Research article

Biomedical Chromatography

Received 12 November 2010, Accepted 15 November 2010 Published online in Wiley Online Library: 14 March 2011

(wileyonlinelibrary.com) DOI 10.1002/bmc.1590

Controlled *ex-vivo* plasma hydrolysis of valaciclovir to acyclovir demonstration using tandem mass spectrometry

Dipanjan Goswami[a]*, Arshad Khuroo[a], Sanjay Gurule[a], Yogesh Modhave[a] and Tausif Monif[a]*

ABSTRACT: Plasma estimation of valaciclovir, an antiviral drug, is challenging due to both *in-vivo* and *ex-vivo* hydrolysis to active metabolite acyclovir. A simultaneous method is described involving the solid-phase ion-exchange extraction procedure requiring 100 µL of plasma volume, a reverse-phase Lichrosphere RP Select B (125 × 6 mm, 5 µm) column and isocratic mobile phase to achieve the desired chromatographic separation. ESI-MS/MS multiple reaction monitoring in positive polarity, detected mass pairs for valaciclovir (*m/z* 325.5 → 152.2), acyclovir (*m/z* 226.3 → 152.2) and respective internal standards valganciclovir (*m/z* 307.1 → 220.3) and acyclovir-d4 (*m/z* 230.2 → 152.0). Fully fledged method validation was evaluated as per current regulatory requirements and results were deemed acceptable. The plasma samples showed extensive hydrolysis of valaciclovir when collected or processed at room temperature, without buffer stabilization prior to storage at −15°C. Our results showed that using prechilled K_3EDTA vacutainers immersed in an iced-water bath during blood sample collection, and addition of 50% *ortho*phosphoric acid solution to plasma samples prior to storage at −50°C for at least 120 days controlled the hydrolysis of valaciclovir to acyclovir. While monitoring drug absorption into systematic circulation, the valaciclovir to acyclovir formation ratio was improved to 1:20 in healthy volunteers for the first time. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: valaciclovir controlled hydrolysis; plasma stability; simultaneous method development/validation; LC-MS/MS; drug: active metabolite formation ratio

Introduction

Ex-vivo hydrolysis of ester drug/s or labile metabolites on the bench top is a frequent occurrence that poses serious challenges to bioanalysis. Bioanalysts have to adroitly revive the analytical steps to minimize such interconversion between drug and metabolites for accurate estimation of pharmacokinetic parameters. One such drug is valaciclovir, L-valine-2-[(2-amino-1, 6-dihydro-6-oxo-9H-purin- 9yl) metonym] ethyl ester, VCV], a pro-drug of the antiviral drug acyclovir (ACV; 9-[2-hydroxy ethoxy]-methyl guanine), which had attracted interest due to its rapid and extensive metabolism in the liver to yield ACV and an essential amino acid L-valine (Ormrod *et al.*, 2000). The pharmacokinetic variability of VCV is highly dependant on pH stabilization as per previous communications (Granero and Amidon, 2006; Soul-Lawton *et al.*, 1995). Many difficulties have to be overcome to analyse both VCV and ACV simultaneously and accurately. Both compounds are highly polar with unavoidable retention problems in reverse-phase columns. To the best of the knowledge of the authors, previous assay methods cannot accurately provide suffucient insight regarding *in-vivo* formation of ACV from VCV, due to the lack of a systematic approach to controlling *ex-vivo* VCV hydrolysis (Weller *et al.*, 1993; Pham-Huy *et al.*, 1999). Goswami *et al.* (2009) showed that in bioanalytical research, the drug–active metabolite formation ratio has a major impact on the merit and accuracy of assay methods. Although analytical methods for monitoring plasma levels of VCV or ACV in biological matrix are available, either ACV is estimated (Brown *et al.*, 2002) or there is a lack of sensitivity in simultaneous determination (Kasiari *et al.*, 2008). Very few authors have reported simultaneous determination of both analytes with a sensitive rugged bioanalytical method (Yadav *et al.*, 2009; Kanneti *et al.*, 2009) that can mimic actual clinical sample analysis. Human plasma contains esterases like butyryl-cholinesterase, paraoxonase, albumin esterase and acetyl cholinesterase which might impart age-, sex- or race- related variability while evaluating the pharmacokinetic profile of an ester labile pro-drug molecule. Kim *et al.* (2003) reported that

* Correspondence to: D. Goswami, Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Laboratories Ltd, Plot no. GP-5, HSIDC, Sector-18, Udyog Vihar Industrial Area, Old Delhi Gurgaon Road, Gurgaon 122 015, Haryana, India. E–mail: dipanjan.goswami@ranbaxy.com

T. Monif, Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Laboratories Ltd., Plot no. GP-5, HSIDC, Sector-18, Udyog Vihar Industrial Area, Old Delhi Gurgaon Road, Gurgaon 122 015, Haryana, India. E–mail: tausif.monif@ranbaxy.com

[a] Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Laboratories Ltd, Plot no. GP-5, HSIDC, Sector-18, Udyog Vihar Industrial Area, Old Delhi Gurgaon Road, Gurgaon 122 015, Haryana, India

Abbreviations used: ACV, acyclovir, 9-[2-hydroxy ethoxy]-methyl guanine; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; RPLC, reverse phase liquid chromatography; VCV, valaciclovir, L-valine-2-[(2-amino-1, 6-dihydro-6-oxo-9H-purin- 9yl) metonym] ethyl ester.

valaciclovir-hydrolysing enzyme (hVACVase), found in human cells, is characterized as an enzyme capable of significant hydrolysis of VCV with its identity with the biphenyl hydrolase-like (BPHL) protein.

Although Yadav *et al.* (2009) tried to control the VCV hydrolysis step on the bench-top, conversion of VCV to ACV during the clinical phase could not be inferred. The researchers would have demonstrated the control effect of addition of buffer to conclude that VCV conversion to ACV is not only controlled but also that there is no impact on ACV samples in the presence of buffer. Unless the control samples are run or extra precautions are taken during the clinical phase, *in-situ* drug degradation of labile ester drugs cannot be arrested due to exposure to room temperature or the absence of stabilizing agents before plasma is frozen prior to analysis. Finally, when samples are packed with dry ice during transportation, carbon dioxide can affect the stability of pH sensitive drugs. Another major concern nowadays is the handling of haemolysed plasma subject samples received from clinics, which is unavoidable in spite of the adoption of precautions during blood collection, separation and transport of plasma samples. Hughes *et al.* (2009) reported that haemolysed sample should not impact clinical data, and rejection of pharmacokinetic data just on the ground of haemolysis is not acceptable. Along with selectivity, matrix effect and precision and accuracy batches need to be run with haemolysed plasma during validation, which has not been detailed in any of the previous reports to the best knowledge of the authors. Therefore an improved LC-MS/MS simultaneous method, achieving high VCV sensitivity (lower limit of quantitation, LLOQ, 4 ng/mL) with adequate recovery and confirming minimal matrix effect in haemolysed and non-haemolysed plasma is delineated. Employing an applied Biosystem API 3000 at unit mass resolution involving 100 µL of plasma volume along with 10 µL of injection load can be deemed as recent progress in simultaneous determination of VCV and ACV in human plasma. Moreover a unique clinical phase, with innovative steps to control *in-situ* metabolism, is envisaged for the first time to accurately evaluate the VCV:ACV formation ratio in terms of the rate of absorption into systemic circulation.

Experimental

Chemicals and reagents

Reference standards of valaciclovir hydrochloride (99.0% purity) and valganciclovir (92.3% purity) were purchased from Vardha Biotec (India). ACV (94.9% purity) was procured from USP and acyclovir-d4 (99.9% purity) was obtained from Toronto Research Centre (Canada). Ammonia solution (32% purity) was purchased from Merck (Goa, India) and *ortho*phosphoric acid (88% purity) was obtained from Qualigens (Mumbai, India). Methanol and acetonitrile (extra pure HPLC grade; purity > 99%) were purchased from S.D. Fine Chemicals (Mumbai, India). Qualigens (India) supplied formic acid (85% purity). HPLC-grade dimethyl sulfoxide (99.9% purity) was obtained from Spectrochem Pvt. Ltd (Mumbai, India). Solid-phase extraction cartridges were sourced by Waters (USA) and HPLC-grade water, used in the entire analysis, was prepared from a Milli-Q water purification system (Millipore, Moscheim Cedex, France). Drug-free K_3EDTA human plasma (one year expiry) was obtained from healthy volunteers from Ranbaxy Clinical Pharmacology Unit (Majeedia, New Delhi, India), and haemolysed plasma (2% label claimed with one year expiry) was procured from Yash Laboratories (Mumbai, India). All categories of plasma samples were stored at −15°C until use. Human plasma batches, free from significant interference, were used to prepare calibration standards (calibrator) and quality control samples (control).

Calibrator and control sample preparation

The preparation of aqueous dilution and spiked samples were carried out under low-light laboratory conditions in an iced-water bath. The calibrators and control samples, prepared in methanol, were spiked in K_3EDTA-anticoagulated drug-free human plasma using separate stock solutions. Eight non-zero calibrators at nominal values of 4.03, 9.19, 22.96, 51.03, 113.41, 252.22, 560.14 and 702.68 ng/mL and 50.08, 124.45, 320.60, 803.52, 2010.17, 4019.00, 8038.94 and 10031.73 ng/mL concentrations for VCV and ACV, respectively, were prepared. Four control samples at nominal values of 4.15, 10.45, 110.00 and 550.00 ng/mL for VCV and 50.78, 125.39, 2006.25 and 8025.00 ng/mL for ACV designated as respective LOQQC (lowest), LQC (low), MQC (middle) and HQC (high) were also prepared. Calibrators and control plasma samples, spiked with 50% *ortho*phosphoric acid solution prepared in HPLC-grade water in a ratio of 96:4 v/v (plasma: buffer) were stored at −50°C until analysis, and aqueous stock solution which were further used for preparation of dilutions were refrigerated at 2–10°C.

Human plasma extraction

All frozen samples (calibrators, control and study samples) were retrieved from the freezer and allowed to thaw in an iced-water bath under low light conditions. A simple sample processing technique using a solid-phase ion exchange extraction principle was followed. The plasma sample of 0.100 µL aliquot was vortex mixed with 50 µL of IS dilution stock (approximately 1000.00 ng/mL of valganciclovir and 2000.00 ng/mL of acyclovir-d4) solution and subsequently buffered with 0.750 mL of 5% *ortho*phosphoric acid solution prepared in HPLC-grade water. The buffered samples were loaded onto Oasis MCX extraction cartridges (30 mg/1 cc) which were earlier preconditioned with 1 mL of methanol, followed by 1 mL of water. The loaded samples were washed with 1 mL of 0.1% *ortho*phosphoric acid solution and eluted with 1.0 mL of freshly prepared 5% ammoniated methanol solution, twice. The eluted contents were dried in a stream of nitrogen at 50 ± 2°C at about 15 psi and reconstituted with 500 µL of 1 mM ammonium acetate buffer (pH 3.2):methanol 35:15 v/v. The reconstituted samples of 10 µL volume were used for injection in the chromatographic system.

Liquid chromatography

VCV, ACV and internal standards (IS) were separated on a Lichrosphere RP Select B (125 × 4.6 mm, 5 µm) analytical column, maintained at 35°C using an HPLC system (Shimadzu LC-VP from Kyoto, Japan). The mobile phase, composed of methanol:1 mM ammonium acetate buffer (pH found 6.2; 70: 30 v/v), was pumped isocratically at a flow rate of 0.8 mL/min. All compounds were eluted within 4.0 min from the column with split ratio of 70:30 v/v and flow directed to the ion source interface was equivalent to 240 µL/min. However the chromatographic run was continued until 5.0 min to show that there are no co-eluting peaks from the endogenous component of plasma even with haemolysed plasma samples and incurred (confirmatory reanalysis) samples.

Mass spectrometry

The ionization and detection were carried out on a triple quadruple mass spectrometer, MDS Sciex API-3000 (Sciex Division of MDS Ontario, Canada), equipped with electrospray ionization and operated in positive ion mode using multiple reaction monitoring (MRM). The instrument response was optimized by syringe pump infusion of all compounds at 100 ng/mL concentration, by constant flow (10 µL/min) into the stream of mobile phase eluting from the LC column. The source-dependant optimized parameters for the analysis were as follows: ion source temperature was set at 550°C, ion spray voltage was set at 1500 V and the interface heater was kept on. Ultrapure nitrogen was used as curtain, nebulizer and collision gas, and all were set at 7 psi pressure.

The compound dependant parameters like declustering potential were optimized at 40, 42, 32 and 41 V for VCV, valganciclovir, ACV and

acyclovir-d4 respectively. The focusing potential was set at 175, 175, 160 and 160 V for VCV, valganciclovir, ACV and acyclovir-d4 respectively. The entrance potential was optimized at 7, 8, 6 and 8 V for VCV, valganciclovir, ACV and acyclovir-d4 respectively. The precursor → product ion transitions were monitored at *m/z* 325.2 → 152.2 (collision energy = 24 eV; cell exit potential = 7 V), 226.2 → 152.2 (collision energy = 17 eV; cell exit potential = 10 V), 307.1 → 220.3 (collision energy = 25 eV; cell exit potential = 9 V) and 230.2 → 152.0 (collision energy = 18 eV; cell exit potential = 10 V) for VCV, ACV, valganciclovir and acyclovir-d4, respectively. Dwell time was 200 ms at unit mass resolution. Analyst software version 1.4.1 was used to control all parameters of LC and MS.

Method validation aspects

A fully fledged complete method validation for both analytes was executed corroborating with USFDA guidance (US Department of Health and Human Services, 2001).

Selectivity

The selectivity of the method towards endogenous plasma matrix components, metabolites and concomitant medications was assessed after screening 12 batches of (six sets of non-haemolysed and six sets of haemolysed) K_3EDTA plasma samples. Control samples at LLOQ analyte concentration with (haemolysed) and without the presence of 2% haemolysed blood (non-haemolysed) were analysed in replicates of six and then compared along with blank haemolysed control sample. Selectivity experiment would ensure null interference at the retention time of all compounds.

Linearity and sensitivity

At least three calibration curves were used to demonstrate the linearity of the method. The ratios of area responses for VCV and ACV to IS were used for regression analysis. Each calibration curve was analysed individually by using least square weighted ($1/x^2$) linear regression (obtained by best fit method). Back-calculations were made from these curves to determine the concentration of VCV and ACV in each calibrator. Correlation coefficient (r) > 0.99 was desirable for all the calibration curves. The sensitivity was demonstrated by checking signal and noise in spiked samples at lowest QC concentration. For determination of signal-to-noise (S/N) ratio, four replicates of LOQQC along with pooled blank matrix samples were processed and analysed using both haemolysed and non-haemolysed plasma samples. The S/N of spiked samples was deemed acceptable when:

$$\text{S/N ratio} = \frac{\text{S/N of LOQQC}}{\text{Mean of S/N of blanks}} \geq 5$$

In addition, the analyte peak of LLOQ sample should be identifiable, discrete and reproducible with a precision (%CV) not greater than 20.0 and accuracy within 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15.0%.

Precision and accuracy

The intra- and inter-day accuracy and precision were performed for both analytes in K_3EDTA non-haemolysed plasma. The precision of the method was determined by calculating the percentage coefficient of variation (%CV) at each level. The deviation at each concentration level from the nominal concentration was expected to be within ±15.0% except for the LLOQ, for which it should not be more than 20.0%. Similarly the mean accuracy should not deviate by ±15.0% except for the LLOQ, where it can be ±20.0% of the nominal concentration. A similar set of single precision and accuracy batches was analysed using haemolysed plasma quantitated against freshly spiked calibrators prepared from non-haemolysed K_3EDTA plasma samples, which was chosen from screening and selectivity acceptable batches. Batch acceptance criteria for control samples should be within ±15% when compared against non-haemolysed plasma except at the LLOQ level where ± 20% deviation from nominal concentration was allowed.

Extraction recovery

The extraction efficiency (recovery) of VCV and ACV was performed at LQC, MQC and HQC levels by comparing the mean area response of six replicates of extracted samples (spiked before extraction) with that of unextracted samples (spiked after extraction) at each QC level. Drug and metabolite of comparable concentration was spiked into previously screened K_3EDTA plasma individual lots and injected with aqueous unextracted samples to determine percentage recovery and CV across QC concentrations. As per the acceptance criteria, the %CV across LQC, MQC and HQC should be within 20% and consistent, precise and reproducible.

$$\text{Percentage recovery} = \frac{\text{mean peak response of extracted samples}}{\text{Mean peak response of unextracted samples}} \text{x} 100$$

Matrix factor, matrix effect and stability

For purine analogue, the matrix effect is relatively high and ion suppression/enhancement in haemolysed plasma needs to be evaluated critically. The enhancement of matrix ions in positive ESI mode was checked using a post-column analyte infusion experiment and phospholipid monitoring was carried out using *m/z* transitions. The matrix factor (*MF*) is defined as the peak response in the presence of matrix ions vs the peak response in the absence of matrix ions, prepared using appropriate concentration.

Since our method involved a terminal drying step, biological matrix samples were prepared by reconstituting the post-extracted blank plasma samples with reference solution containing VCV and ACV at concentrations representing the QC concentration (at LQC, MQC and HQC level) and were quantified with their respective IS. The control sample was the reference solution prepared in mobile phase. An absolute matrix factor (IS normalized) was evaluated using six different (drug-free) blank plasma lots (both haemolysed and nonhaemolysed state) and calculated by comparing the peak area response and analyte/IS peak area ratio of biological matrix sample against the peak area response and analyte/IS peak area ratio of reference solution as represented by:

$$MF = \frac{\text{peak response in presence of matrix ions}}{\text{peak response in absence of matrix ions}}$$

where $MF = 1$ signifies no matrix effects and *MF* value <0.8 indicates ion suppression; $MF > 1.2$ indicates ion enhancement (and can also be caused by analyte loss in the absence of matrix during analysis) as per our in-house procedure.

The matrix effect was evaluated using six lots of matrix (both haemolysed and non-haemolysed state), processed in duplicate samples at HQC and LQC level and the area response of analyte/IS was used to check batch acceptability. The standard deviation for each lot was calculated, along with percentage CV and percentage accuracy at each level. The deviation of the standards should not be more than ±15.0% and at least 90% of the lots at each QC level should be within the aforementioned criteria. A stability experiment was carried out to examine the analyte and IS stability in stock solutions and in plasma samples under different conditions. All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison standards with identical concentration. The percentage stability was determined as follows:

$$\text{Percentage stability} = \frac{\text{mean peak area ratio of stability samples}}{\text{mean peak area ratio of freshly spiked comparison samples}} \text{x} 100$$

Stock solutions of VCV, ACV and IS prepared in methanol were checked for short-term stability at room temperature and long-term

stability at 2–10°C. The solutions were considered stable if the deviation from nominal value was within ±15.0%. Autosampler stability (wet extract from solid-phase extraction) and freeze–thaw stability were tested at LQC and HQC using six replicates at each level. Freeze–thaw stability was evaluated by successive cycles of freezing (at −50°C for 24 h) and thawing in an iced-water bath. The long term stability of spiked plasma samples stored at −50°C was also studied at LQC and HQC levels. To authenticate the ruggedness of the method, one precision and accuracy batch was analysed separately using different instruments, but the mass spectrometric model remained unchanged.

Plasma stability in buffered solution

A long-term stability experiment was performed at LQC and HQC level using plasma samples fortified with *ortho*phosphoric acid solution and unbuffered plasma samples, both frozen at −15 and −50°C. The procedure adopted was spiking of VCV in replicates (free from ACV as well as in the presence of ACV) and after 120 days stability was evaluated against freshly spiked calibrators and control samples. The purpose was to trace the degradation of VCV when unbuffered plasma samples worked up in an iced-water bath were stored at −15 and −50°C, compared with buffered plasma samples worked up in iced-water bath and stored at −50°C.

A similar set of long-term storage samples was prepared with ACV replicate controls spiked with *ortho*phosphoric acid solution and was kept with unbuffered plasma samples. The unbuffered plasma samples were run against controls, using ACV plasma in the presence or absence of VCV to assess the impact on ACV from VCV during long-term storage conditions. Moreover ACV sample free from VCV and ACV + VCV (at LQC and HQC concentration level for both analytes) were kept at −15 and −50°C to derive percentage conversion of VCV to ACV with respect to ACV total response/peak area (total represents = ACV + VCV converted to ACV). The mean LQC and HQC were taken to demonstrate percentage degradation of VCV as well as simultaneous conversion to ACV from the above two sets of samples. The samples were monitored in a mass spectrometer simultaneously after a 120 day storage period.

Clinical phase: pharmacokinetic study

A bioavailability study was carried out in accordance with the Principles of Good Clinical practices defined in the Schedule Y, Drug and Cosmetics Act (2005), the ICH E6 'Guidance for Good Clinical Practice' and the principles enunciated in the Declaration of Helsinki (as per the 59th Amendment, 2008). All the subjects were informed about the aim and risks involved in the study and informed consents were obtained. An ethics committee approved the study protocol. A single-dose biostudy was conducted with 1000 mg valaciclovir hydrochloride test (T) and reference (R) tablet formulation administered to eight healthy fasted male volunteers. Blood samples (4 mL each) were collected in prechilled K_3 EDTA vacutainers immersed partially in an iced-water bath pre-dose and at 0.167, 0.250, 0.333, 0.417, 0.500, 0.667, 0.883, 1.000, 1.250, 1.500, 1.750, 2.000, 2.250, 2.500, 2.750, 3.000, 3.500, 4.000, 5.000, 6.000, 8.000, 10.000, 12.000, 16.000, 20.000 and 24.000 h post-dose in each period. Heparin was used for continuous sampling. All post-dose samples were collected, processed and analysed using a cold chain sample handling technique by maintaining ice cold water bath under low light conditions. The buffer solution (50% *ortho*phosphoric acid in HPLC-grade water) was added to plasma (separated by centrifugation of blood samples at 4000 rpm for 15 min at 4 °C) in a ratio of 4: 96 v/v and then samples were vortex mixed. Finally the buffered plasma samples maintained in an iced-water bath even in the post centrifugation phase were frozen at −50°C.

The samples were extracted, quantified for pharmacokinetic evaluation using a validated PK software WinNonlin version 4.0 (Pharsight Corp. Mountain View, CA, USA). An incurred sample reanalysis (ISR) was performed on 40 sample points from eight different subjects selected randomly from VCV study population. Our predefined criteria for choosing sampling time points were at least two time points from each period of these identified eight subjects of which one time point was the T_{max} point and the second was the last time point at which the concentration obtained is at least three times the LLOQ. The basic objective of ISR was to reconfirm the initial values and to demonstrate that the assay is reproducible. The conformity of the original result with the ISR sample is calculated as percentage difference. The percentage difference should be within 20% for at least 67% or two-thirds of the total reanalysed incurred samples (Viswanathan *et al.*, 2007).

$$\text{Percentage difference ISR} = \frac{\text{absolute (reanalysed value} - \text{original value)}}{\text{average of reanalysed and original value}} \times 100$$

Results and discussion

Bio-analytical method development – tuning MS parameter

Method development was focused to suitably optimize the chromatographic and mass spectrometric conditions. The inherent selectivity of MS/MS detection was expected to be beneficial in developing a selective and sensitive method. An intense stable response was derived in ESI positive ionization MRM mode with a signal-to-noise ratio ≥ 95 for all analytes, even in haemolysed plasma samples when compared with negative mode of ionization. The MS scans at *m/z* 226 and 325 for ACV and VCV respectively, with mass fragmentation pattern, are presented in Figs 1 and 2. The ion spray voltage set at 1500 V displayed protonated guanine species at *m/z* 152.2 as the most abundant ion in comparison to 146.3 (Fig. 1) and 135.1 (Fig. 2) product ion mass spectra of VCV and ACV respectively in positive ESI ionization. The occurrence of product ions was typical for the purine analogue used for antiviral treatment and corresponds to cleavage of 2-amino-3-methylbutanoic ethylene ester to yield finally *m/z* transition at 152 amu, thus corroborating previous reports (Yadav *et al.*, 2009). All precursor ions were fragmented using collision activated dissociation gas and by applying collision energy in the range of 17–25 eV to get the most stable *m/z* 152.2 as the fragment ion. Moreover declustering potential, collision energy and collision activated dissociation gas were suitably optimized to fragment the solvent and other interfering ions. The optimized curtain gas set at 7 psi helped in avoiding solvent ions entering the mass interface.

Chromatography optimization

The compounds were separated by isocratic elution and resolution was improved using various combinations of methanol–acetonitrile, acidic buffers and additives like formic acid on different reversed-phase columns. Different set of columns were tried, such as Gemini C_{18}, porous graphitized carbon PGC column Hypersil Gold, Hypurity Advance C_{18} columns and Discovery C_8 columns and limitations were evaluated. While using PGC and C_{18} columns, ion enhancement could not be reduced below 1.5 (deemed as significant matrix factor) and poor separation of analytes was also observed. The problem encountered with the Gemini C_{18} column was peak tailing, probably due to elution of the protonated form of VCV (protonated amino group of the valine moiety). Sequant ZIC-HILIC (100 × 4.6 mm, 5 µm) was also used due to the highly polar nature of analytes with little or no retention in RP columns. The zwitterionic ZIC®-HILIC stationary phase is attached to porous silica and separation is achieved by a hydrophilic partitioning mechanism superimposed on weak electrostatic interactions. Although retention of analytes was found, interference from

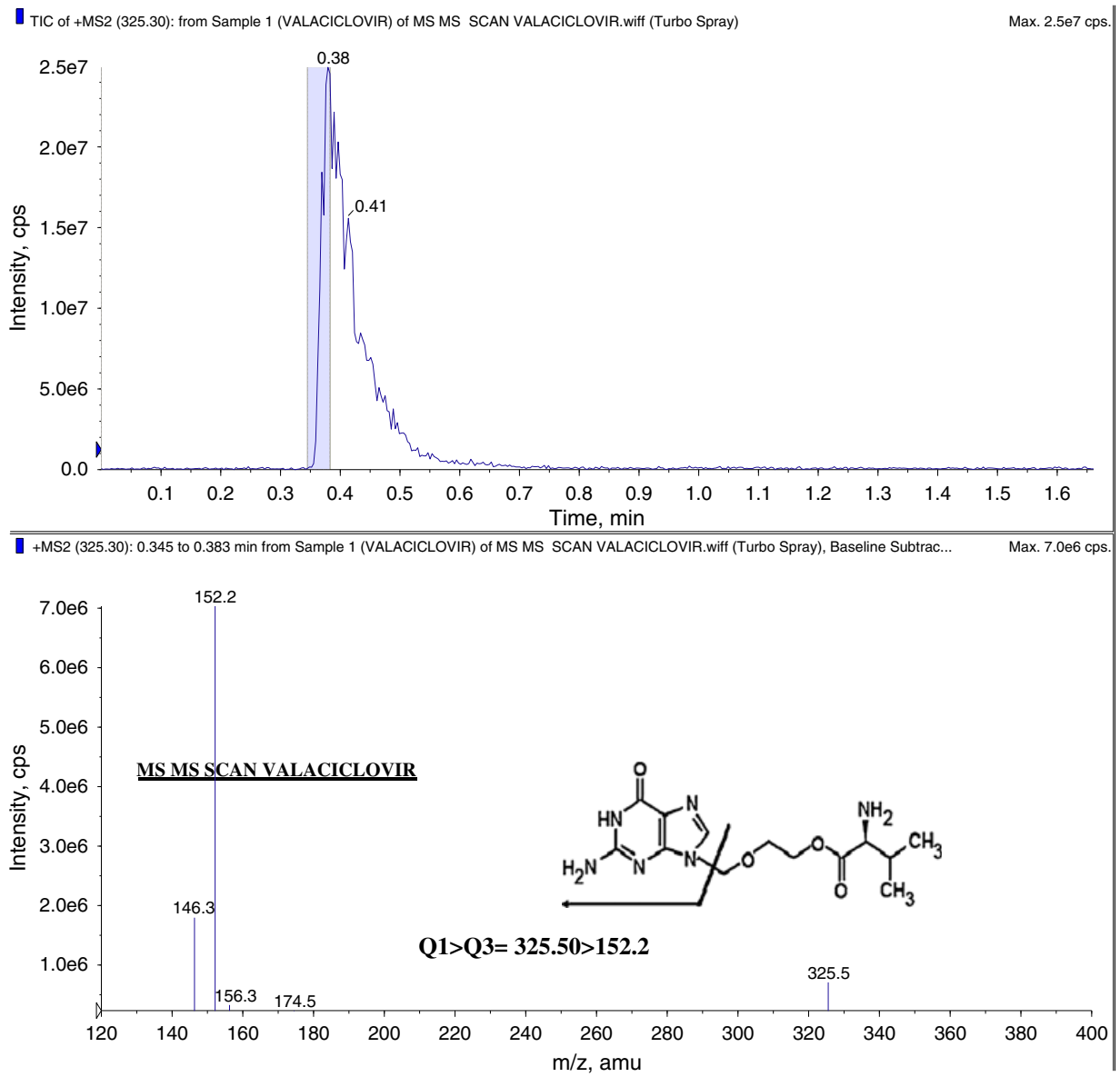

Figure 1. MS/MS scan and fragmentation pathway of valaciclovir.

endogenous compounds could not be eliminated completely, especially while using haemolysed plasma.

The interference at retention time of metabolite could not be avoided with change of mobile phase composition, flow rate or selecting different dimensions of ZIC®-HILIC. Although a previous report recommends polar columns, due to the molecule nature, the problem with chromatography and endogenous compound interference could not be overcome with the use of a HILIC column as depicted in Fig. 3(a). For the RPLC mechanism, generally group B, a newer generation C_{18} column with low silanol activity and high retentivity, was used. A lichrosphere column was therefore tried due to the large proportion of highly acidic silanols, although they are probably isolated non-hydrogen-bonded silanols. The best results in terms of reproducibility, resolution and symmetric peak shape were obtained with the Lichrosphere RP Select B (125 × 4.0 mm, 5 µm) C_{18} column (Fig. 3b) compared with all other options. Moreover to allow matrix elution at around 1 min (especially with haemolysed plasma), the LC parameter was further modified such that the retention time of VCV was shifted from 3 min (Fig. 3b) to 3.5 min (Fig. 4).

This result may be attributed to mobile phase composition optimization to produce acceptable precision and accuracy batches that were analysed using haemolysed plasma samples. The mobile phase with 1 mM ammonium acetate buffer (pH ~6.2) buffer and methanol (30:70, v/v) was found most suitable for eluting VCV, ACV and IS within 4 min. A flow-rate of 0.8 mL/min with 70% flow splitting produced acceptable peak shape and the analytical run was continued for 5 min to show that there was no post elution of matrix components after all compounds were eluted. The final extract was reconstituted with 500 µL of 1 mM ammonium acetate buffer (pH adjusted at 3.2 with formic acid) mixed with methanol (35:15 v/v) in order to stabilize VCV, existing primarily as a cationic moiety. The aqueous component predominated in reconstitution solution to stabilize VCV and minimized *ex-vivo* conversion to ACV. Since mobile phase cation concentration and pH are relevant parameters that can affect elution of analyte cations, an increase in the pH of the mobile

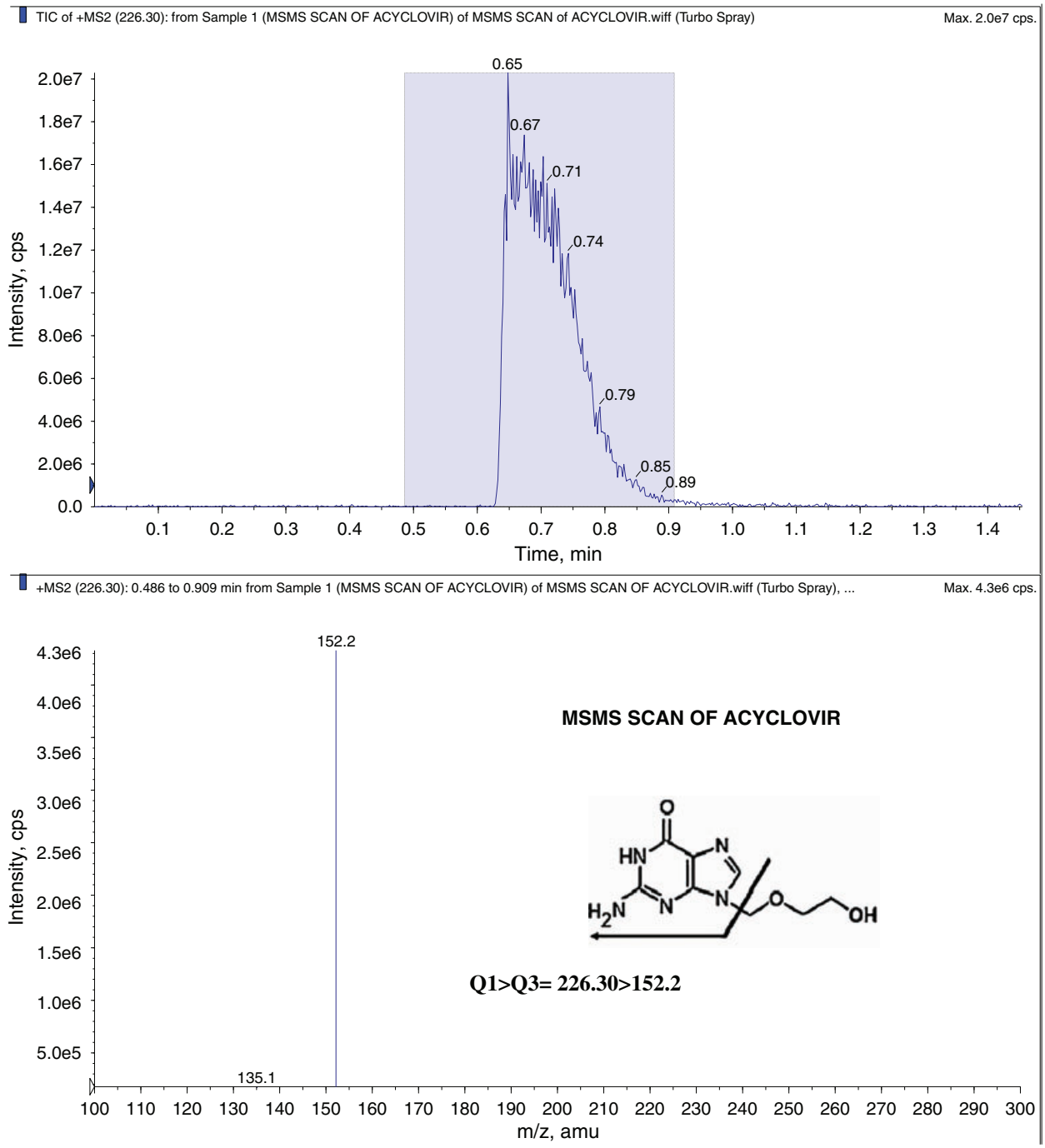

Figure 2. MS/MS scan and fragmentation pathway of acyclovir.

phase, (buffer pH ~6.2) increases retention of sample cations because it favours ionization of acidic silanol groups of the Lichrosphere RP Select B column. This could be the reason for achieving the desired retention and separation of all analytes using a Lichrosphere RP Select B column within 4 min.

Plasma extraction

Owing to the instability of VCV at pH ≥ 4 and its rapid conversion/degradation to ACV (Granero and Amidon, 2006), it was difficult to extract quantitatively both the analytes from human plasma. Protein precipitation and liquid–liquid extraction technique both showed inconsistent recovery with ion suppression (greater than 15% CV) for both the analytes. Then solid-phase extraction was tried. Addition of strong acid (hydrochloric acid) like hydrochloric acid during sample preparation helped maintain the analyte in the ionized form but Waters Oasis HLB was unable to retain the ionized drug. The optimization of the solid-phase extraction process was initiated using the ionic mode of extraction employing MCX cartridges. Moreover the use of strong acid during processing led to inconsistency in our data; probably the equilibration/stabilization of ionic compound was not achieved completely. Finally the use of *ortho*phosphoric acid buffer during spiking of calibrators and control samples was evaluated. The optimization of *ortho*phosphoric acid

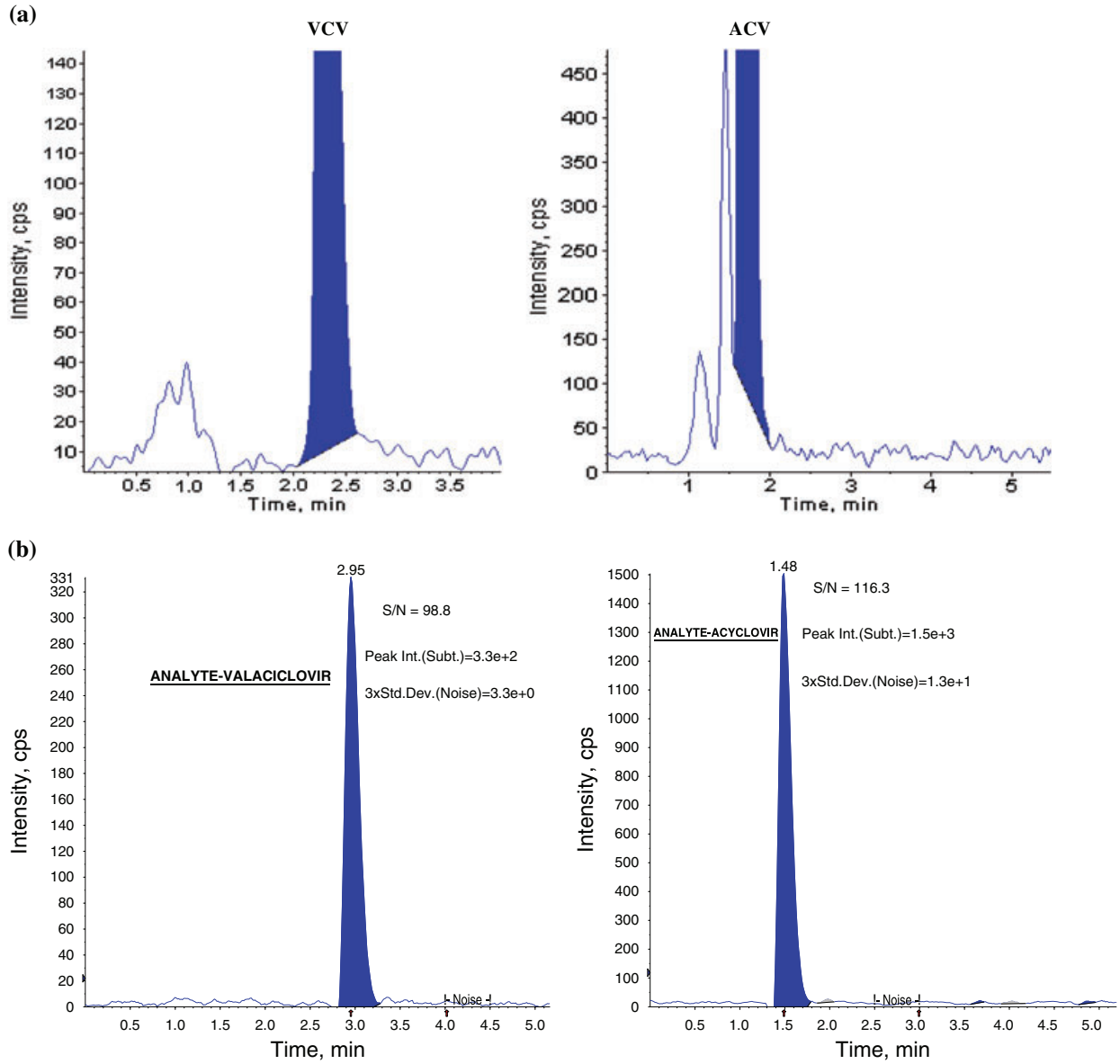

Figure 3. Matrix effect removal by changing HILIC to Licrosphere RP Select B column. (a) Column: Sequant ZIC-HILIC (100 × 4.6 mm, 5 µm); matrix ion interference and poor chromatography noted. (b) Column: Licrosphere RP Select B (125 × 4.0 mm, 5 µm) with improved chromatography. VCV spiked LOQ (4 ng/mL), S/N ratio = 98.8; ACV spiked LOQ (50 ng/mL), S/N ratio = 116.3.

strength was carried in the range of 25–75% and finally consistent recovery, with minimum matrix ion interference, especially at LLOQ level, was observed using 50% buffer strength.

Addition of acidic buffer in pre-extraction phase with cold chain sample work-up procedure was the prime step in method optimization. We observed that, without using cold chain processing, degradation of VCV increased with time. Moreover washing and elution steps were modified for maximum recovery without any effect of ion suppression/enhancement. The fluconazole/non-labelled compounds were tested as IS but the precision and accuracy batch failed to meet acceptance criteria for haemolysed plasma samples. The purine group of compounds as IS were found best suited due to similar physicochemical characteristics to analytes. The reason might be that the purine chemical class of compound showed a significant matrix effect, which could be compensated by using valganciclovir and acyclovir-d4, the best possible structural analogue of VCV and ACV. However use of deuteriated VCV (1 – 2% of acyclovir present in standard) led to unavoidable interference at the retention time of ACV and might distort *ex-vivo* hydrolysis results. Therefore deuteriated VCV was not used and valganciclovir was used in our method. The use of acyclovir-d4 as IS for acyclovir significantly reduced matrix ion suppression and degree of variability encountered in haemolysed plasma samples.

Linearity and lower limit of quantification

The calibration curves analysed during the course of validation were linear for the standards at 4.03–702.68 and 50.08 – 10031.73 ng/mL for VCV and ACV, respectively. The within- and between-batch accuracy and precision for VCV calibrators ranged from 95.4 to 103.4% and from 96.9 to 100.1%, respectively. The within- and between-batch precision (CV) ranged from 1.2 to 9.0% and from 1.6 to 6.0% for VCV. The within- and between-batch

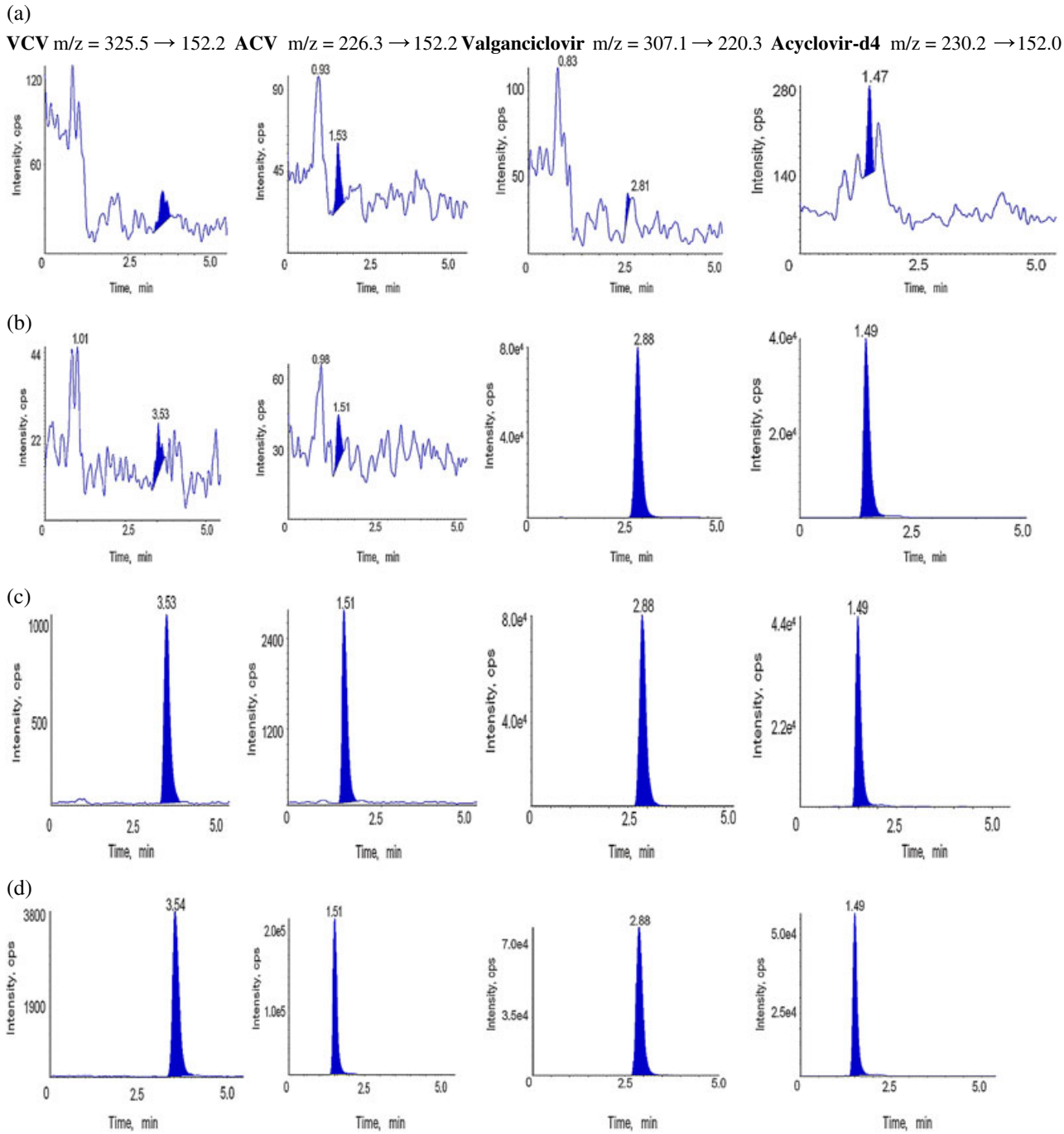

Figure 4. Typical MRM chromatograms from incurred study samples: (a) double blank; (b) blank + IS; (c) LLOQ; (d) real subject at 4.0 h after intake of 1000 mg of valaciclovir tablet.

accuracy for ACV calibrators ranged from 96.3 to 101.1% and from 97.1 to 99.0%, respectively. The within- and between-batch precisions were 0.4–3.4% and 1.6–3.0% for ACV, respectively. The LLOQ achieved was 4.03 ng/mL for VCV and 50.08 ng/mL for ACV at a signal-to-noise ratio ≥90 during the entire course of validation and clinical sample analysis.

Recovery

The mean recovery at LQC, MQC and HQC levels was 100.5, 91.2 and 91.2%, and 71.6, 80.4 and 73.1% for VCV and ACV, respectively. The recoveries of valganciclovir and acyclovir-d4 were 82.6 and 69.8% respectively. The mean recoveries for VCV (5.7%CV) and ACV (6.3%CV) were 94.3 and 75.0%, respectively (Table 1), and result was deemed acceptable.

Ion-suppression and matrix effect

Post-column infusion and post-extraction spiked method indicated no ion suppression or enhancement at the retention time of VCV and ACV. The matrix factor for the intended method was assessed using chromatographically screened human

plasma. The mean matrix factors were 0.93 ± 0.01 and 0.98 ± 0.01 for VCV and ACV, respectively, which were within the acceptance limit (Table 1). The results for haemolysed plasma batches for both VCV and ACV were presented for the first time in Tables 2 and 3. The precision (%CV) in haemolysed plasma was 2.4–3.7 and 1.2–1.9% for VCV and ACV respectively. The accuracy (% nominal) in haemolysed plasma was 97.8–101.8 and 102.2–103.6% for VCV and ACV respectively. Moreover there was no change in response for haemolysed plasma for both drug and IS. Thus the results strongly indicate that the newly developed bioanalytical method was reasonably free from the effects of endogenous substances like purine analogues (normal plasma), hemoglobin (for haemolysed) and bilirubin (lipemic) in plasma.

Stability results for valaciclovir, acyclovir and IS

Stock solutions for short-term and long-term stability of VCV, ACV and IS were stable at room temperature for a minimum period of 8.25 h and between 2 and 10°C for 8 days. The bench-top stability of plasma samples in an iced-water bath was found to be stable at least for 6.25 h and three freeze and thaw cycles passed successfully. As compared with using acid addition to stabilize analytes on the bench-top (Yadav *et al.*, 2009), our approach is simple and degradation of VCV was observed when an iced-water bath was not used during the entire sample processing stage. The auto-sampler stability of the spiked quality control samples maintained at 10°C was determined up to 54.37 h. For all stability exercises the accuracy and precision were within 5% (Table 4), which proved that our method is reliable and unique. The long-term stability of the spiked quality control samples, buffered with *ortho*phosphoric acid and stored below −50°C was determined up to 120 days. Finally based on the results obtained from ISR, it was observed that more than 90% of sample points for VCV and ACV were within ±15% of the initial concentration value after a time span of 3 months. From incurred study samples the typical chromatograms obtained from blank plasma, blank spiked with IS, LLOQ plasma sample and a subject's plasma sample collected at 4.0 h post dose of 1000 mg valaciclovir tablet are presented in Fig. 4.

Table 1. Recovery and Matrix factor determination for valaciclovir and acyclovir

	Valaciclovir (%)	Acyclovir (%)
Recovery-QC		
LQC	100.5	71.6
MQC	91.2	80.4
HQC	91.2	73.1
Mean	94.3	75.0
SD (±)	5.4	4.7
CV (%)	5.7	6.3
Matrix factor		
LQC	0.92	0.99
MQC	0.94	0.96
HQC	0.94	0.98
Mean	0.93	0.98
SD (±)	0.01	0.01
CV (%)	1.4	1.5

Controlled *ex vivo* hydrolysis of VCV

The comparative analysis of buffered and unbuffered samples after long-term storage of plasma sample for VCV and ACV is presented and discussed in detail. The unbuffered samples showed a decrease in VCV response (area ratio of drug/IS) to the extent of 38.6% (plasma storage at −50°C) and 48.8% (plasma storage at −15°C) in comparison to buffered samples (plasma storage at −50°C) where VCV response decreased to only 2.3% at LQC and HQC levels after 120 days, plotted in three dimensions (Fig. 5).

Moreover the plasma ACV samples fortified with VCV, when compared against ACV (free from VCV), showed that VCV to ACV conversion was 0.5% for buffered plasma stored at −50°C, while unbuffered replicates revealed 6.2 and 7.7% conversion for plasma samples storage at −50 and −15°C respectively after same time period of 120 days (Fig. 5). The results from the above two sets of simultaneous stability tests using replicates of LQC and HQC plasma samples under similar storage conditions to 120 days marked the ester hydrolysis of VCV, termed here as 'VCV degradation' as well as 'simultaneous conversion to ACV', which was further supported by the increase in ACV response in unbuffered plasma samples.

It had been reported (Soul-Lawton *et al.*, 1995) that, after oral administration of VCV, only 54% of the dose was absorbed, due to the conversion, and hence the impact of physicochemical properties of both compounds need to be highlighted. VCV has three pK_a values equal to 1.90, 7.47 and 9.43 that correspond to the amine attached to the C_2 in the purine, the N-terminal

Table 2. Validation for valaciclovir and acyclovir in haemolysed and non-haemolysed plasma from a freshly spiked precision and accuracy batch. Calibrators back-calculated concentrations using non-haemolysed plasma

Back-calculated CC concentrations in non-haemolysed plasma									Slope	Intercept
Valaciclovir									$r = 0.9978$	
Nominal concentration (ng/mL)	4.04	8.98	22.46	56.15	140.38	280.76	561.53	701.91		
Calculated concentration (ng/mL)	4.25	8.24	20.6	53.93	144.52	293.99	579.36	732.17	0.0026	−0.0011
Percentage nominal	105.3	91.8	91.7	96.1	102.9	104.7	103.2	104.3		
Acyclovir									$r = 0.9986$	
Nominal conccentration (ng/mL)	50.66	128.25	320.63	801.57	2003.93	4007.85	8015.71	10019.63		
Calculated conccentration (ng/mL)	52.67	117.99	306.14	773.01	2047.78	4110.76	8227.61	10491.6	0.0009	−0.0007
Percentage nominal	104.0	92.0	95.5	96.4	102.2	102.6	102.6	104.7		

Table 3. Validation for valaciclovir and acyclovir haemolysed and non-haemolysed plasma from a freshly spiked precision and accuracy batch. Precision and accuracy batch using above calibrators

	Non-haemolysed plasma		Haemolysed plasma	
	LQC (ng/mL)	HQC (ng/mL)	LQC (ng/mL)	HQC (ng/mL)
Valaciclovir, N = 6				
Nominal concentration	10.46	523.22	10.46	523.22
Mean	10.22	535.85	10.23	532.48
SD (±)	0.300	19.459	0.243	19.955
CV (%)	2.9	3.6	2.4	3.7
Percentage nominal	97.7	102.4	97.8	101.8
Acyclovir, N = 6				
Nominal concentration	136.60	7589.08	136.60	7589.08
Mean	142.51	7879.59	141.54	7758.50
SD (±)	3.218	102.738	1.690	145.361
CV (%)	2.3	1.3	1.2	1.9
Percentage nominal	104.3	103.8	103.6	102.2

Table 4. Plasma stability determination for valaciclovir and acyclovir

Stability exercises	Storage conditions	Comparison QC concentration (ng/mL)	Stability QC concentration (ng/mL)	Stability (%)	Precision (%)	Accuracy (%)
Bench-top	6.25 h when kept	10.58[a]	10.49[a]	103.0	3.1	101.7
stability, VCV	on an ice-cold	528.83[b]	524.64[b]	104.2	4.1	102.6
Bench-top	water bath under	129.88[a]	129.99[a]	98.2	3.5	100
stability, ACV	low light conditions	7379.38[b]	7386.02[b]	100.9	4.0	101.2
Three freeze–thaw	Kept on an ice-cold	10.58[a]	10.49[a]	98.2	4.3	101.5
cycle stability, VCV	water bath under	528.83[b]	524.64[b]	100.9	4.4	99.3
Three freeze–thaw	low light conditions	129.88[a]	129.99[a]	99.3	0.7	98.6
cycle stability, ACV		7379.38[b]	7386.02[b]	100.3	5.5	100.4
In-injector		10.58[a]	10.49[a]	97.8	4.4	98.8
stability, VCV	10°C for 54.37 h	528.83[b]	524.64[b]	101.7	4.0	99.8
In-injector		129.88[a]	129.99[a]	100.2	2.9	100.6
stability, ACV		7379.38[b]	7386.02[b]	101.4	3.0	100.0

[a]LQC; [b]HQC.

amine in valine and the NH-acidic N_1 in the purine, respectively. Since VCV is an aminoacyl ester with pK_a 7.47, the protonation of the amino group of the valine moiety is unavoidable, leading to enhanced rate of hydrolysis of VCV to ACV in plasma. Therefore pH needs to be lowered and at low pH conditions VCV exists primarily as a cationic moiety. Thus the best VCV stability was found in the pH zone below 4. The long-term stability of VCV in buffered plasma (pH ~3.00) and unbuffered (pH ~7.8) human plasma after separation from blood sample was elucidated for the first time. There was no detectable degradation of ACV at plasma pH <4 when buffered plasma spiked with VCV and ACV simultaneously was compared with VCV-free ACV-spiked samples and stored for 120 days, both being analysed against freshly spiked ACV samples free from VCV. It could be predicted that degradation kinetics is nonlinear when plasma storage temperature and pH were taken as variables as per Fig. 5 (depicting the conversion VCV to ACV), provided blood samples were collected in prechilled K_3EDTA-anticoagulated vacutainers immersed partially in an iced-water bath. Based on the results it can be proposed that esterase activity could lead to significant degradation of VCV in blood/plasma samples under conditions likely to be encountered during clinical studies and during assay preparation.

Bioavailability assessment of valaciclovir

The validated method was successfully applied for the assay of VCV and ACV in healthy male volunteers under fasting condition and pharmacokinetic results are presented in Table 5. Finally time point selection and study design should be such that VCV, undetectable in plasma after 3 h (Granero and Amidon, 2006), and availability in systemic circulation should be accurately captured. Our method was sensitive and rugged enough to monitor VCV and ACV plasma concentration up to 24 h. A day-to-day continuous run in mass spectrometry was sustained for 3 months without any problem and ISR analysis met our in-house acceptance criteria. Further, there was no serious adverse event during the course of the study.

VCV is a BCS class-I drug (high permeability and high solubility) and *in-vivo* metabolite formation is not governed by

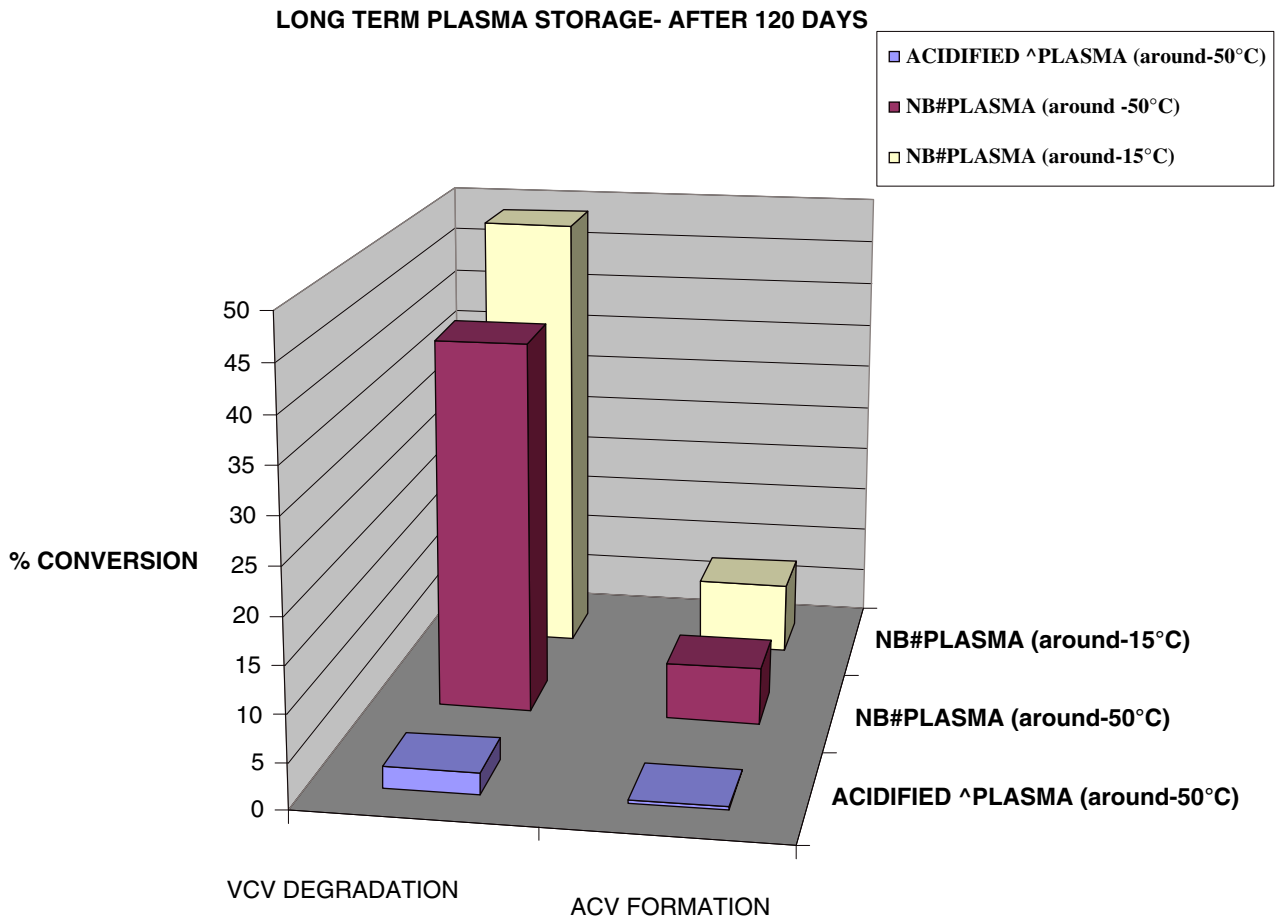

Figure 5. *Ex-vivo* controlled hydrolysis of VCV into ACV at three different conditions: (a) acidified buffered condition → plasma storage around −50°C; (b) NB#, no buffer addition → plasma storage around −50°C; (c) NB#, no buffer addition → plasma storage around −15°C.

Table 5. Pharmacokinetic parameter for valaciclovir and acyclovir from a bioequivalence study with 1000 mg oral dosage administered to eight healthy male fasted subjects

	T_{max} (h)		Cmax (ng/mL)		$AUC_{last\ 0-72}$ (h ng/mL)		$AUC_{0-\infty}$ (h ng/mL)		Half life (h)		K_{el} (1/h)	
Acyclovir, N = 8												
Sequence	R	T	R	T	R	T	R	T	R	T	R	T
Mean	2.5	2.3	2914.5	3468.6	12755.2	14160.5	13199.2	14552.4	5.1	4.1	0.16	0.17
SD	0.48	0.52	1231.14	852.37	5090.72	4134.14	5023.2	4159.8	2.83	0.76	0.06	0.04
Min	2	1.75	1194.2	2094.3	6182	8756.4	6679.2	9142.3	3.02	2.59	0.06	0.13
Median	2.5	2.25	3022.1	3589.4	13167.3	14183.5	13478.6	14653.2	4.05	4.22	0.17	0.16
Max	3.5	3	4380.9	4842.9	19997.1	20680.5	20373.2	21095.3	11.55	5.18	0.23	0.27
CV%	19.3	22	42.2	24.6	39.9	29.2	38.1	28.6	55.9	18.4	35.5	23.3
Valaciclovir, N = 8												
Sequence	R	T	R	T	R	T	R	T	R	T	R	T
Mean	1.8	1.3	152.6	176.3	312.8	340	318.4	345.1	0.65	0.69	1.13	1.07
SD	0.85	0.43	52	49.26	65.78	50.98	65.34	51.58	0.2	0.19	0.28	0.26
Min	0.67	0.67	88.8	123.3	222.6	277	230.3	281.7	0.46	0.5	0.66	0.67
Median	1.75	1.25	153.9	173.4	292	324.7	296.7	329.3	0.59	0.64	1.17	1.08
Max	3.5	2	247.84	262.2	403	420.6	407.4	426.8	1.05	1.03	1.51	1.39
CV%	46.4	32.8	34.1	27.9	21	15	20.5	14.9	30	27.2	24.2	24.6

the CYP group but by valaciclovir hydrolase (Kim *et al.*, 2003). Thus we could infer that the metabolizer effect (variability in pharmacokinetics due to genotype effect) could be ruled out. Our data showed a ratio of ACV to VCV of ~20:1, but recent work by Yadav *et al.* (2009) showed ACV:VCV ≈25:1 (as deduced) when the rate of absorption to systemic circulation was considered for Indian volunteers. Moreover their work reflected higher t_{max} for VCV [2.0 ± 0.8 (R) h and 1.9 ± 0.6 h (T)] as compared with ours

[1.8 ± 0.85 h (R) h and 1.3 ± 0.43 h (T)]. Therefore it is evident that VCV release is complete, yet ACV rate and extent of absorption (Kanneti *et al.*, 2009; Yadav *et al.*, 2009) indicated higher conversion compared with ours although the population effect was nullified due to participation of healthy Indian volunteers in both cases. This could be due to *ex-vivo* conversion of VCV to ACV in the clinic as proper precautions have not been reported to date to the best of the knowledge of the authors.

Therefore our current study, delineating controlled *ex-vivo* degradation of VCV, rests on the following critical parameters: protecting from light and maintaining an iced-water bath from blood samples collection in prechilled K_3EDTA vacutainers until plasma storage at −50°C. The analytical processing in an iced-water bath until the sample drying step and allowing similar treatment of acidic buffer addition to bulk spiked calibrators, control samples and biostudy samples were the critical parameters that made the bioanalytical method an improved version over previously reported simultaneous methods. Our results were consistent without any possibility of underestimation of plasma VCV concentrations when expressed in terms of drug–metabolite ratio formation ratio into systemic circulation. The source of bias due to uncontrolled hydrolysis during long-term storage could distort all pharmacokinetic parameters, especially parameters related to the absorption phase, such as the maximum concentration achieved and the absorption rate constant of VCV. The degradation of VCV by plasma esterase activity was not completely arrested, even when samples were kept continuously on ice. Moreover maintenance of the cold chain and rapid processing of samples cannot always be guaranteed under clinical study conditions. We therefore recommend future studies of the clinical pharmacokinetics of VCV to incorporate the use of inhibitors of plasma esterase activity to avoid these potential problems of in-situ hydrolysis. This investigation showed the role of clinical chemistry for labile active moiety, prone to undergo ester hydrolysis under both analytical and normal clinical conditions.

Conclusion

A validated bioanalytical method, for the *ex-vivo* stabilization of VCV in human plasma samples, in both haemolysed and non-haemolysed state, is discussed with successful clinical outcome. VCV was found to be unstable in plasma and must be stabilized immediately upon separation from blood samples to inhibit esterase activity of plasma enzymes, weakening the valine ester bond. The stabilizing agent was 50% *ortho*phosphoric acid solution, which might be suitable for drugs that are required to be stabilized in the acidic zone as blood pH was found to be in the region of 7.5–8 from collection until separation of plasma (data collated from our previous experience). The results obtained when using the present method for the analysis of VCV in human plasma demonstrated that the plasma concentrations previously reported (Kanneti *et al.*, 2009; Yadav *et al.*, 2009) were clearly underestimated due to uncontrolled degradation reaction, and not by stabilizing VCV before plasma storage. This assay will be helpful to accurately measure concentrations of VCV and ACV in human plasma in day-to-day routine clinical trials. A new approach to achieve hydrolytic stability for aminoacyl ester drug to accurately estimate the bioavailability factors is described for the first time that will strongly support exploratory pharmacokinetic analysis of clinical samples in the near future.

Acknowledgement

The authors would like to thank Ranbaxy Laboratories Ltd for giving permission to write this paper and to use in-house data. The authors would like to acknowledge the Formulation and Clinical Department's endeavours to support us during different stages of this investigation.

Declaration of Conflict of Interest

The authors further declare no conflict of interest for publication of this research work.

References

Brown SD, White CA and Bartlett MG. Hydrophilic interaction liquid chromatography/electrospray mass spectrometry determination of acyclovir in pregnant rat plasma and tissues. *Rapid Communications in Mass Spectrometry* 2002; **16**: 1871–1876.

Goswami D, Gurule S, Khuroo AH and Monif T. Bioequivalence model for evaluation of Losartan in human plasma with special reference to drug–metabolite ratio. *Clinical Research and Regulatory Affairs* 2009; **26**: 101–112.

Granero GE and Amidon GL. Stability of valacyclovir: implications for its oral bioavailability. *International Journal of Pharmaceutics* 2006; **317**: 14–18.

US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. *Guidance for Industry: Bioanalytical Method Validation*. Rockville, MD, 2001, Available from: http://www.fda.gov/CVM

Hughes NC, Bajaj N, Fan J and Wong EYK. Assessing the matrix effects of haemolysed samples in bioanalysis. *Bioanalysis* 2009; **1**: 1057–1066.

Kanneti R, Rajesh R, Raj JRA and Bhatt PA. An LC-MS-MS method for the simultaneous quantification of acyclovir and valacyclovir in human plasma. *Chromatographia* 2009; **70**: 407–414.

Kasiari M, Gikas E, Georgakakou S, Kazanis M and Panderi I. Selective and rapid liquid chromatography/negative-ion electrospray ionization mass spectrometry method for the quantification of valacyclovir and its metabolite in human plasma. *Journal of Chromatography B: Biomedical Sciences and Applications* 2008; **864**: 78–86.

Kim I , Chu XY, Kim S, Provoda CJ, Lee KD and Amidon GL. Identification of a human valacyclovirase: biphenyl hydrolase-like protein as valacyclovir hydrolase. *The Journal of Biological Chemistry* 2003; **278**: 25348–25356.

Ormrod D, Scott L J and Perry CM. Valaciclovir A Review of its Long Term Utility in the Management of Genital Herpes Simplex Virus and Cytomegalovirus Infections. *Drugs* 2000; **59**: 839–863.

Pham-Huy C, Sathoulopoulou F, Sandouk P, Scherrmann J-M, Palombo S and Girre C. Rapid determination of valaciclovir and acyclovir in human biological fluids by high-performance liquid chromatography using isocratic elution. *Journal of Chromatography B: Biomedical Sciences and Applications* 1999; **732**: 47–53.

Soul-Lawton J, Seaber E, On N, Wootton R, Rolan P and Posner J. Absolute bioavailability and metabolic disposition of valaciclovir, the L-valyl ester of acyclovir, following oral administration to humans. *Antimicrobial Agents and Chemotherapy* 1995; **39**: 2759–2764.

Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG and Weiner R. Workshop/Conference Report – quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *American Association of Pharmaceutical Scientists Journal* 2007; **9**: E30–E42.

Weller S, Blum MR, Doucette M, Burnette T, Cederberg DM, de Miranda P and Smiley ML. Pharmacokinetics of the acyclovir pro-drug valaciclovir after escalating single- and multiple-dose administration to normal volunteers. *Clinical Pharmacology And Therapeutics* 1993; **54**: 595–605.

Yadav M, Upadhyaya V, Singhal P, Goswami S and Shrivastav PS. Stability evaluation and sensitive determination of antiviral drug, valacyclovir and its metabolite acyclovir in human plasma by a rapid liquid chromatography–tandem mass spectrometry method. *Journal of Chromatography B: Biomedical Sciences and Applications* 2009; **877**: 680–688.