shape and the retention time was not affected.

The aqueous phase was also studied in terms of its concentration and pH. A buffer of high concentration gave a peak of better shape and of a shorter retention time, but it caused an increase in the pumping pressure; thus, the optimum strength for the buffer was 20 mM. Regarding the pH, a broad peak was noticed for a pH greater than 4.00 and no peak improvement was observed at lower pH. Therefore, the buffer was adjusted at pH 4.00 as an optimum pH and this can be elaborated that the pKa of ganciclovir is 2.2.

Regarding temperature, the higher the temperature, the lower the retention time. This can be explained that when the temperature increases, the viscosity of the mobile phase decreases. Accordingly, 30°C was selected as the optimum temperature as it was enough to improve the peak shape and decreases retention time. Knowing that raising the temperature more than that did not improve the peak any further.

For the flow rate it was 0.5mL/min and it was ideal to have a short run time with no increasing the pressure of the pump.

The optimum wavelength was 253 nm, which is the $\lambda_{max}$of ganciclovir at which the drug has its highest absorption as shown in the UV-Spectrum in **Figure 3**. The optimum chromatographic conditions were represented in **Table 2**. By applying these optimum conditions, the peak for ganciclovir appeared at retention time (tR) equals 1.447 min as shown in **Figure 4**. Next, this analytical method was validated.

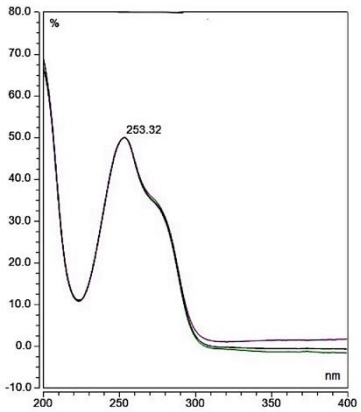

Figure 3: The ultraviolet spectrum (UV) ofGanciclovir

Table 2: Optimum chromatographic conditions

Parameters	Optimized condition
Column	Acclaim™ RSLC 120 C18 column 2.2 µm (2.1 × 100 mm)
Elution Mode	Isocratic
Mobile phase	Methanol:Phosphate buffer pH (4.00) (60:40; *v/v*)
Temperature	30°C
Flow rate	0.5 mL/min
Detector	PDA
Detection wavelength	253 nm
Injection volume	10 µL
Retention time	1.447 min
Run time	5 min

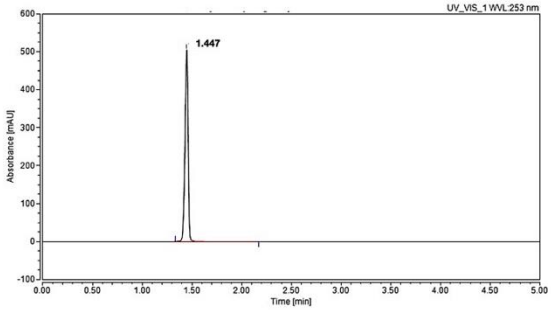

Figure 4: **Chromatogram of standard working solution 60 µg/mL ganciclovir under the optimum conditions.**

3.2. Validation of the developed method

3.2.1. Linearity, limit. of detection and limit. of quantification

The method was linear in a range from 20.00 to 300.00 µg/mL as represented by the calibration curve graph shown in **Figure 5**. The slope, intercept, and R^2 were obtained from the regression equation y = 0.2815x + 1.8393. The slope was 0.2815, intercept was 1.8393, and the R^2 was 0.9999, therefore the method was found to be linear according to the R^2 value. LOD was 7 µg/mL and LOQ was 20 µg/mL.

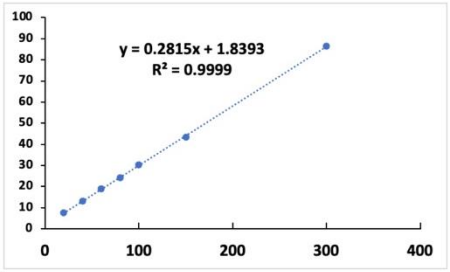

Figure 5: Calibration curve of ganciclovir

3.2.2. Precision

The % RSD for intra-day and inter-day precisions were ranged from 0.38 to 1.10 % and 0.56 to 2.08 %, respectively as shown in **Table 3**. The lower % RSD indicates the higher intra-day and inter-day precision of the method.

3.2.3. Accuracy

Accuracy was evaluated using % R and the results showed that the method is accurate as the % R was ranged from 98.71 to 101.02 % and the % RSD was ranged from 0.38 to 1.10 % as shown in **Table 4**.

3.2.4. System Suitability parameters

The results showed that the method passed the system suitability parameters as shown in **Table 5**

3.3. Assessment of Greenness of the Method: The Analytical Eco - Scale

Analytical Eco-Scale is a semi quantitative method used to assess the greenness of analytical methodologies to assist in selecting the greenest method to be applied. Method assessment is based on determining the amount of the reagents consumed, Hazardous reagents, energy consumption and waste production. An ideal green analytical method expected to have a score of 100. Total penalty points are calculated according to this equation ***Analytical Eco-Scale. = 100. - total penalty*** points. Score higher than 75 represents. indicates. green analysis, while greater than 50.represents acceptable green analysis, less than 50 indicates. inadequate analysis[42]. The Eco-Scale results are shown in the **Table.6** was 79. Therefore, the method showed excellent green analysis.

3.4. Application on pharmaceutical preparations

The method was able to analyze ganciclovir in its tablet dosage form Valcyte® as shown in **Figure 6** where, the retention time was 1.447 min. The % R was 100.01 % and the % RSD was 0.51 %. The method was also applied for the analysis of Ganvir® 0.15 % eye gel as presented in **Figure 6** where, the retention time was 1.443 min. The % R was 99.09 % and the % RSD was 0.84 %. It worth mentioning that the excipients in both Valcyte® and Ganvir® did not interfere with ganciclovir as shown in the dosage forms and the blank chromatograms presented in **Figure 6**, . A comparison of the newly developed validated green method in this work with some of the reported chromatographic conditions for the determination of Ganciclovir is presented in **Table 7.**

4. Conclusion

A green RP-UPLC method was developed and. validated according for the analysis of ganciclovir. The method successfully passed the validation parameters and in turn it was used for the analysis of ganciclovir in its two dosage forms, the first one was Valcyte® tablets and the second one was Ganvir® eye gel.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

Special thanks to Mahy Ezzat and Manar Awad for their help during work.

Table 3: Intra-day and inter-day precision of the proposed method

Concentration (µg/mL)	Intra-day precision		Inter-day precision	
	Peak area*	% RSD**	Peak area*	% RSD**
20.00	7.46	0.82	7.50	0.57
40.00	13.21	0.46	13.00	1.41
60.00	18.83	1.08	18.74	0.56
80.00	24.27	0.38	24.72	2.08
100.00	30.28	1.10	29.80	1.46
150.00	43.52	0.79	43.93	0.80
300.00	86.47	0.80	85.40	1.16

* Average of 3 measurements
**RSD: Relative standard deviation

Table 4: Accuracy of the proposed method.

Theoretical concentration (µg/mL)	Actual concentration (µg/mL)	% RSD*	% Recovery*
20.00	19.95	0.82	99.75
40.00	40.38	0.46	100.96
60.00	60.35	1.08	100.58
80.00	79.67	0.38	99.59
100.00	101.02	1.10	101.02
150.00	148.07	0.79	98.71
300.00	300.65	0.80	100.22

* Average of 3 measurements
**RSD: Relative standard deviation

Table 5: System suitability parameters

Parameters	Standard(Ganciclovir)	Reference value
Retention time (t_R) (min)	1.447	------
Tailing factor (T_f)	1.10	*Less than 2*
Capacity Factor (K)	4.05	*>1*
Number of theoretical plates (N)	2980	*>2000*
Height equivalent of the Theoretical plates (HETP)	0.0003	*The smaller the value the higher the column efficiency*

Table 6: Penalty points to calculate Analytical Eco-Scale

Type of reagent	Penalty point
Methanol	(More than 100ml) = 18
Energy consumption	(Less than or equal to 0.1k Wh per sample) = 0
Waste production	(1-10ml) = 3
Total penalty points	21
Analytical Eco-Scale total score	79
Greenness Evaluation	Excellent green analysis

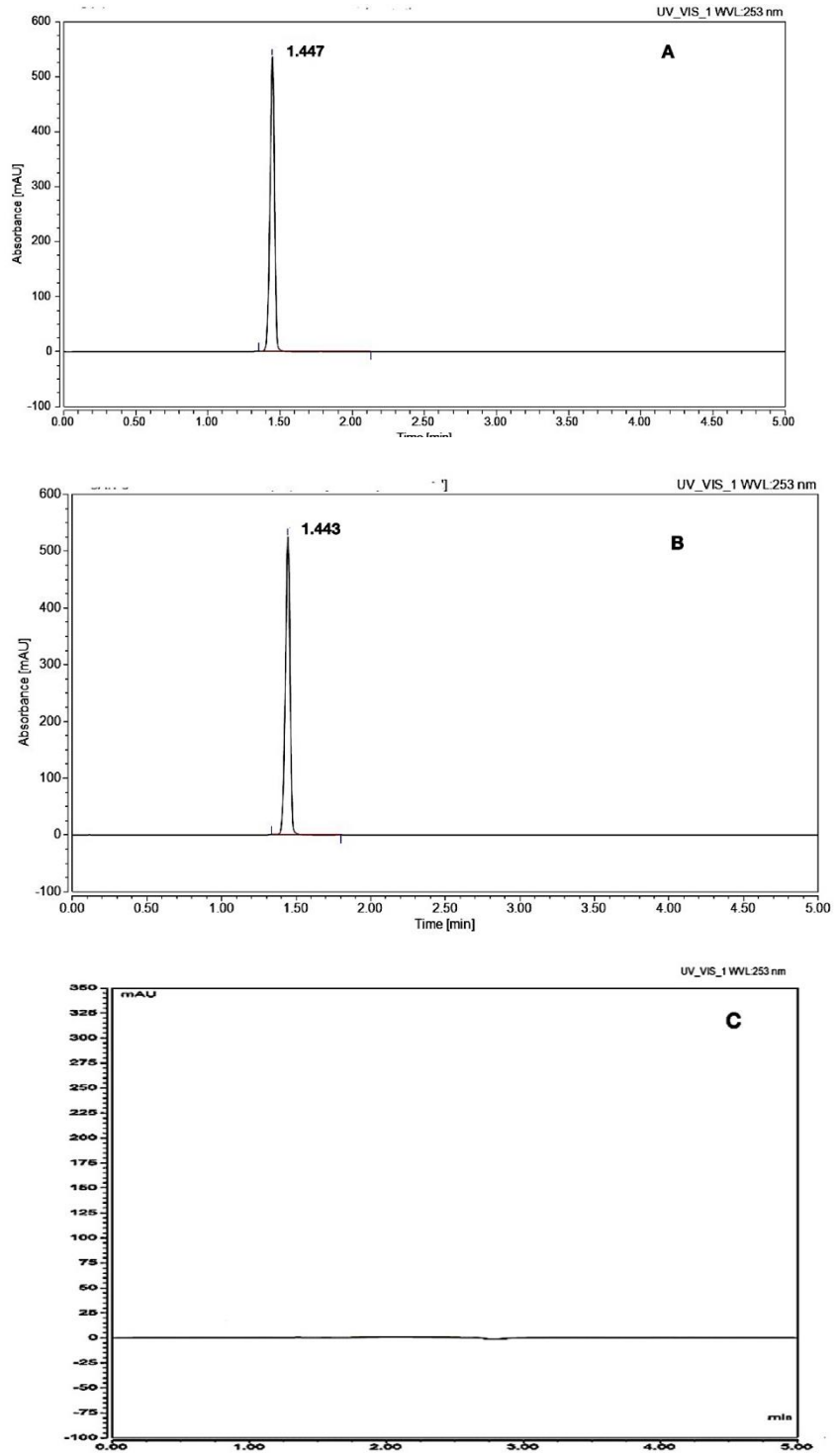

Figure 6: **The chromatogram of 60 µg/mL valcyte® tablets (A), Ganvir® eye gel (B) and blank (C) under the optimum conditions.**

Table 7: Comparison of the current newly developed chromatographic method in this work and reported chromatographic methods for determination of Ganciclovir

Sample / Application	Stationary phase	Mobile phase	Detector	Green Assessment	Limit of detection	Sensitivity	References
Dried Blood spots	Phenomenex®Synergi TM Polar-RP (100 × 2 mm), 2.5 µm	0.1% formic acid in water: methanol (99:1; *v/v*)	MS/MS	ND	ND	10 ng/mL	[32]
Bulk and capsule dosage form	Grace smart RP-C18 column (250 × 4.6 mm), 5 µm	Methanol : (0.05M) Citrate buffer (pH 5.2) with potassium hydroxide (70:30; *v/v*)	UV at 254 nm	ND	0.25 µg/mL	ND	[43]
Gancyclovir and its derivatives in human cells	Waters Atlantis® HILIC Silica (150 × 2.1mm), 5 µm	Phase A: 2 mM/L ammonium formate (pH 3.0) and phase B: Acetonitrile: 2 mM/L ammonium formate (pH 3.0) (90:10; *v/v*)	MS/MS	ND	1 µg/mL	ND	[44]
Bulk drug and tablet dosage form	Inertsil ODS C18 (250 × 4.6 mm), 5 µm	0.025 M Ammonium acetate buffer (pH 6.8):acetonitrile (90:10; *v/v*)	UV at 245 nm	ND	4 ng/mL	ND	[45]
Infant Plasma	Cadenza C18 (150 × 4.6 mm), 3 µm	(25 mM) phosphate buffer (pH 2.5) containing 1% methanol: acetonitrile (4:3; *v/v*)	Fluorescence at excitation and emission wavelengths of 265 and 380 nm, respectively	ND	ND	0.02 µg/mL	[46]
Gancyclovir and its related substances	A Hypersil ODS column (250 × 4.6 mm), 5 µm	0.02 M potassium dihydrogen phosphate buffer (pH 6.0): methanol (92:8; *v/v*)	UV at 254 nm	ND	0.04 µg/mL	ND	[47]
Bulk drug, tablet and gel dosage forms	Acclaim™ RSLC 120 C18 (100 × 2.1 mm), 2.2 µm	Methanol: 20 mM phosphate buffer, pH (4.0) (60: 40; *v/v*)	Photodiode array at 253 nm	Excellent green analytical method	7.00 µg/mL	11.50 µg/mL	The current study

UV: Ultra-violet detector
MS/MS: Mass Spectrometer/ Mass spectrometer
ND: Not determined

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