Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 864 (2008) 78–86
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Selective and rapid liquid chromatography/negative-ion electrospray ionization mass spectrometry method for the quantification of valacyclovir and its metabolite in human plasma

Maria Kasiari, Evagelos Gikas, Sofia Georgakakou, Michael Kazanis, Irene Panderi *

University of Athens, School of Pharmacy, Division of Pharmaceutical Chemistry, Panepistimiopolis, Zografou 157 71, Athens, Greece

Received 20 September 2007; accepted 27 January 2008
Available online 7 February 2008

Abstract

A rapid, sensitive and specific method was developed for the quantification of valacyclovir and acyclovir in human plasma. Sample preparation was performed by protein precipitation with acetonitrile followed by filtration. Valacyclovir, acyclovir and ganciclovir (internal standard) were separated isocratically on a reversed-phase porous graphitized carbon analytical column (2.1 mm × 125.0 mm i.d., particle size 5 μm), using a mobile phase of acetonitrile/water with 0.05% (v/v) diethylamine (50:50, v/v) at a flow rate of 0.15 mL min^{-1} in 4.0 min. Detection was performed by negative electrospray ionization using the selected ion monitoring mode of the deprotonated molecular ions at *m/z* 323.0 for valacyclovir, 224.0 for acyclovir and 254.0 for ganciclovir. The assay had linear calibration curves over the range 0.020–0.800 μg mL^{-1} for valacyclovir and 0.100–20.00 μg mL^{-1} for acyclovir. Accuracy and precision were within the acceptance limit of 15%. The method was successfully applied to the analysis of plasma samples obtained from patients after oral administration of valacyclovir.

Keywords: LC–ESI/MS; Valacyclovir; Acyclovir; Ganciclovir; Human plasma

1. Introduction

Valacyclovir, L-valine-2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9yl) methoxy]ethyl ester, is the L-valyl ester pro-drug of the antiviral drug acyclovir that exhibits activity against herpes simplex virus types, 1 (HSV-1) and 2 (HSV-2) and varicella-zoster virus (VZV) [1]. The mechanism of action of acyclovir involves the highly selective inhibition of herpes virus DNA replication, via enhanced uptake in herpes virus-infected cells and phosphorylation by viral thymidine kinase. The substrate specificity of acyclovir triphosphate for viral, rather than cellular, DNA polymerase contributes to the specificity of the drug [2,3]. After oral administration, valacyclovir is rapidly and extensively converted to acyclovir as a result of first-pass intestinal and hepatic metabolism through enzymatic hydrolysis [4]. The oral bioavailability of valacyclovir is low while the oral bioavailability of acyclovir is higher after administration of valacyclovir relative to acyclovir itself [5,6]. Following single 1000 mg doses of oral valacyclovir, peak acyclovir concentrations average 5.0–6.0 μg mL^{-1} and occur approximately 2 h post-dose. Valacyclovir 2000 mg four times daily, provides steady-state peak to trough acyclovir concentrations of 8.4 and 2.5 μg mL^{-1}, respectively. Peak plasma concentrations of valacyclovir are only 4% of acyclovir levels [7–9].

In previous studies acyclovir was analysed by high-performance liquid chromatography with UV detection in human plasma [10–14] serum [15] and maternal plasma, amniotic fluid, fetal and placental tissues [16]. High-performance capillary electrophoresis has also been used for the determination of acyclovir in urine [17] and plasma [18]. A sensitive and selective LC–MS/MS method based on hydrophilic interaction liquid chromatography has been reported for the determination of acyclovir in pregnant rat plasma and tissues [19]. Only one assay has been reported for the simultaneous determination of valacyclovir and acyclovir in human serum and urine but this method utilizes less sensitive UV detection and has a 12 min run time [20]. Recently, the chemical and enzymatic stability of valacyclovir has been investigated by HPLC with UV detection [21]. Valacyclovir has also been quantified in pharmaceutical preparations using acyclovir as internal standard by HPLC with

* Corresponding author. Tel.: +30 210 7274820; fax: +30 210 7274747.
E-mail address: ipanderi@pharm.uoa.gr (I. Panderi).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.01.046

UV detection [22]. To our knowledge no report has been mentioned in the literature for the simultaneous determination of valacyclovir and acyclovir in human plasma.

Valacyclovir and acyclovir are synthetic purine nucleoside analogues that share structural similarity with endogenous compounds. This fact makes the analysis of those compounds complicated and requires the use of selective analytical methodology. In the present work, we tried to combine the advantages of the highly sensitive and selective MS detection technique with those of the porous graphitized carbon packing material that is able to retain polar compounds so as to achieve selective and sensitive quantitation of valacyclovir and acyclovir in human plasma. The proposed LC–MS method utilizes negative-ion electrospray ionization procedure that reduces significantly the signal-to-noise ratio and allows the quantitation of the analytes at low concentration levels. The proposed method, compared to the already existing methodology, provides a useful insight into the quantification of the analytes in human plasma as it is selective, offers higher sensitivity and has a short analytical run time (less than 4.0 min).

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, methanol, formic acid and trifluoroacetic acid of HPLC grade were purchased from Merck (Darmstadt, Germany) and diethylamine of analytical reagent grade was obtained from Fluka (Buchs, Switzerland). Water was deionised and further purified by means of a Milli-Q Plus Water Purification System, Millipore, Waters (Milford, MA, USA) and was filtered through a 0.22 μm filter prior to liquid chromatographic procedure.

Valacyclovir hydrochloride, acyclovir and the internal standard, ganciclovir, were USP reference standards, USP (Rockville, MD, USA). Acrodisk® GHP membrane syringe filters (13 mm, pore size 0.45 μm) were obtained from Pall life sciences (Ann Arbor, MI, USA).

2.2. Liquid chromatographic and mass spectrometric conditions

The HPLC system included a SpectraSeries Model P100 isocratic pump SP ThermoSeparation products (San Jose, CA, USA) and a Rheodyne Model 7725i injector, Rheodyne (California, CA, USA) with a 20-μL loop. The analytical column was a porous graphitized carbon PGC column, 2.1 mm × 125.0 mm, particle size 5 μm Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A hypercarb guard column, Thermo Fisher Scientific, Inc. (Waltham, MA, USA) was also used to prolong column lifetime. The mobile phase consisted of 50% acetonitrile in water containing 0.05% (v/v) diethylamine and pumped at a flow rate of 0.15 mL min^{-1}. Chromatography was performed at 25 ± 2 °C with a chromatographic run time of 4 min; samples were injected into a 20 μL loop.

Mass spectrometry was performed using a ThermoQuest Finnigan AQA (Manchester, U.K.) single quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source. The detection of valacyclovir, acyclovir and ganciclovir was performed by electrospray negative-ion mode. The ESI probe temperature was set at 270 °C, the cone voltage (AQA_{max}) was set at 20.0 V and the capillary voltage was set at 4.5. A Nitrox-N2 Model UHPLC-MS12E nitrogen generator, Domnick hunter (Gateshead, England) was used to provide highly pure nitrogen that utilized as sheath and nebulizing gas. Data acquisition and analysis were performed using the Xcalibur (v. 1.2) IBM data system running under Windows 2000 on a Pentium V computer. The SIM mode was chosen for the quantitative determination of the analytes.

2.3. Stock and working standard solutions

Stock standard solutions of valacyclovir, 200.0 μg mL^{-1}, acyclovir, 500.0 μg mL^{-1}, and ganciclovir, 500.0 μg mL^{-1} were prepared by dissolving the appropriate amounts of the compounds in 50% aqueous acetonitrile. These solutions were stored in the dark at −20 °C and were found to be stable for at least 1 month.

A series of mixed working standard solutions of valacyclovir and acyclovir were prepared by subsequent dilution of the above-mentioned stock standard solutions in acetonitrile to reach concentration ranges of 0.50–100.0 μg mL^{-1} for acyclovir and of 0.02–4.0 μg mL^{-1} for valacyclovir. A working standard solution of the internal standard, ganciclovir, 100.0 μg mL^{-1} was also prepared by subsequent dilution of the above-mentioned stock standard solution in acetonitrile. The working standard solutions were freshly prepared every week and stored in the dark at −20 °C.

2.4. Calibration spiked plasma standards and quality control samples

Six different lots of drug-free human plasma were obtained from Sotiria General Hospital, Athens, Greece and were used to prepare pooled human plasma. Calibration spiked plasma standards were prepared fresh daily by spiking the appropriate amounts of the above-mentioned mixed working standard solutions into 0.5 mL of pooled human plasma. The calibration curve ranges were 0.02–0.80 μg mL^{-1} for valacyclovir and 0.10–20.00 μg mL^{-1} for acyclovir. Each calibration sample contained 2.5 μg mL^{-1} of the internal standard, by the addition of 50 μL of the 100.0 μg mL^{-1} ganciclovir working standard solution.

Quality control (QC) samples were also prepared in human plasma at three concentration levels for valacyclovir (0.02, 0.32 and 0.80 μg mL^{-1}) and at four concentration levels for acyclovir (0.10, 0.50, 8.00 and 20.00 μg mL^{-1}). All QC samples were freshly prepared for each run. Separate analyte stock solutions were used for the preparation of calibration standard solutions and QC samples.

2.5. Sample preparation procedure

Cleanup of human plasma samples was carried out by protein precipitation. For the isolation of valacyclovir and

acyclovir from 0.5 mL of plasma samples, 50 μL IS solution (100.0 μg mL^{-1}) along with 1450 μL of acetonitrile were added to the plasma sample, and the mixture was vortex mixed for a few seconds and centrifuged at 16,000 × *g* and 4 °C for 15 min. The supernatant was filtered through a 13 mm GHP Acrodisk® membrane syringe filter (pore size 0.45 μm) prior to the injection into the LC–ESI/MS system.

2.6. Validation procedures

Spiked plasma calibration standards at nine different concentration levels 0.10, 0.50, 1.00, 2.00, 4.00, 8.00, 10.00, 15.00 and 20.00 μg mL^{-1} for acyclovir and at eight different concentration levels 0.02, 0.04, 0.08, 0.16, 0.32, 0.40, 0.60 and 0.80 μg mL^{-1} for valacyclovir were prepared and analysed in duplicate in five different analytical runs. Quantitation was performed using the peak area ratio of each analyte to that of the internal standard, ganciclovir. Weighted ($1/y^2$) least squares linear regressions were used to obtain the equation of the calibration curves.

Quality control samples were processed in five replicates at three concentration levels for valacyclovir and four concentration levels for acyclovir. To assess intra- and inter-assay accuracy and precision, QC samples were analysed for five different analytical runs.

The recovery of the sample preparation procedure was evaluated at three concentration levels for valacyclovir (0.02, 0.32 and 0.80 μg mL^{-1}) and for acyclovir (0.50, 8.00 and 20.0 μg mL^{-1}) and at 2.50 μg mL^{-1} for the internal standard. Recovery of the cleanup procedure was determined by comparing the analytical response obtained from the quality control samples after the sample preparation procedure with the analytical response of blank plasma samples that were processed according to the sample preparation procedure and then spiked with equivalent concentrations of the analytes and the IS.

The stability of the analytes in spiked human plasma samples was investigated under various storage conditions. In particular, plasma samples were spiked at two concentration levels for valacyclovir (0.08 and 0.60 μg mL^{-1}) and for acyclovir (2.00 and 15.00 μg mL^{-1}) and were stored at ambient temperature for 4 h and at −20 °C for 3 weeks. Freeze–thaw stability was also evaluated by successive cycles of freezing and thawing; three complete freeze–thaw cycles were performed with samples frozen at −20 °C for 7 days per cycle and thawed (without warming) at room temperature. Absolute peak area measurements obtained from the analysis of the stored samples were compared to the absolute peak area measurements that were obtained from the analysis of freshly prepared spiked plasma samples. The analyte was considered stable in the biological matrix when 80–120% of the initial concentration was found.

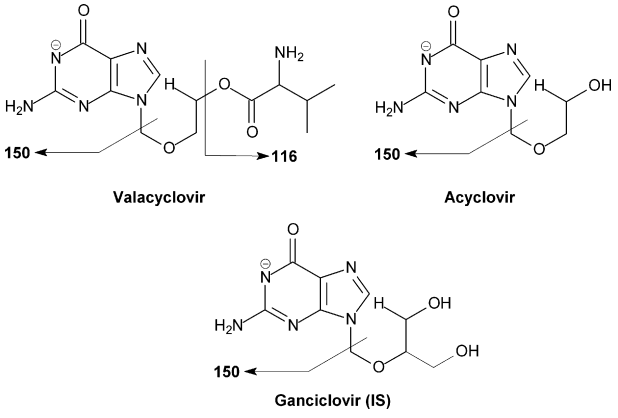
3. Results and discussion

3.1. Optimization of MS detection

Mass spectrometric parameters were optimized so as to achieve the maximum abundance of the product ions of valacyclovir, acyclovir and ganciclovir (IS). Acquisition parameters were determined by direct infusion into the mass spectrometer of a 25.0 μg mL^{-1} solution (in mobile phase) of each one of the compounds at a flow rate of 80 μL min^{-1}. Variable mass spectrometric conditions (ESI probe temperature, capillary voltage and cone voltage) were investigated. ESI probe temperature was set at the minimum acceptable value (270 °C) regarding the mobile phase flow rate (0.15 mL min^{-1}) to avoid thermal degradation of the compounds. Capillary voltage was kept at 4.5 kV for the detection of the analytes.

The electrospray ionisation mass spectral behaviour of the analytes and the IS were thoroughly investigated by cone voltage fragmentation in the single quadrupole mass spectrometer. Mass spectra of valacyclovir, acyclovir and ganciclovir obtained in ESI negative-ion mode and under the optimal cone voltage conditions (20 V) are presented in Fig. 1. The ESI mass spectrum of valacyclovir in negative-ion mode (cone voltage 20 V) is dominated by the deprotonated molecular ion, $[M-H]^{-1}$, at *m/z* 323; chlorine ion adduct, $[M-Cl]^{-1}$, is also present at *m/z* 359. An increase in the cone voltage to 50 V causes concomitant increase in the fragmentation of valacyclovir. The low-abundance ion at *m/z* 150 in the spectrum of valacyclovir presumably results via the losses of formaldehyde (30u) and of 2-amino-3-methylbutanoic ethylene ester (143u), as a result of a γ-hydrogen rearrangement reaction. The latter group (143u) is further fragmented by the loss of an ethylene group (28u) to give an intense 2-amino-3-methyl-butanoic acid anion at *m/z* 116.0. Analogous fragmentation patterns were observed for the other two substances (acyclovir and ganciclovir). Thus, an increase in the cone voltage up to 50 V increases the fragmentation process generating an ion peak at *m/z* 150.0 for both acyclovir and ganciclovir. The chemical structures of the analytes and the IS along with the masses of the ion fragments in ESI negative-ion mode are presented in Scheme 1.

The monitoring of all analytes in selected ion monitoring SIM mode by their dominant mass peaks was found to be crucial as it improves significantly the sensitivity of the procedure. Thus, a cone voltage value of 20.0 V was chosen as the optimum to obtain the maximum abundance of the deprotonated molecular ions. In particular, valacyclovir, acyclovir and the



Scheme 1. Chemical structures and masses of the ion fragments for valacyclovir, acyclovir and ganciclovir (IS).

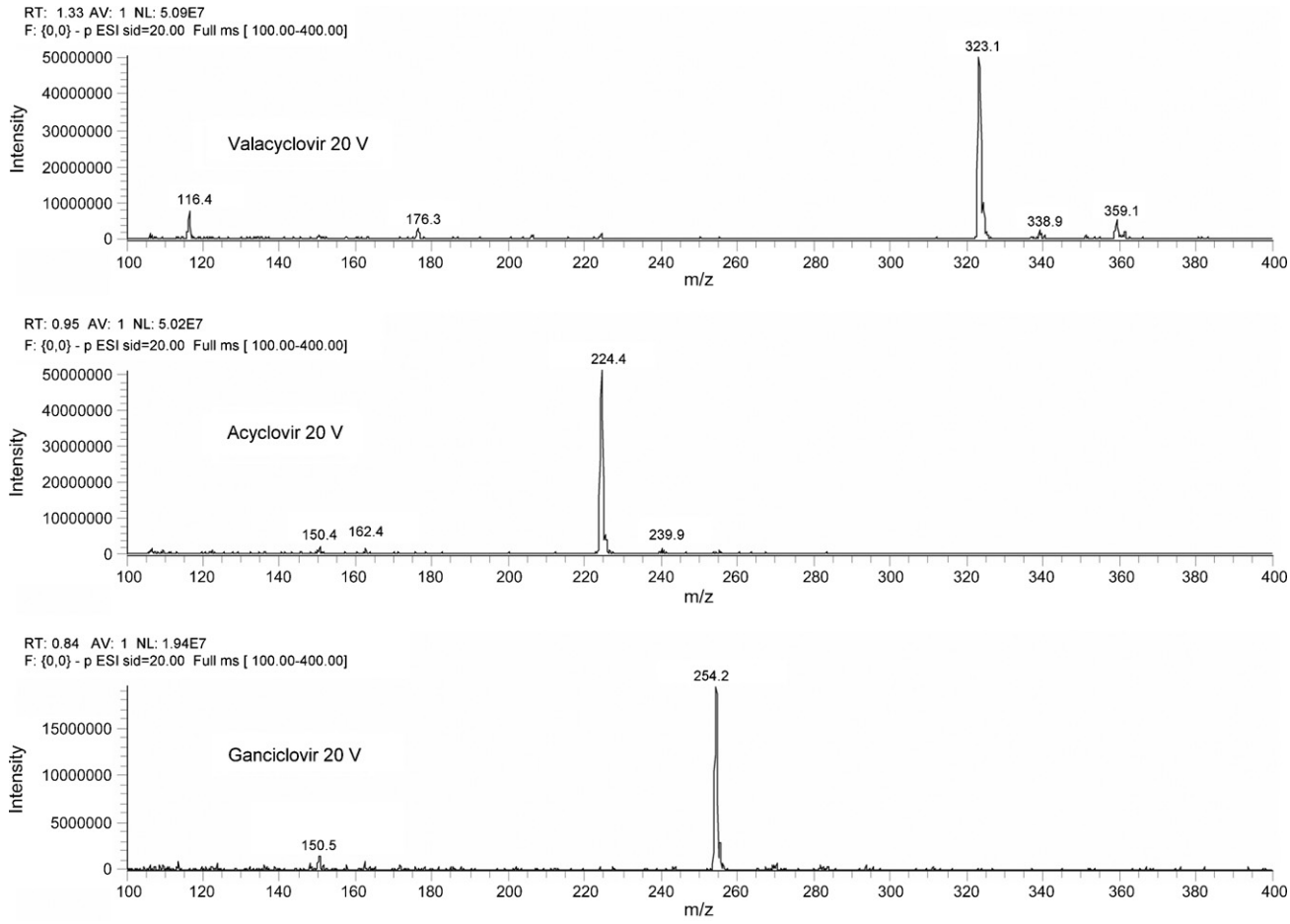


Fig. 1. ESI electrospray mass spectra of 25.0 μg mL^{-1} valacyclovir standard solution, 25.0 μg mL^{-1} acyclovir standard solution and 25.0 μg mL^{-1} ganciclovir standard solution prepared in a mixture of 50% acetonitrile in water containing 0.05% (v/v) diethylamine under the optimal cone voltage conditions of 20 V. MS conditions: negative ESI mode; ESI probe temperature 270 °C; capillary voltage 4.0 kV; flow rate 50 μL min^{-1}.

IS were evidenced in ESI negative-ion mode by the deprotonated molecular ions at *m/z* 323.0, 224.0 and 254.0, respectively. Moreover, under the optimum mass spectrometric conditions the deprotonated molecular ion of valacyclovir at *m/z* 323.0 is abundantly generated and no in source fragmentation of valacyclovir to acyclovir is observed. Experiments by injecting valacyclovir alone and monitoring acyclovir indicate that there is no acyclovir formation under the optimum mass spectrometric conditions.

3.2. Chromatography

Valacyclovir and acyclovir are highly polar compounds that were eluted close to the solvent front with the classical reversed-phase stationary phases that were tested (C_{18} and C_8). Porous graphitized carbon (PGC) columns are most applicable to the separation of polar ionic molecules that are not retained with classical reversed-phase HPLC columns. Moreover, the unique chemical stability of PGC columns over the full pH range allows separation with strongly acidic and basic mobile phases and makes those columns an excellent choice for the combination with mass spectrometric detection. Various combinations of acetonitrile or methanol as organic modifiers and water with changed content of each component and the percentage of basic modifiers (triethylamine and diethylamine) in the aqueous component of the mobile phase were investigated to identify the optimal mobile phase composition that produced the best sensitivity and peak shape for all the analytes. It was found that the use of methanol as organic modifier improves the retention of acyclovir but showed wide peaks for valacyclovir and increases greatly the backpressure of the LC system. On the other hand, acetonitrile as organic modifier improves the chromatographic retention and peak shapes of both analytes and the IS. In particular, an increase in the content of acetonitrile as organic modifier in the mobile phase could improve peak shape, whereas an increase in water content broadened the peak. The effect of different electronic modifiers on the retention and MS signal of the analytes was thoroughly investigated. Triethylamine, as basic modifier in the mobile phase, improves the chromatography of valacyclovir, but lead to broad assymetrical peaks for acyclovir. Diethylamine was chosen over triethylamine as it improves the chromatography of both compounds without the disadvantage of ionization suppression that is caused be the use of triethylamine in the mobile phase [23,24].

A mobile phase consisting of 50% acetonitrile in water containing 0.05% diethylamine was chosen as the optimum mobile phase composition. Each chromatographic run was

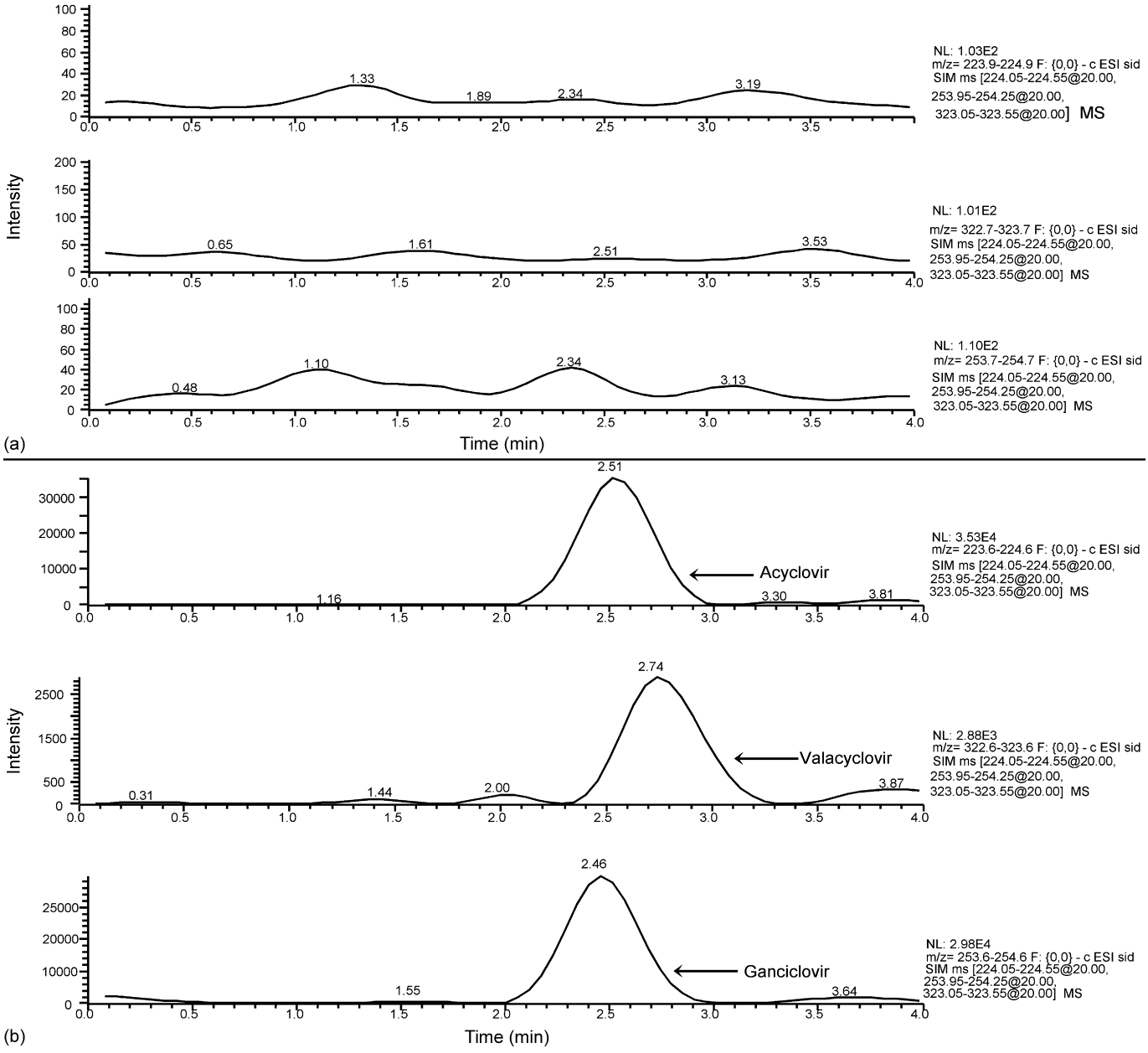


Fig. 2. (a) Smoothed and normalized mass chromatogram (SIM mode) of a blank plasma extract (top), (b) along with a smoothed and normalized mass chromatogram (SIM mode) of a calibration plasma sample spiked with 0.32 μg mL^{-1} valacyclovir, 8.00 μg mL^{-1} acyclovir and 2.5 μg mL^{-1} of the IS; the retention times of valacyclovir, acyclovir and ganciclovir are 2.74, 2.51 and 2.46 min, respectively.

completed within 4.0 min. A representative MS chromatogram obtained from the analysis of blank plasma sample along with a chromatogram obtained from the analysis of a sample spiked with 0.32 μg mL^{-1} valacyclovir and 8.0 μg mL^{-1} acyclovir are presented in Fig. 2a and b, respectively. Under the current chromatographic conditions valacyclovir, acyclovir and ganciclovir were eluted at 2.74, 2.51 and 2.46 min, respectively.

3.3. Statistical analysis of data

Spiked plasma calibration standards containing valacyclovir and acyclovir were analysed in duplicate in five different analytical runs for the calibration procedure. Linear relationships between the ratios of the peak area signals of the analytes to that of the internal standard and the corresponding concentrations were observed as shown by the equations presented in Table 1. In all cases, a weighting factor of $1/y^2$ was used due to data heteroscedascity and because of the consistently better results regarding to other weighting schemes ($1/y$, $1/x$, $1/x^2$) and to unweighted linear regression that were also tested. Back-calculated concentrations in the calibration curves were within 15% of the nominal values, which are in agreement with international guidelines [25] and indicate that the linear model acceptably describes the relationship between concentration and response. The average regression equations of five calibration curves over a period of 1 month, the standard deviation values of

Table 1
Analytical concentration parameters of the calibration equations for the determination of valacyclovir and its metabolite, acyclovir, by LC–ESI/MS

Compound	Concentration range ($\mu g\,mL^{-1}$)	Regression equations[a]	r[b]	Standard deviation		S_r[c]	α/S_α[d]
				Slope	Intercept		
Representative calibration data for one of the runs							
Valacyclovir	0.020–0.800	$R_{Valcv} = 0.3094 \times C_{Valcv} - 0.00122$	0.997	0.0095	5.2×10^{-4}	0.068	2.34
Acyclovir	0.100–20.00	$R_{Acv} = 0.1108 \times C_{Acv} + 0.00146$	0.998	0.0024	7.6×10^{-4}	0.057	1.92
Mean of five calibration curves over a period of 1 month							
Valacyclovir	0.020–0.800	$R_{Valcv} = 0.3172 \times C_{Valcv} - 0.00133$	≥0.997	0.0062	3.6×10^{-4}	≤0.068	≤2.38
Acyclovir	0.100–20.00	$R_{Acv} = 0.1159 \times C_{Acv} + 0.00088$	≥0.997	0.0034	4.9×10^{-4}	≤0.076	≤1.92

[a] Ratios of the peak areas signals of valacyclovir, R_{Valcv}, and acyclovir, R_{Acv}, to that of the internal standard (ganciclovir) vs. the corresponding concentration of valacyclovir, C_{Valcv}, and acyclovir, C_{Acv}, respectively.
[b] Correlation coefficient.
[c] Standard error of the estimate.
[d] Theoretical value of t at $P = 0.05$ level of significance for valacyclovir $f = n - 2 = 6$ df, 2.45; for acyclovir $f = n - 2 = 7$ df, 2.36.

the slopes and intercepts along with the correlation coefficients are also presented in Table 1.

A Student's t-test was performed to determine whether the experimental intercepts (α) of the above-mentioned regression equations were significantly different from the theoretical zero value. The test is based on the calculation of the quantities $t = \alpha/S_\alpha$, where α is the intercept of the regression equations and S_α is the standard deviation of α, and their comparison with tabulated data of the t-distribution. The calculated t-values are also presented in Table 1; these values do not exceed the 95% criterion of $t_p = 2.45$ for $f = 6$ degrees of freedom for valacyclovir and of $t_p = 2.36$ for $f = 7$ degrees of freedom for acyclovir, which denotes that the intercept of all regression lines are not significantly different from zero. The insignificance of intercepts indicates that there is no matrix effect.

The limits of detection, LOD, and the limits of quantitation, LOQ, for valacyclovir and acyclovir were determined according to the definitions of ICH Topic Q2B [26]. In particular, the LOD was calculated using the equations $y - \alpha = 3.3 \times S\alpha$ and $y - \alpha = b \times \text{LOD}$, while the limit of quantitation, LOQ, was attained using the equations $y - \alpha = 10 \times S\alpha$ and $y - \alpha = b \times \text{LOQ}$ (where b is the slope and $S\alpha$ is the standard deviation of the intercept of the regression line). In particular, LOD and LOQ were calculated taking under consideration data obtained from the calibration equations presented in Table 1. For valacyclovir, average values of LOD and LOQ were found to be $0.0066 \pm 0.0016\,\mu g\,mL^{-1}$ and $0.0199 \pm 0.0050\,\mu g\,mL^{-1}$, respectively. For acyclovir average values of LOD and LOQ were found to be $0.0198 \pm 0.0035\,\mu g\,mL^{-1}$ and $0.092 \pm 0.011\,\mu g\,mL^{-1}$, respectively.

One-way analysis of variance (ANOVA) was used to evaluate the intra- and inter-assay precision. Results presented in Table 2 indicate that intra-assay coefficients of variations, % CV, were between 3.3 and 4.5% for valacyclovir and between 3.6 and 3.9% for acyclovir. The inter-assay % CVs were lower than 4.1% for valacyclovir, while for acyclovir were lower than 2.1%. The overall accuracy was assessed by the relative percentage error, absolute % E_r that was ranged from −2.4 to 0.4% for valacyclovir, and from 0.6 to 4.4% for acyclovir.

Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. In particular blank samples were injected twice after high concentration calibration plasma samples containing $0.80\,\mu g\,mL^{-1}$ valacyclovir, $20.00\,\mu g\,mL^{-1}$ acyclovir and $2.50\,\mu g\,mL^{-1}$ of the IS. In all cases peak areas of the analytes in the blank sample that follows the high concentration sample were less than 20% of the peak areas of the analytes in calibration plasma samples in the LOQ level containing $0.02\,\mu g\,mL^{-1}$ valacyclovir, $0.10\,\mu g\,mL^{-1}$ acyclovir and $2.50\,\mu g\,mL^{-1}$ of the IS. Moreover, no ghost peaks appears during the analysis of blank samples.

The selectivity of the proposed method towards endogenous plasma compounds was tested in six different lots of drug-free human plasma by analysing blanks (non-spiked plasma samples) and plasma samples spiked with valacyclovir and acyclovir at their corresponding LOQ levels and internal standard at $2.50\,\mu g\,mL^{-1}$. Mass chromatograms of six batches of drug-free plasma contained no co-eluting peaks greater than 15% of the area of each one of the analytes at the LOQ level, and no co-eluting peaks greater than 5% of the area of the internal standard ganciclovir. The concentrations of the analytes obtained after the analysis of the six different lots of human plasma were found to be $0.0189 \pm 0.0014\,\mu g\,mL^{-1}$ and $0.1026 \pm 0.0033\,\mu g\,mL^{-1}$ for valacyclovir and acyclovir, respectively and indicate that the matrix effect does not appreciably affect the assay.

The suppression or enhancement of ionization of the analytes and the IS by the presence of matrix components in plasma samples (matrix effect) was also evaluated by analyzing plasma extracts from different sources (lots) spiked with the analytes and the IS after the sample preparation procedure. The MS response (peak areas) of the analytes and the IS at any given concentration spiked after the sample preparation procedure into a plasma extract was compared to the MS response of the analytes and the IS in acetonitrile:water (3:1, v/v) [27]. For this purpose, six individual lots of drug-free human plasma were processed according to the sample preparation procedure and then spiked with $0.32\,\mu g\,mL^{-1}$ valacyclovir, $8.00\,\mu g\,mL^{-1}$ acyclovir and IS at $2.50\,\mu g\,mL^{-1}$. Subsequently, standard solutions of both analytes and the IS in equivalent concentration were prepared

Table 2
Accuracy and precision evaluation of quality control samples for valacyclovir and acyclovir ($n = 5$ runs, five replicates per run)

Compound	Concentration ($\mu g\,mL^{-1}$)		
	0.02000	0.320	0.800
Valacyclovir			
Run 1 (mean ± S.D.)	0.01858 ± 0.00035	0.307 ± 0.019	0.794 ± 0.041
Run 2 (mean ± S.D.)	0.01858 ± 0.00045	0.312 ± 0.013	0.798 ± 0.019
Run 3 (mean ± S.D.)	0.02028 ± 0.00091	0.317 ± 0.013	0.800 ± 0.026
Run 4 (mean ± S.D.)	0.02011 ± 0.00095	0.315 ± 0.011	0.816 ± 0.015
Run 5 (mean ± S.D.)	0.02008 ± 0.00076	0.319 ± 0.013	0.808 ± 0.028
Overall mean	0.01953	0.314	0.803
Intra-assay CV (%)[a]	3.7	4.5	3.3
Inter-assay CV (%)[a]	4.1	N.V.[b]	N.V.[b]
Overall accuracy, % E_r[c]	−2.4	−1.9	0.4

Compound	Concentration ($\mu g\,mL^{-1}$)			
	0.1000	0.500	8.00	20.00
Acyclovir				
Run 1 (mean ± S.D.)	0.1026 ± 0.0033	0.517 ± 0.016	7.91 ± 0.41	19.8 ± 1.1
Run 2 (mean ± S.D.)	0.1050 ± 0.0042	0.538 ± 0.022	8.23 ± 0.41	19.71 ± 0.72
Run 3 (mean ± S.D.)	0.1010 ± 0.0051	0.519 ± 0.014	8.40 ± 0.22	19.78 ± 0.73
Run 4 (mean ± S.D.)	0.1009 ± 0.0037	0.514 ± 0.018	8.26 ± 0.11	20.71 ± 0.32
Run 5 (mean ± s.d.)	0.0974 ± 0.0032	0.521 ± 0.025	8.19 ± 0.18	20.57 ± 0.50
Overall mean	0.1014	0.522	8.20	20.12
Intra-assay CV (%)[a]	3.9	3.8	3.6	3.6
Inter-assay CV (%)[a]	2.1	N.V.[b]	1.5	1.7
Overall accuracy, % E_r[c]	1.4	4.4	2.5	0.6

[a] Coefficient of variation; intra- and inter-assay CVs were calculated by ANOVA.
[b] N.V.: No significant additional variation was observed as a result of performing the assay on different runs.
[c] Relative percentage error = [(overall mean assayed concentration − added concentration)/(added concentration) × 100].

in acetonitrile:water (3:1, v/v) and analysed according to the proposed method. Matrix factor was used to quantitatively measure the matrix effect and is defined as the ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions. Matrix factors obtained from the analysis of six different lots of human plasma were found to be 0.77 ± 0.02, 0.87 ± 0.01 and 0.90 ± 0.06, for valacyclovir, acyclovir and ganciclovir, respectively. The variability in matrix factors was expressed by coefficients of variation that were found to be 9.3, 7.9 and 8.1% for valacyclovir, acyclovir and ganciclovir, respectively, and are in agreement with international guidelines [28].

The absolute recovery of the proposed extraction procedure was determined by calculating the ratio of the analytical responses of QC samples to the analytical response of blank plasma samples that were processed according to the sample preparation procedure and then spiked with equivalent concentrations of the analytes and the IS that represent 100% recovery. The data presented in Table 3 indicate average recovery of more than 98.1% for valacyclovir and more than 97.4% for acyclovir. The recovery for ganciclovir (IS) was found to be 99.2 ± 1.9% at 2.50 $\mu g\,mL^{-1}$.

The stability results presented in Table 4 indicate that the analytes can be considered stable under the various conditions investigated. In particular, valacyclovir concentrations deviates by no more than −6.2% relative to the reference while acyclovir concentrations deviates by no more than −8.2% relative to the reference, while no degradation products were observed for any of the tests. Calibration plasma samples containing valacyclovir and its metabolite (acyclovir) may therefore be kept for up to 4 h at ambient temperature, 21 days at −20 °C, and after three freeze–thaw cycles (7 days per cycle) at −20 °C without any significant degradation.

3.4. Sample analysis

The method was applied to the analysis of plasma samples obtained after oral administration of valacyclovir. The local ethics committee approved the studies on human patients. A male and a female patient were used in the study, aged 34 and

Table 3
Recovery data of the extraction procedure for valacyclovir, acyclovir and ganciclovir

Compound	Concentration levels ($\mu g\,mL^{-1}$)	% Recovery (mean ± S.D., $n = 3$)
Valacyclovir	0.02	97.7 ± 1.1
	0.32	97.8 ± 1.0
	0.80	98.7 ± 1.7
Acyclovir	0.50	97.0 ± 1.2
	8.00	97.2 ± 1.0
	20.00	97.9 ± 1.6
Ganciclovir	2.50	99.2 ± 1.9

Table 4
Stability data for valacyclovir and acyclovir in human plasma under various storage conditions

Storage conditions/time	Calculated concentration ($\mu g\,mL^{-1}$), mean ± S.D. ($n = 3$)				% E_r*			
	Valacyclovir		Acyclovir		Valacyclovir		Acyclovir	
	0.080	0.60	2.00	15.00	0.080	0.60	2.00	15.00
Ambient temperature/4 h	0.0735 ± 0.0020	0.589 ± 0.026	1.948 ± 0.062	14.87 ± 0.19	−2.3	−2.0	−1.8	−3.4
−20 °C/20 days	0.0731 ± 0.0011	0.583 ± 0.013	1.928 ± 0.028	14.72 ± 0.28	−2.6	−2.4	−5.6	−4.5
−20 °C/1 freeze–thaw cycles	0.0758 ± 0.0018	0.564 ± 0.028	1.958 ± 0.093	14.35 ± 0.45	−4.6	−4.1	−3.9	−3.0
−20 °C/3 freeze–thaw cycles	0.0646 ± 0.0019	0.540 ± 0.023	1.912 ± 0.013	14.06 ± 0.10	−6.1	−8.2	−5.6	−6.2

%E_r: Relative percentage error = (overall mean assayed concentration − added concentration)/(added concentration) × 100.

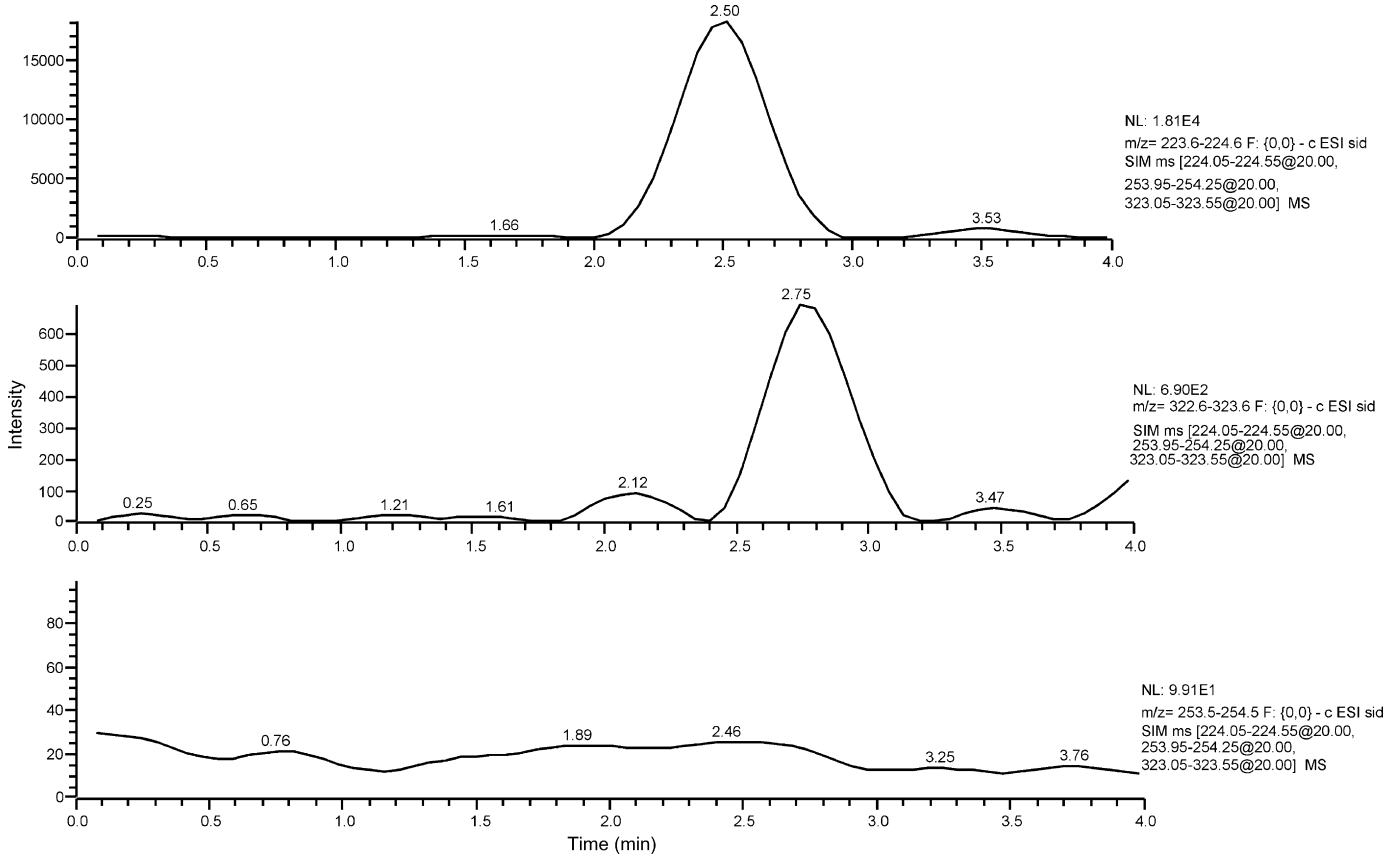


Fig. 3. Smoothed and normalized mass chromatogram (SIM mode) obtained from the analysis of a clinical sample containing valacyclovir and acyclovir. No IS was added to the extracted plasma sample. Valacyclovir and acyclovir are eluted at 2.75 and 2.50 min, respectively.

40 years old, respectively. The patients were receiving 1000 mg valacyclovir two times daily (Valtrex® 500 mg, Glaxo Wellcome) for a weekly treatment. Samples were collected during the fourth day of the treatment. Blood samples were collected 2 h after dosing in Venoject® tubes containing lithium heparin as anticoagulant. Immediately after drawing, the samples were shaken gently and centrifuged at 4000 rpm for 10 min at 4 °C. All plasma samples were analysed in triplicate 2 days after storage at −20 °C, according to the sample preparation procedure. Each plasma sample contained 2.5 $\mu g\,mL^{-1}$ of the internal standard, by the addition of 50 μL of the 100.0 $\mu g\,mL^{-1}$ ganciclovir working standard solution. Plasma sample obtained from the female patient exhibited valacyclovir and acyclovir plasma concentrations 0.233 ± 0.015 and 6.30 ± 0.16 $\mu g\,mL^{-1}$, respectively. Plasma sample of the male patient exhibited valacyclovir and acyclovir plasma concentrations 0.228 ± 0.021 and 7.04 ± 0.28 $\mu g\,mL^{-1}$, respectively. Fig. 3 illustrates a MS chromatogram obtained from the analysis of a patient's plasma sample without IS addition so as to demonstrate that there is no interference at the retention time of ganciclovir (IS).

4. Conclusions

The proposed LC/ESI-MS method enables a rapid, accurate and selective assay for the determination of valacyclovir and its metabolite (acyclovir) in human plasma with a run time lower than 4.0 min. The method consists of a simple sample pretreatment with protein precipitation and a reversed-phase

liquid chromatographic procedure that was performed on a porous graphitized carbon column. Negative-ion electrospray mass spectrometric detection increases the sensitivity and selectivity of the proposed method. The proposed method with a LOQ of 0.02 $\mu g\,mL^{-1}$ for valacyclovir and 0.10 $\mu g\,mL^{-1}$ for acyclovir is suitable to support a wide range of pharmacokinetic or bioequivalence studies.

Acknowledgment

The Special Account for Research Grants, University of Athens, Greece supported this work financially.

References

[1] D. Ormrod, L.J. Scott, C.M. Perry, Drugs 59 (2000) 839.
[2] J.J. O'Brien, D.M. Campoli-Richards, Drugs 37 (1989) 233.
[3] C.P. Landowski, D. Sun, D.R. Foster, S.S. Menon, J.L. Barnett, L.S. Welage, C. Ramachandran, G.L. Amidon, J. Pharmacol. Exp. Ther. 306 (2003) 778.
[4] K.R. Beutner, Antiviral Res. 28 (1995) 281.
[5] A.E. Thomsen, M.S. Christensen, M.A. Bagger, B. Steffansen, Eur. J. Pharm. Sci. 23 (2004) 319.
[6] D.D. Phan, P. Chin-Hong, E.T. Lin, P. Anderle, W. Sadee, B.J. Guglielmo, Antimicrob. Agents Chemother. 47 (2003) 2351.
[7] F.G. Hayden, Goodman & Gilman. The pharmacological basis of therapeutics, vol. 50, McGraw Hill, New York, 2001, p. 1313.
[8] B.S. Anand, S. Katragadda, A.K. Mitra, J. Pharmacol. Exp. Ther. 311 (2004) 659.
[9] C. Dias, Y. Nashed, H. Atluri, A. Mitra, Curr. Eye Res. 25 (2002) 243.
[10] D. Teshima, K. Otsubo, T. Yoshida, Y. Itoh, R. Oishi, Biomed. Chromatogr. 17 (2003) 500.
[11] R. Boulieu, C. Gallant, N. Silberstein, J. Chromatogr. B 693 (1997) 233.
[12] J.M. Poirier, N. Radembino, P. Jaillon, Ther. Drug Monitor. 21 (1999) 129.
[13] R.A. Bangaru, Y.K. Bansal, A.R.M. Rao, T.P. Gandhi, J. Chromatogr. B: Biomed. Sci. Appl. 739 (2000) 231.
[14] M. Fernández, J. Sepúlveda, T. Aránguiz, C.V. Plessing, J. Chromatogr. B: Biomed. Sci. Appl. 793 (2003) 357.
[15] Gh. Bahrami, Sh. Mirzaeei, A. Kiani, J. Chromatogr. B: Biomed. Sci. Appl. 816 (2005) 327.
[16] S.D. Brown, C.A. White, C.K. Chu, M.G. Bartlett, J. Chromatogr. B: Biomed. Appl. 772 (2002) 327.
[17] S.S. Zhang, H.X. Liu, Y. Chen, Z.B. Yuan, Biomed. Chromatogr. 10 (1996) 256.
[18] H.C. Vo, P.A. Henning, D.T. Leung, S.L. Sacks, J. Chromatogr. B: Biomed. Appl. 772 (2002) 291.
[19] S.D. Brown, C.A. White, M.G. Bartlett, Rapid Commun. Mass Spectrom. 16 (2002) 1871.
[20] C. Pham-Huy, F. Stathoulopoulou, P. Sandouk, J.M. Scherrmann, S. Palombo, C. Girre, J. Chromatogr. B 732 (1999) 47.
[21] G.E. Granero, G.L. Amidon, Int. J. Pharm. 317 (2006) 14.
[22] M.L. Palacios, G. Demasi, M.T. Pizzorno, A.I. Segall, J. Liq. Chromatogr. Rel. Technol. 28 (2005) 751.
[23] S.Å. Gustavsson, J. Samskog, K.E. Markides, B. Långström, J. Chromatogr. B: Biomed. Appl. 937 (2001) 31.
[24] J. Xing, A. Apedo, A. Tymiak, N. Zhao, Rapid Commun. Mass Spectrom. 18 (2004) 1599.
[25] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[26] ICH Topic Q2B, Pharmaeuropa, 8 (1996) 108.
[27] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[28] C.T. Viswanathan, S. Bansai, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J 9 (1) (2007) E10.