Journal of Chromatography B, 879 (2011) 436–442

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of Ganciclovir and its prodrug Valganciclovir by hydrophilic interaction liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 10 September 2010
Accepted 28 December 2010
Available online 4 January 2011

Keywords:
Ganciclovir (GCV)
Valganciclovir (VGC)
Valcyte®
Plasma
Liquid chromatography–tandem mass spectrometry (LC–MS/MS)
Hydrophilic interaction liquid chromatography (HILIC)
Dried blood spots (DBS)

ABSTRACT

This manuscript describes the determination of Ganciclovir (GCV), active component of the antiviral drug Valcyte®, and its ester prodrug Valganciclovir (VGC) in human and rat plasma, using liquid chromatography coupled to tandem mass spectrometry. Protein precipitation with acetonitrile was followed by hydrophilic interaction liquid chromatography on a silica column with 4 min run time. After electrospray ionization, the compounds were detected in positive ion selected reaction monitoring (SRM) mode. The lower limits of quantification (LLOQ) were 16 ng/mL for GCV and 4 ng/mL for VGC in human and rat plasma. Inter-day and intra-day precisions and inaccuracies were below 15% and between 85 and 115%, respectively. Five-fold deuterated GCV and VGC were used as internal standards and compensated for any matrix effect. The method was successfully applied to samples from a rat pharmacokinetic study. The feasibility of blood analysis as dried blood spots (DBS) was investigated.

1. Introduction

Valganciclovir (VGC, Valcyte®) is the diastereomeric mono-L-valylester prodrug of the antiviral nucleoside analogue Ganciclovir (GCV), which is widely used for the treatment or prevention of cytomegalovirus (CMV) infection in immunodeficient individuals and transplantation patients [1–3]. Fig. 1 displays the structures of the prodrug and active compound. Determination of GCV and VGC in human and animal plasma is required in therapeutic drug monitoring, further clinical development (i.e. for special patient populations and infants) and in mechanistic studies in animals. VGC is rapidly converted into GCV by enzymes in the organism and also by chemical hydrolysis at higher pH [3]. Appropriate sample storage and assay conditions have to be considered during analysis to preserve the sensitive ester function. The diastereomers undergo rapid interconversion and do not exhibit different pharmacokinetics and pharmacological properties; therefore, a mixture is applied, and separation during analysis is not mandatory [3]. Historically, chromatography with UV, fluorescence or electrochemical detection has been used during the main part of drug development; the sensitivity was sufficient to detect the relatively large therapeutic drug levels [4–6]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has now become a routine tool to measure VGC and GCV [4,7–9]. Its sensitivity and selectivity allowed to simplify sample preparation and shorten the chromatographic run time. Protein precipitation was performed using trichloroacetic acid [7,8] or organic solvent [9], and separations were performed on RP columns. Because of issues to sufficiently retain the polar GCV long columns were used with highly aqueous mobile phases, but still the analytes eluted near the void volume, and peak shapes were not optimal which made automated integration difficult. Our attempts to reproduce the RP methods reliably in-house failed as we observed almost no retention for GCV and unacceptable peak shape for VGC. Literature data on the structural analogues acyclovir and its prodrug valacyclovir suggested the use of analytical columns with large surface area [10] or diethylamine modifier to improve the retention in RP mode [11], or performing the separation in hydrophilic interaction mode (HILIC) [12].

This manuscript describes the determination of Ganciclovir and Valganciclovir in rat and human plasma by LC–MS/MS using reversed phase or hydrophilic interaction LC. HILIC was finally utilized which provided good retention, symmetrical peak shape and sufficient separation from matrix constituents. Because no need existed to decrease the LLOQ, sensitivity was only slightly better or comparable to previous methods but significantly lower plasma volumes (10 μL *versus* at least 50 μL) were used. A wider linear calibration range of more than three orders of magnitude was obtained. For the first time, stable isotope labelled VGC and GCV were used as internal standards which contributed to good quality and robustness. The method was successfully validated according

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doi:10.1016/j.jchromb.2010.12.033

to regulatory requirements and employed for analysis of rat plasma samples from a pharmacokinetic study. The method was extended to dried blood spots analysis, which may be employed in therapeutic drug monitoring of VGC.

2. Experimental

2.1. Compounds, reagents and solvents

Valganciclovir (VGC) was synthesized at F. Hoffmann-La Roche Ltd., Ganciclovir (GCV) was purchased from Sigma and the internal standards VGC-d5 and GCV-d5 (five-fold deuterated drugs) were obtained from Toronto Research Chemicals, (North York, Canada). The structures are shown in Fig. 1. Ethanol and methanol (Lichrosolv for HPLC) were obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, UK). Formic acid (p.a.) was purchased from Fluka (Buchs, Switzerland), ammonium formate and hydrochloric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). The water used for the preparation of all solutions was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA). Rat blank EDTA plasma was supplied by internal laboratories. Human blank EDTA plasma was purchased from TRINA Bioreactives (Nänikon, Switzerland).

2.2. Calibration, quality control samples and internal standards

Stock solutions of the analytes and internal standards were prepared by dissolving the appropriate amounts with 1 mM HCl to obtain concentrations of 1 mg/mL free base. The analyte stock solutions were spiked to blank rat or human EDTA plasma to obtain calibration standards in the concentration range 4 ng/mL to 10 μg/mL for VGC and 16 ng/mL to 40 μg/mL for GCV; quality control (QC) samples were prepared to contain 12, 300 or 7500 ng/mL VGC and 48, 1200 or 30,000 ng/mL GCV.

The internal standard working solution was prepared by diluting the stock solutions with water/acetonitrile 10:90 (v/v) containing 5 mM ammonium formate and 0.2% formic acid to obtain final concentrations of 10 ng/mL VGC-d5 and GCV-d5.

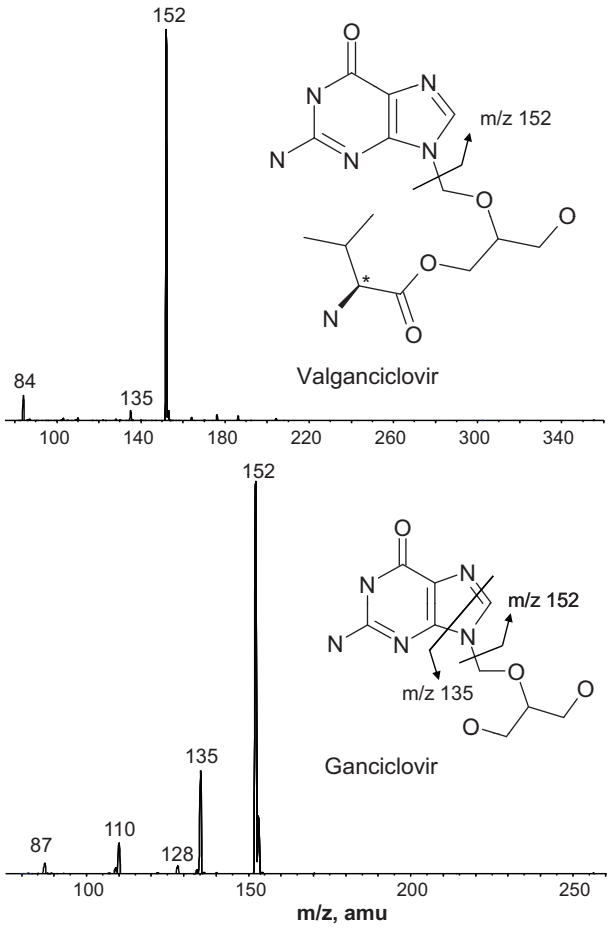

Fig. 1. Structures and product ion spectra of analytes.

2.3. Sample preparation

Samples were thawed at approximately 4 °C and worked upon ice or alternatively, only if preparation within 3 h could be achieved, at ambient temperature. To 10 μL of sample, which was pipetted into a 96 deep well rack, 1 mL of internal standard working solution was added using the automated pipettor Tecan Genesis 100/4 (Tecan Schweiz AG, Männedorf, Switzerland) or a Multipette Pro (Eppendorf, Hamburg, Germany). The sample solutions were mixed at 1500 rpm for 5 min (Mix Mate, Eppendorf) at ambient conditions and centrifuged for approximately 10 min at 5000 × *g* and 20 °C.

2.4. Liquid chromatography–mass spectrometry

The HPLC system, controlled by the software of the mass spectrometer, consisted of a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a 1200-series binary pump SL (Agilent, Waldbronn, Germany). The analytical column was a Luna Silica (2), 3 μm, 2.0 mm × 50 mm with a SecurityGuard Silica 2.0 mm × 4.0 mm pre-column (Phenomenex, Torrance, USA). Mobile phases were water/methanol 15/85 (v/v) (A) and water/acetonitrile 10/90 (v/v) (B), both containing 5 mM ammonium formate and 0.2% formic acid. Aliquots of 10 μL sample solution were injected, and gradient separation was performed as shown in Table 1. The flow was diverted to waste after 1.8 min using a 6-port 2-position valve (VICI Valco Instruments, Houston, USA).

An API5000 triple quadrupole mass spectrometer (ABSciex, Toronto, CAN), equipped with a Turboionspray source and operated in the positive ion mode, was used with Analyst 1.4.2 software. The sprayer voltage was set at 5500 V, the temperature at 500 °C and the entrance potential at 5 V. Nitrogen was used as nebulizing, auxiliary, curtain and collision gas with flow settings of 60, 50, 14 and 4, respectively. Data acquisition was carried out at unit mass resolution (peak width for Q1 and Q3 set to 0.7 amu using a dwell time of 150 ms. Selected reaction monitoring (SRM) mode was performed at the transitions m/z 355 → 152 for VGC, m/z 360 → 152 for VGC-d5, m/z 256 → 135 for GCV and m/z 261 → 152 for GCV-d5 at optimized collision energies between 21 and 47 eV. Product ion spectra for the analytes are shown in Fig. 1. Phospholipids were monitored via parent scan of a common fragment ion at m/z 184 applying 25 eV collision energy.

2.5. Calibration and validation

The calibration curves ($y = a + bx$) were calculated by weighted linear least-squares regression (weighting factor $1/x^2$) of the measured peak area ratios analyte/ISTD (y) *versus* the nominal analyte concentration (x). The calibration curves were then used to calculate the concentrations of the analytes in QC and study samples from the measured peak area ratios. Method validation according to regulatory requirements [13,14] and departmental standard operating procedures was performed. Inter-day precision and accuracy were evaluated by analyzing samples at the LLOQ, three QC levels and at the upper limit of quantification against a calibration curve on five independent occasions. Intra-day precision and accuracy were determined by analyzing three QC levels in five replicates