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Rapid determination of valaciclovir and acyclovir in human biological fluids by high-performance liquid chromatography using isocratic elution

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Abstract

A rapid high-performance liquid chromatographic assay with isocratic elution is developed for the simultaneous quantification of valaciclovir (VACV) prodrug and its active converted compound, acyclovir (ACV), in biological fluids of treated patients. For serum, the samples are deproteinized with perchloric acid in presence of 1-methylguanosine as the internal standard (IS). For urine and dialysis liquid, the samples are diluted with a mobile phase containing the IS, then filtered. VACV, ACV and the IS are separated on a SymmetryShield™ RP-8 column with acetonitrile–ammonium phosphate buffer as the mobile phase and detected at 254 nm. The chromatographic time is about 12 min. The relative standard deviations (RSD) of VACV and ACV standards are between 0.5 and 3.5%. Most endogenous nucleosides and their metabolites, psychotropic drugs and drugs of abuse are shown not to interfere with this technique. The method has been applied to study the pharmacokinetics of VACV and ACV in serum, dialysis liquid and urine of renal failure patients on continuous ambulatory peritoneal dialysis (CAPD) under oral treatment of VACV. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Valaciclovir; Acyclovir

1. Introduction

Valaciclovir (VACV), the L-valyl ester of acyclovir, is an oral prodrug that is rapidly and extensively metabolized by enzymatic hydrolysis probably in the liver and the intestine to acyclovir (ACV) and L-valine, an essential amino acid [1,2].

VACV is used for the treatment of herpesvirus infections predominantly caused by the herpes simplex virus (HSV-1, HSV-2) and the varicella zoster virus (VZV) [1,3,4]. ACV, a nucleoside analog [9-(2-hydroxyethoxymethyl) guanine], is the active antiviral component of VACV and has shown also good activity against the Epstein–Barr virus and moderate efficacy against the cytomegalovirus [1]. The bioavailability of ACV from oral VACV is between

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three and five times greater than that obtained after oral ACV administration [1,5]. Therefore, it is suitable to know the biotransformation rate of VACV to ACV in biological fluids for pharmacokinetic study and drug monitoring therapy of this prodrug.

In the literature, most assays using either high-performance liquid chromatography (HPLC) [6–15] or radioimmunoassay (RIA) [16] quantified only ACV and not VACV. Recently, only one HPLC method using a gradient mobile phase mode can analyse VACV and ACV simultaneously [17].

The purpose of this report is to develop a rapid method for the simultaneous determination of VACV and its active metabolite, ACV, in human biological fluids by reversed-phase HPLC with isocratic elution utilizing 1-methylguanosine as the internal standard (IS) and a SymmetryShield™ RP-8 column. The method has been applied to study the pharmacokinetics of VACV and ACV in serum, dialysis liquid and urine of renal failure patients on continuous ambulatory peritoneal dialysis (CAPD).

2. Experimental

2.1. Chemicals

Valaciclovir (VACV) and acyclovir (ACV) were kindly provided by the Glaxo Wellcome Laboratory (Paris, France), 1-methylguanosine used as the IS was purchased from Sigma (St. Louis, MO, USA). Other chemicals and solvents were of analytical purity and were all obtained from Merck (Darmstadt, Germany). Deionized water was purified by Milli-Q UV Plus system (Millipore, Milford, MA, USA).

2.2. Apparatus and chromatographic conditions

The Merck liquid chromatographic system consisted of an isocratic pump Model L-6000 connected with an Intelligent Auto-Sampler Model AS-4000, an UV-VIS detector Model L-4250 and an integrator Model D-2500. The chromatographic separation was performed at ambient temperature (18–20°C) using an analytical column, SymmetryShield™ RP-8, 5 µm, 250×4.6 mm I.D., (Waters, Milford, MA, USA) connected with a guard column packed with the same bonded phase (20×3.9 mm I.D.).

The reversed-mobile phase was obtained by mixture of acetonitrile–monoammonium phosphate buffer 0.025 M, adjusted with 10% diluted phosphoric acid to pH 4.0 (2:98, v/v). Mobile phase was filtered through 0.2 µm Millipore filters and degassed by sonication before use, then pumped at a flow-rate of 1.0 ml/min. Injection volumes of samples and standards (50 µl) were performed with an automatic sample injector. The detector was set at 254 nm.

2.3. Preparation of standards

Three stock standard solutions were obtained by dissolving 1.0 mg/ml of VACV, ACV and 1-methylguanosine (IS) individually in methanol–water (50:50, v/v). The ACV and the IS stock solutions were stable for several weeks at 4°C, and the VACV stock solution was stored at –20°C to avoid hydrolysis. Standard calibration solutions were prepared by spiking drug-free human serum or urine with stock standard solutions, which were then further diluted to achieve final concentrations of between 0.5 and 20.0 µg/ml of VACV and of ACV, and then stored at –20°C. Two serum and urine quality controls (2.0 and 4.0 µg/ml) were also prepared for controlling the assay. An IS working solution at 20 µg/ml was obtained by dilution of an aliquot of 1-methylguanosine stock solution with the mobile phase.

2.4. Preparation of samples

For the serum, 200 µl of the thawed biological samples or the standard calibration solutions were added into a 5-ml centrifuge glass tube, then 50 µl of the IS and 200 µl of 5% perchloric acid in water were added successively to it. The tubes were tightly capped and vortex-mixed for 1 min. After centrifugation for 10 min at 1500 g, the upper layer was transferred into a 0.4 ml autosampler vial. For urine and dialysis liquid, 200 µl of samples previously diluted with the mobile phase (from 1:5 to 1:10) for the urine, but undiluted for the dialysis liquid, were added with 50 µl of the IS into a 2-ml microfiltration tube with a 0.20-µm porosity filter (Polylabo, Paris, France), then centrifuged and transferred into an autosampler vial. A 50-µl aliquot of each of the

samples was then automatically injected onto the column.

2.5. Validation of the method

This was performed by assaying three human serum and urine standards and two patient samples determined in a day ($N=6$) and from replicate analysis on six separate days in order to achieve the intra-day and inter-day relative standard deviations (RSD), respectively. The linearity of the standard calibration curves of the biological fluids was performed by simple measures of the spiked standard samples over six days in the range of 0.5–20.0 $\mu\text{g/ml}$. To calculate the recovery, the spiked serum samples (1 and 5 $\mu\text{g/ml}$) were deproteinized as described above. Peak areas from deproteinized serum samples ($N=6$) were compared to peak areas from injection of the appropriate stock standard solutions diluted in the same conditions with the mobile phase.

The interference with the assay was determined by the injection of different standard endogenous nucleosides and their metabolites, some psychotropic drugs and drugs of abuse diluted in the mobile phase directly onto the column.

2.6. Application of the method

Eight renal failure patients on CAPD received orally a single dose of 500 mg VACV in the morning. A number of serum, dialysis liquid and urine samples were collected at different times in the day (t_0 – t_{32} h) and used to test the applicability of this method for pharmacokinetic study. All biological samples were frozen immediately after collection at -20°C before use.

2.7. Calculation

The VACV and ACV concentrations in the biological samples were calculated on the peak area ratio of each analyzed compound to the IS. The ratio values found were reported on a standard calibration curve performed under the same conditions as described above.

3. Results and discussion

3.1. Chromatography

Typical chromatograms of drug-free human serum, the standard calibration serum and treated patient serum are shown in Fig. 1. The retention times were approximately 7.1, 9.7 and 12.1 min for ACV, VACV and the IS, respectively. Their peak form was sharp and symmetrical.

3.2. Statistical data

The standard calibration curves of the serum, dialysis liquid and urine exhibited good linearity for VACV and ACV over the range of concentrations tested with correlation coefficients greater than 0.999 for both compounds (Fig. 2A and B and Table 1). The intra-day and inter-day RSD ($N=6$) of the three serum and urine standard samples (1, 5 and 10 $\mu\text{g/ml}$ of VACV and ACV) and of the serum, dialysis liquid and urine samples from two treated patients are shown in Tables 1 and 2. The accuracy of VACV and ACV of two quality control (QC) samples and their recoveries from the two serum, and urine standards were good and shown in Table 1. For the IS, its recovery was about 94–95%. The RSD in the study samples were in general slightly higher than the values of the spiked standard samples because the last samples were prepared from normal human serum purchased from Sigma, versus the study samples, which were obtained from renal failure patients. In this disease, the levels of some endogenous compounds, such as proteins, creatinine, urea etc, are higher than those in healthy subjects; therefore, these compounds could slightly increase the coefficient of variation. However, this difference is not significant. The detection limit (signal-to-noise ratio >3) of the assay after serum deproteinization was about 50 ng/ml for ACV and 70 ng/ml for VACV. The lower limits of quantification were about 0.20 $\mu\text{g/ml}$ for ACV and 0.25 $\mu\text{g/ml}$ for VACV with RSD of about 4.5%.

3.3. Interferences

Most endogenous nucleosides and their metabolites were shown not to interfere with ACV, VACV

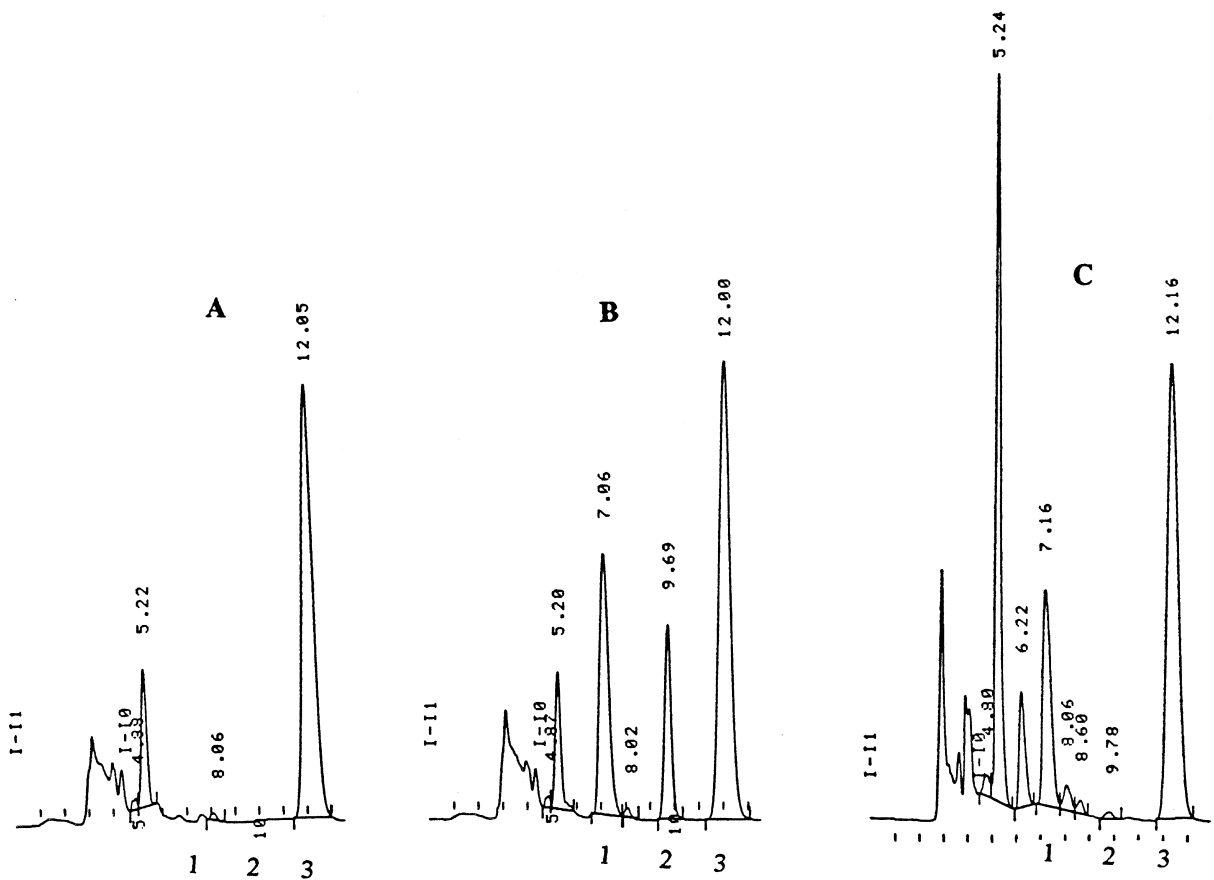


Fig. 1. Chromatograms of acyclovir (ACV) and valaciclovir (VACV) in human serum after deproteinization. (A) Blank serum, (B) human serum standard spiked with 5 $\mu\text{g}/\text{ml}$ of ACV and VACV, (C) patient serum 2 h after oral administration of 500 mg of VACV. Peaks 1,2,3=ACV, VACV and the IS, respectively.

and the IS. Their retention times were about 8.2 min for guanosine, 9.0 min for xanthosine, 14 min for adenosine and from 3.5 to 6.0 min for ATP, ADP, AMP, hypoxanthine, xanthine, guanine and adenine, respectively. This is also true for caffeine and theophylline, which had retention times of 39 and 26 min, respectively. Most psychotropic drugs, such as benzodiazepines, barbiturates, tricyclic antidepressants, and drugs of abuse, like morphine, codeine, cocaine, methadone, were not eluted with this mobile phase after 30 min. In the spiked serum, no endogenous peak was observed after the chromatogram time of 12 min. However, in some renal failure patient samples, a little parasite peak was observed at 14 min; therefore, the autosampler time was programmed at 15 min. For control, one mobile phase

sample followed by two QC samples were placed in the carousel after a set of six to eight samples. No peak was observed with the mobile phase sample.

3.4. Application of the method

The results of the pharmacokinetic study of VACV and ACV determined by this method in serum, dialysis liquid and urine of eight renal injury patients on CAPD treated with a single dose of VACV 500 mg will be published elsewhere. In brief, the VACV levels in the serum and dialysis fluid were either not found or very low (up to 0.40 $\mu\text{g}/\text{ml}$) from 30 min to 2 h after oral post-dose and undetectable in all consecutive samples. For ACV, the maximum serum concentration (C_{max}) varied from 3.5 to 7.0 $\mu\text{g}/\text{ml}$

TABLE NO. = 1
 NO. OF LEVELS = 6
 CALIB TYPE = LINEAR

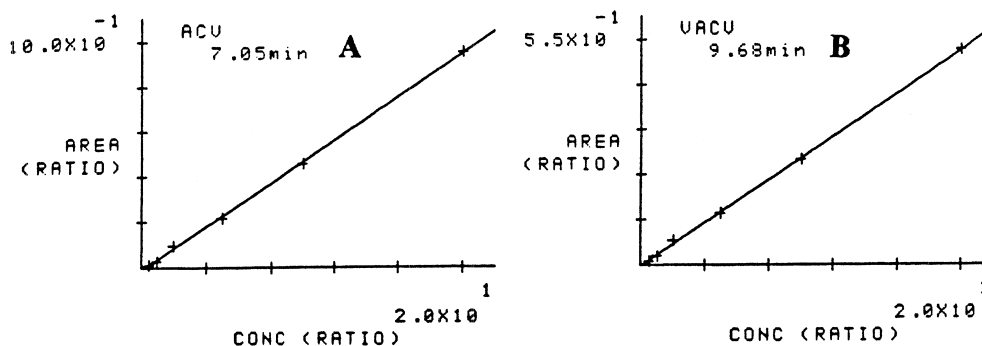


Fig. 2. Calibration curves of the human serum standards spiked with 0.50–20 $\mu\text{g/ml}$ of acyclovir (ACV) and valaciclovir (VACV) after deproteinization and automatic determination by the integrator (Merck, Model D-2500). (A) ACV calibration curve, (B) VACV calibration curve.

and the time to maximum concentration (T_{max}) ranged from 1.5 to 2.0 h. The VACV pharmacokinetic study in these patients shows that this prodrug is rapidly and completely metabolized to the active ACV form. The results of these pharmacokinetic parameters are consistent with those reported by others [2,18].

During the development of this method, it has been realized that VACV was partially hydrolyzed in aqueous solution to ACV after incubating at 37°C for several hours or storing at 4°C for several weeks. However, spiked serum and urine standards with concentrations of between 0.5 and 20 $\mu\text{g/ml}$ of VACV and ACV were found to be stable for about

one month at -20°C . A previous publication [2] reported that the conversion of VACV to ACV can be achieved rapidly by in vitro enzymatic biotransformation, but also slowly by chemical hydrolysis at neutral pH. For accurate determination of VACV and ACV in biological fluids, it is important to freeze the patient samples as soon as possible. To avoid the hydrolysis of VACV after blood withdrawal and during the separation of serum or plasma, it is preferable to centrifuge the blood samples at $3 \pm 1^\circ\text{C}$ after rapid clotting at low temperature ($10\text{--}12^\circ\text{C}$). For dialysate, the lack of all enzymes in this liquid could avoid this biotransformation.

The method described in this paper shows an

Table 1
 Precision, accuracy, linearity and recovery of valaciclovir (VACV) and acyclovir (ACV) standards ($N=6$)

| Compound | Precision, RSD (%) | | | | | | Accuracy of QC (Mean \pm SD) | | Linearity of standards (0.5–20.0 $\mu\text{g/ml}$) | Recovery (%) | |
|--------------|--------------------|-----------|--------------------|-----------|---------------------|-----------|--------------------------------|--------------------|---|--------------------|--------------------|
| | 1 $\mu\text{g/ml}$ | | 5 $\mu\text{g/ml}$ | | 10 $\mu\text{g/ml}$ | | 2 $\mu\text{g/ml}$ | 4 $\mu\text{g/ml}$ | | 1 $\mu\text{g/ml}$ | 5 $\mu\text{g/ml}$ |
| | Intra-day | Inter-day | Intra-day | Inter-day | Intra-day | Inter-day | | | Inter-day | | |
| <i>Serum</i> | | | | | | | | | | | |
| ACV | 0.75 | 3.06 | 0.45 | 2.75 | 1.05 | 2.46 | 2.03 \pm 0.04 | 4.04 \pm 0.07 | $r=0.9997$ | 90.4 \pm 2.3 | 93.5 \pm 2.7 |
| VACV | 1.12 | 3.50 | 1.15 | 3.16 | 1.25 | 2.86 | 2.06 \pm 0.06 | 4.10 \pm 0.12 | $r=0.9992$ | 91.2 \pm 3.4 | 90.4 \pm 1.8 |
| <i>Urine</i> | | | | | | | | | | | |
| ACV | 0.45 | 0.72 | 1.82 | 2.24 | 0.95 | 2.12 | 1.98 \pm 0.04 | 3.98 \pm 0.06 | $r=0.9997$ | 96.0 \pm 2.1 | 97.5 \pm 2.6 |
| VACV | 1.04 | 2.55 | 0.91 | 3.38 | 1.15 | 3.16 | 2.03 \pm 0.06 | 4.02 \pm 0.10 | $r=0.9995$ | 94.8 \pm 3.1 | 95.2 \pm 3.6 |

Table 2
Precision of acyclovir (ACV) and valaciclovir (VACV) in the biological fluids of two treated patients (N=6)

| Compound | Patient 1 | | | Patient 2 | | |
|-------------------------|---------------------------------------|---------------|-----------|---------------------------------------|---------------|-----------|
| | Concentration ($\mu\text{g/ml}$) | RSD (%) (N=6) | | Concentration ($\mu\text{g/ml}$) | RSD (%) (N=6) | |
| | | Intra-day | Inter-day | | Intra-day | Inter-day |
| <i>Serum</i> | | | | | | |
| ACV | 4.6800 | 2.52 | 3.20 | 7.090 | 2.14 | 2.90 |
| VACV | 0.0941 | 4.62 | 8.63 | NF | ND | ND |
| <i>Dialysate (CAPD)</i> | | | | | | |
| ACV | 1.4000 | 2.77 | 3.60 | 3.300 | 1.72 | 3.50 |
| VACV | 0.4020 | 3.75 | 5.82 | 0.301 | 4.50 | 7.80 |
| <i>Urine</i> | | | | | | |
| ACV | 18.8000 | 1.92 | 3.94 | 11.900 | 1.25 | 3.80 |
| VACV | NF | ND | ND | NF | ND | ND |

NF=Not found, ND=not determined.

improvement over existing HPLC assays concerning either the simultaneous determination of VACV and ACV [17] or the quantification of ACV alone [6–15] in the following aspects. The present HPLC technique using isocratic elution for the simultaneous determination of VACV and ACV is simpler and faster (less than 15 min) than the one [17] using gradient elution, which has a chromatographic time longer than 27 min. These improved performances are due to the use of the new SymmetryShield™ RP-8 column and of the described preparation of the mobile phase. Most HPLC procedures for ACV quantification alone did not employ an internal standard, thereby reducing the precision and the reproducibility of the analysis [6–10,12,14,15]. Moreover, the ACV limit of detection (LOD) of some techniques [6,9–11] were reported to be between 100 and 300 ng/ml versus the LOD of 50 ng/ml of the present method. A solid–liquid extraction procedure [14] has also been tested after slight modifications by using a polymeric Oasis™ extraction column (Waters) and methanol as the eluting solvent. The corresponding recoveries of VACV and ACV were approximately less than 43% and this extraction procedure was more time consuming than the deproteinization technique using perchloric acid. For the quantification of ACV only, a flow-rate of 1.20 ml/min can be used by the present technique; this allows the chromatographic time to

be reduced to about 10 min without influencing precision and specificity.

In conclusion, because of its rapidity, simplicity and specificity, the present procedure is suitable for the simultaneous determination of valaciclovir and acyclovir in therapeutic monitoring and in the pharmacokinetic studies of patients treated either with this prodrug or with acyclovir only. It is also applicable to metabolism studies of valaciclovir in animal experiments and cell cultures in order to explore the conversion mechanism of valaciclovir to acyclovir, which remains unclear.

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