Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 852 (2007) 420–429
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of aciclovir and ganciclovir in human plasma by liquid chromatography–spectrofluorimetric detection and stability studies in blood samples

N. Perrottet [a], A. Beguin [a], P. Meylan [b], M. Pascual [c], O. Manuel [c], T. Buclin [a], J. Biollaz [a], L.A. Decosterd [a,*]

[a] *Division de Pharmacologie Clinique, Département de Médecine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*
[b] *Institut de Microbiologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*
[c] *Centre de Transplantation d'Organes, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*

Received 21 September 2006; accepted 29 January 2007
Available online 3 February 2007

Abstract

A sensitive HPLC method has been developed for the assay of aciclovir and ganciclovir in human plasma, by HPLC coupled with spectrofluorimetric detection. Plasma (1000 μl), with 9-ethyl-guanine added as internal standard, is submitted to protein precipitation with trichloroacetic acid solution 20%. The supernatant, evaporated to dryness at 37 °C, is reconstituted in 100 μl of a solution of sodium heptanosulfonate 0.4% adjusted with acetic acid to pH 2.60 and a 30 μl volume is then injected onto a Nucleosil 100–5 μm C18 column. Aciclovir and ganciclovir are analysed by spectrofluorimetric detection set at 260 nm (excitation) and 380 nm (emission) using a gradient elution program with solvents constituted of acetonitrile and a solution of sodium heptanosulfonate 0.4% adjusted to pH 2.60. The calibration curves are linear between 0.1 and 10 μg/ml. The mean absolute recovery of aciclovir and ganciclovir are 99.2 ± 2.5 and 100.3 ± 2.5%, respectively. The method is precise (with mean inter-day C.V.s within 1.0–1.6% for aciclovir and 1.2–3.5% for ganciclovir), and accurate (range of inter-day deviations −1.6 to +1.6% for aciclovir and −0.4 to −1.4% for ganciclovir). The method has been applied in stability studies of ganciclovir in patients' blood samples, demonstrating its good stability in plasma at −20 °C and at room temperature. The distribution of ganciclovir and aciclovir in plasma and red blood cells was also investigated in vitro in spiking experiments with whole blood, which showed an initial drop of ganciclovir and aciclovir levels in plasma (about −25%) due to the cellular uptake of aciclovir and ganciclovir by red blood cells. The method has been validated and is currently applied in a clinical study assessing the ganciclovir plasma concentration variability after administration of valganciclovir in a population of solid organ transplant patients.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Aciclovir; Ganciclovir; Stability; HPLC

1. Introduction

Cytomegalovirus (CMV) is the most important pathogen affecting transplant recipients [1]. CMV is known to cause both direct and indirect effects, including acute and chronic allograft rejection [2]. To prevent the burden of this infection in solid organ transplant (SOT) patients, antiviral drugs are commonly used for both CMV prophylaxis and treatment. Intravenous administration of ganciclovir (Fig. 1), an acyclic guanosine analogue, has been the gold standard for the *treatment* of established infection, while valaciclovir (a pro-drug of aciclovir, Fig. 1) and oral ganciclovir were administered for CMV *prophylaxis*. Valganciclovir, the valyl ester of ganciclovir, has been recently developed and is characterised by a near ten-fold higher bioavailability than ganciclovir. Valganciclovir thus offers the perspective of replacing suboptimal oral prophylactic (valaciclovir or oral ganciclovir) and intravenous therapeutic regimens.

Valganciclovir is hydrolysed in the intestinal wall and liver to L-valine and ganciclovir. Ganciclovir, after activation via triphosphorylation by virus and host cell enzymes, inhibits viral DNA polymerase and blocks viral DNA synthesis. The

* Corresponding author at: Division de Pharmacologie Clinique, Laboratoire BH 18-218, Département de Médecine, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne CHUV, Switzerland. Tel.: +41 21 314 42 72; fax: +41 21 314 42 88.
E-mail address: LaurentArthur.Decosterd@chuv.ch (L.A. Decosterd).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.01.045

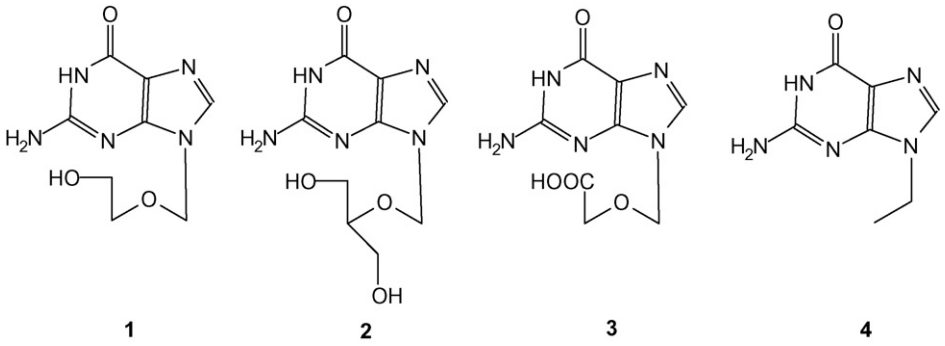

Fig. 1. Aciclovir (1), ganciclovir (2), 9-carboxymethoxymethylguanine (CMMG) (3) and 9-ethyl-guanine (I.S.) (4) chemical structure.

efficacy of valganciclovir has been formally validated by randomised controlled studies in the treatment of CMV retinitis in HIV patients and in the prophylaxis of CMV infection among high-risk (donor CMV seropositive/recipient CMV seronegative, D+/R−) kidney, liver and heart transplant recipients [3,4].

A number of pharmacokinetic studies of the parent drug ganciclovir are already available and suggest, according to Scott et al. [5], that the routine therapeutic drug monitoring of ganciclovir in SOT patients is of limited clinical usefulness. By contrast, little is known about the pharmacokinetics of ganciclovir after administration of its pro-drug valganciclovir in SOT patients. Pescovitz et al. have studied the pharmacokinetics over 24 h of a single dose of oral valganciclovir in comparison to oral and intravenous ganciclovir in 28 liver transplant recipients [6]. Wiltshire et al. have analysed the pharmacokinetic profile of ganciclovir after administration of valganciclovir in high-risk (D+/R−) kidney, liver and heart transplant recipients during prophylaxis regimens [7]. Yet, the usefulness of *routine clinical monitoring* of ganciclovir plasma levels in SOT patients under prophylaxis or treatment with valganciclovir has never been evaluated. For example, ganciclovir is extensively eliminated by the kidney and patients with renal failure require dose adjustment. Ganciclovir is a substrate of renal tubular organic anion transporters, an active clearance system which is increasingly recognised as an important target in renal drug interactions [8]. In addition, there are no data on valganciclovir pharmacokinetics in transplant patients with cystic fibrosis, who are known to have gastrointestinal absorption problems and enhanced renal clearance, leading possibly to reduced systemic exposure to ganciclovir.

Nevertheless, a number of analytical methods have been proposed for the measurement of ganciclovir in biological fluids by high performance liquid chromatography (HPLC), but only a few enable the measurement of aciclovir and/or ganciclovir with the same assay, and none have considered the influence of the metabolite of aciclovir, 9-carboxymethoxymethylguanine (CMMG) [9–11] that can potentially interfere with ganciclovir. In fact, CMMG, which has been already analysed together with aciclovir by Svensson et al. [12], is at risk of co-eluting with ganciclovir when the latter is used as internal standard, or when previously or subsequently used in the same patient.

In addition, there remained some uncertainties on the stability of ganciclovir in blood samples. While most stability studies of ganciclovir have been performed in plasma – where it was generally found stable – one report raised the concern that ganciclovir may not be stable in whole blood left at room temperature [13], which resulted in stringent recommendations to store blood samples on ice immediately after blood sample collection prior to their transportation and centrifugation at low temperature. As ganciclovir is known to be subjected to active intra-erythrocyte uptake via transmembrane transport proteins [14], it was therefore necessary to ascertain whether those mechanisms would affect ganciclovir plasma levels during *in vitro* experiments, and *in vivo*, in patients blood samples left at room temperature. We describe here, a sensitive method for the assay of ganciclovir and aciclovir in human plasma, by HPLC coupled with spectrofluorimetric detection. This assay reaches the required level of sensitivity and reproductibility for routine clinical application and has also been applied in *in vitro* studies focusing on ganciclovir stability in blood samples.

This assay is currently applied in a research protocol aimed at assessing the inter-individual and residual intra-individual variability of ganciclovir after administration of valganciclovir in solid organ transplant patients.

2. Materials and methods

2.1. Chemicals

Zovirax™ vials (sodium aciclovir, corresponding to aciclovir 250 mg) and Cymevene™ vials (sodium ganciclovir, corresponding to ganciclovir 500 mg) were obtained from GlaxoSmithKline AG (Münchenbuchsee, Switzerland) and from Roche Pharma (Reinach, Switzerland), respectively. Aciclovir and ganciclovir stock solution (1 mg/ml) in ultrapure H_2O was prepared as follow: each extract was reconstituted with 10.0 ml H_2O in the vial, yielding a solution of aciclovir 25 mg/ml and ganciclovir 50 mg/ml: an aliquot of 2.0 ml of aciclovir 25 mg/ml and 1.0 ml of ganciclovir 50 mg/ml were subsequently diluted to 50.0 ml with ultrapure H_2O. Stock solution of 9-ethyl-guanine (Internal Standard, I.S.) at a concentration of 100 μg/ml was obtained by dissolution of 9-ethyl-guanine (Sigma, Switzerland) (3 min sonication) in 89 ml purified H_2O onto which 1.0 ml of acetic acid and 10.0 ml of methanol have been added. This solution was diluted down to 5 μg/ml before use with ultrapure H_2O. Acetonitrile (MeCN) for chromatog-

raphy LiChrosolv®, 100% acetic acid (AcOH), trichloroacetic acid solution (TCA) 20% and heptane-1-sulfonic acid sodium salt LiChrosolv® ($C_7H_{15}NaO_3S$) were from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore).

2.2. Chromatographic system

The chromatographic system consisted of a Hewlett-Packard Series 1050 (Agilent, formerly Hewlett-Packard, Germany) pump equipped with an HP 1100 on line degasser, an HP 1050 autosampler and connected via an HP 35900 AC/DC interface to a spectrofluorimetric detector LC240 (Perkin-Elmer, Boston, USA) set at 260 nm (excitation) and 380 nm (emission). The separation was performed at room temperature (RT) on a ChromCart® cartridge column (250 mm × 4 mm, i.d.) filled with Nucleosil 100–5 μm C18 (Macherey-Nagel, Düren, Germany) and equipped with a guard column (8 mm × 4 mm i.d.) filled with the same packing material. The injection volume was 30 μl.

The HP-ChemStation A.06.03 software was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms) throughout the method validation. Baselines were visually inspected and were manually adjusted (in general, base line to base line) using peak start and end features of the HP-ChemStation software.

2.3. Mobile phase solutions

Solution A was prepared, prior to each series of analysis, by dissolution of 4.0 g sodium heptanosulfonate with ultrapure H_2O up to 1000.0 ml with pH carefully adjusted to 2.60 with 100% acetic acid. Solvent B consisted of pure MeCN. The mobile phase was delivered at 1 ml/min and the gradient elution program was: solvent A, 100% at 0.00 min → 93% at 19.00 min → 86% at 31.00 min → 0% at 31.01 min → 0% at 36.00 min → 100% at 36.01 min → 100% at 42.00 min.

2.4. Stock solution, working solution, plasma calibration and control samples

Stock solution of aciclovir and ganciclovir at 1 mg/ml in H_2O was further diluted with H_2O for the preparation of working solutions at concentrations of 1–100 μg/ml. Plasma calibration samples at 0.1, 0.25, 0.50, 1.0, 5.0 and 10.0 μg/ml, together with plasma quality control samples at 0.75, 3.0 and 8.0 μg/ml, were prepared by 1:10 dilution of the respective working solution with blank plasma from outdated transfusion bags (total added volume ≤10% of the biological sample volume), in accordance with the recommendations on bioanalytical method validation [15,16].

The calibration standards and control samples were prepared in batches at the same occasion and were stored at −20 °C as 1.2 ml aliquots in 5 ml-polypropylene Eppendorf tubes, and thawed on the day of analysis.

2.5. Samples collection

According to a study protocol previously approved by the Ethics Committee of University Hospital, blood samples were taken from solid organ transplant patients under prophylaxis or treatment with valganciclovir during their hospital stay and for their subsequent routine follow-up at their scheduled medical visits. Blood samples (5.5 ml) were collected in Monovettes® (Sarstedt, Nümbrecht, Germany), with K-EDTA as anticoagulant. Samples were sent without delay to the laboratory and were centrifuged at 1850 × *g* (3000 rpm) for 10 min at 4 °C (Beckmann Centrifuge, Model J6B) and the plasma was separated and transferred into 5 ml-polypropylene test tubes before being stored at −20 °C up to the time of analysis.

2.6. Sample preparation

On the day of analysis, calibration, quality control and patient samples were thawed, allowed to equilibrate at RT and vortex-mixed. Aliquots (1000 μl) of plasma samples (calibration, control, patients) with 250 μl of I.S. solution (5 μg/ml) was vortexed in an Eppendorf vial before protein precipitation with 250 μl of TCA 20%. After being vortex-mixed the suspensions were centrifuged for 10.0 min on a benchtop centrifuge at 20,000 × *g* (14,000 rpm) at 4 °C (Hettich® Benchtop Universal 16R centrifuge, Bäch, Switzerland). The supernatants were collected and evaporated to dryness under a nitrogen steam at 37 °C for approximately 2.5 h. The residues were then reconstituted in 100 μl of mobile phase solution A. The resulting solutions were carefully vortexed twice, transferred to Eppendorf microvials and then centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatents were introduced into 0.5 ml HPLC autosampler vials (Laubscher Labs, Switzerland) and a volume of 30 μl was used for HPLC analysis.

2.7. Calibration curves

Quantitative analysis of aciclovir and ganciclovir was performed using the Internal Standard (9-ethyl-guanine) method.

The calibration curves were fitted by least-squares linear regression using 1/concentration ($1/x$) as weighting factor of the peak-area ratio of aciclovir and ganciclovir to I.S. versus the ratio of the injected amount of the respective aciclovir and ganciclovir to I.S., in each standard samples. The calibration was established over the clinically relevant range 0.1–10.0 μg/ml for aciclovir and ganciclovir.

The 9-ethyl-guanine was chosen as internal standard, because this synthetic guanine derivative is a structural analogue of aciclovir and ganciclovir, and is unlikely to be present in patient samples.

2.8. Analytical method validation

The validation of the method was based on the guidelines published on-line by the FDA [17] as well as on the recommendations of the Conference Report of the Washington Conference on "Analytical methods validation: Bioavailability, Bioequiva-

lence and Pharmacokinetic studies" [15] and of the Arlington Workshop "Bioanalytical Methods Validation—A revisit with a Decade of Progress" [16].

Each level of the calibration curve was established after two injections of each calibration samples: one at the beginning and the second at the end of the run. Throughout patient sample analysis, control samples at three concentrations levels (low, medium and high, i.e. 0.75, 3.0 and 8.0 μg/ml) were assayed at least every five samples.

Replicate analysis ($n = 6$) of quality control samples were used for the determination of the precision and accuracy of the assay, the three concentrations were chosen to encompass the range of the calibration curve corresponding to aciclovir and ganciclovir levels expected to occur in patient samples. Precision being calculated as the coefficient of variation (C.V. %) within a single run (intra-assay) and between different assays (inter-assay), and the accuracy as the percentage of deviation between nominal and measured concentration.

Both experimental lower limit of quantification (LLOQ) and limit of detection (LOD) were determined by diluting the calibration samples. The LLOQ for aciclovir and ganciclovir in plasma was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the conference report on analytical method validation [15,16] and the FDA [17] recommending that the deviation between measured and nominal concentration at LLOQ should not deviate more than ±20%. The limit of detection was considered as the concentration of aciclovir and ganciclovir that provides a signal corresponding to three times the HPLC background signal.

2.9. Recovery

The efficiency of the sample preparation by protein precipitation with TCA 20% was determined with quality control samples at three levels (0.75, 3.0 and 8.0 μg/ml of aciclovir and ganciclovir, $n = 3$ for each level). The absolute recovery of aciclovir and ganciclovir from plasma was obtained as the peak-area response of the processed sample, expressed as a percentage of the response of the same amount of aciclovir and ganciclovir, calculated to be contained into the 30 μl-injection volume reconstituted in solution A, which corresponds to the 100% recovery.

2.10. Stability of aciclovir and ganciclovir

Stability studies of aciclovir and ganciclovir included:

1. Long-term stability of plasma samples kept frozen at −20 °C: six series of calibration and quality control plasma samples spiked with aciclovir and ganciclovir were prepared. Three series were either immediately analysed (i.e. without being frozen) while the three remaining series were stored during 4 months at −20 °C. The slopes of the calibrations curves were compared (Student's *t*-test).
2. Samples stability at 4 °C and at room temperature
   (a) Stability of aciclovir and ganciclovir in *plasma* samples at 4 °C and at room temperature: six series of calibration and quality control plasma samples spiked with aciclovir and ganciclovir were prepared. Two series were immediately frozen at −20 °C. Two series were kept at RT for 24 h before being frozen at −20 °C. Two series were stored for 24 h at 4 °C and then frozen at −20 °C. The slopes of aciclovir and ganciclovir calibration curves in both groups were compared (Student's *t*-test).
   (b) Stability of aciclovir and ganciclovir in *blood* samples at 4 °C and at room temperature: six series of calibration and quality control citrated blood samples spiked with aciclovir and ganciclovir in 0.9% NaCl were prepared. Two series were immediately centrifuged at 1850 × *g* (3000 rpm) for 10 min at +4 °C. The plasma was collected and frozen at −20 °C. Two other series of blood samples were kept at RT for 48 h, then centrifuged and frozen at −20 °C. Two series of blood were stored for 48 h at 4 °C before being centrifuged and frozen at −20 °C. The slopes of aciclovir and ganciclovir calibration curves in both groups were compared (Student's *t*-test).
   (c) Kinetics (concentration versus time profile) of the distribution of aciclovir and ganciclovir in plasma and red blood cells from *blood* samples left at room temperature were also assessed: a 100 ml citrated blood sample spiked with aciclovir and ganciclovir at 7000 ng/ml in 0.9% NaCl were prepared. At 0, 1, 2, 3, 4, 6, 8, 24, 30 and 48 h, two samples (3 ml) were centrifuged at 1850 × *g* (3000 rpm) for 10 min at +4 °C. Plasma and remaining cellular components were separated and frozen at −20 °C prior to analysis. For the determination of aciclovir and ganciclovir in red blood cells (RBC), haemolysed cellular samples were analysed using calibration and quality control prepared with haemolysed RBC pellets samples instead of plasma.
   (d) Stability of ganciclovir in *patients' blood* samples: six blood samples collected from patients at the occasion of their medical visits were divided in two aliquots. One blood aliquot was immediately centrifuged at 1850 × *g* (3000 rpm) for 10 min at 4 °C and the plasma was collected and frozen at −20 °C. The other blood aliquot was kept at RT for 24 h (in three patients) or 48 h (in three other patients) prior to centrifugation and plasma storage at −20 °C. The variations of ganciclovir plasma concentrations over time were expressed as a percentage of the levels determined in samples immediately centrifuged.
3. Stability of plasma samples after multiple freeze-thaw cycles: aliquots of plasma spiked with aciclovir and ganciclovir at 0.75, 3.0 and 8.0 μg/ml, underwent three freeze-thaw cycles: frozen samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen. Aciclovir and ganciclovir levels were measured in aliquots from the three consecutive freeze-thaw cycles and were analysed in the same series, to eliminate the inter-assay variability. The variations of aciclovir and ganciclovir concentrations were

expressed in percentage of the levels of samples not subjected to the freeze-thaw cycles.

4. Stability of plasma extracts into HPLC vials at room temperature: processed calibration and quality control samples spiked with aciclovir and ganciclovir (i.e. reconstituted in solution A) were analysed in duplicate either immediately after preparation, or after being left 24 and 48 h at room temperature in the auto-sampler rack. The variation of aciclovir and ganciclovir concentrations were expressed in mean percentage of change of the initial concentration.
5. In case some samples would require HIV viro-inactivation, the stability of aciclovir and ganciclovir in plasma under the recommended thermisation process (60 °C for 60 min) [18–21] was assessed as follows: four series of calibration samples at the six concentrations reported above (0.1 up to 10.0 μg/ml) were analysed in parallel. Two were heated at 60 °C for 60 min, while the thermisation procedure was omitted for the two other series. The slope was compared between the resulting calibration curves (Student's *t*-test).

2.11. Selectivity

The selectivity of our analytical method was determined by injecting onto the HPLC column blank plasma from 25 different subjects and the following drugs: acenocoumarol, acetaminophen, acetylcysteine, acetylsalicylic acid, allopurinol, amlodipin, amoxicillin, amprenavir, atazanavir, atenolol, atorvastatin, azathioprin, azithromycin, bromazepam, cafein, candesartan, ciclosporin, cefepim, ceftazidim, chlortalidone, cilastatin, ciprofloxacin, cisaprid, clavulanic acid, codein, diazepam, diclofenac, efavirenz, enalapril, fludrocortisone, fluconazol, folic acid, furosemide, guaifenesin, ibuprofen, imipenem, indinavir, lamivudin, levofloxacin, loperamid, lopinavir, lorazepam, mefenamic acid, meropenem, metamizol, metoprolol, mycophenolate mofetyl, nelfinavir, nevirapine, omeprazol, oxazepam, phytomenadion, piperacillin, prednison, risedronat, spironolactone, sulfamethoxazol, rifampicine, ritonavir, saquinavir, sulfasalazine, tacrolimus, tazobactam, tipranavir, torasemid, tramadol, trimethoprim, uric acid, vancomycin and zidovudin.

2.12. Applications of the HPLC method

This method is currently applied in a research protocol, approved by the local Ethics Committee, aimed at assessing the inter-individual and residual intra-individual variability of ganciclovir after administration of valganciclovir in solid organ transplant patients who require prophylaxis or treatment of cytomegalovirus infection, using a population pharmacokinetic approach, and at determining the relation between ganciclovir plasma concentration and the virological (viremic charge) and clinical (CMV disease) outcomes.

3. Results and discussion

3.1. Chromatograms

The proposed HPLC method enables the measurement of aciclovir and ganciclovir in plasma with fluorimetric detection at 260 nm (emission) and 380 nm (detection). With the gradient program used, the retention times for ganciclovir, aciclovir and 9-ethyl-guanine are 13, 15 and 30 min, respectively. This gradient elution program yields sharp peaks without producing any significant drift of the baseline. The entire HPLC run (including the rinsing and re-equilibration step) lasts 42 min to achieve an excellent separation of aciclovir and ganciclovir from endogenous plasma components with satisfactory selectivity towards the aciclovir metabolite CMMG (see below).

The chromatograms of a blank plasma and of the quality control at 3.0 μg/ml of aciclovir and ganciclovir (onto which the 9-ethyl-guanine has been added) are shown in Figs. 2 and 3, respectively. The potential interference between ganciclovir and the aciclovir metabolite CMMG (Fig. 1) has also been studied. Using the elution conditions proposed with the solution A in the mobile phase adjusted to pH 2.60, the separation of CMMG and ganciclovir was not found optimal. However, a minor pH modification of solution A to pH 2.90 enabled, with a slight change of their retention time, a satisfactory resolution of CMMG and ganciclovir (difference in retention time of ≈0.6 min). Fig. 4 shows the chromatogram of a plasma quality control sample of aciclovir, ganciclovir (750 ng/ml) spiked with the aciclovir metabolite CMMG at a concentration of 1500 ng/ml using the proposed gradient elution program at pH 2.90. Thus, in the rare clinical instances where aciclovir and ganciclovir would be administered to a same patient (i.e. during treatment switch), CMMG and ganciclovir can be nevertheless efficiently separated by a slight pH modification. More generally however, caution should be exercised if ganciclovir is considered as internal standard for aciclovir determination [10,22,23].

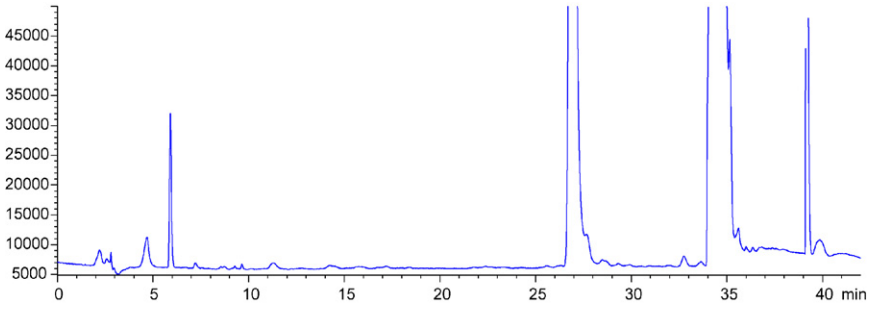

Fig. 2. Chromatographic profile of a blank plasma.

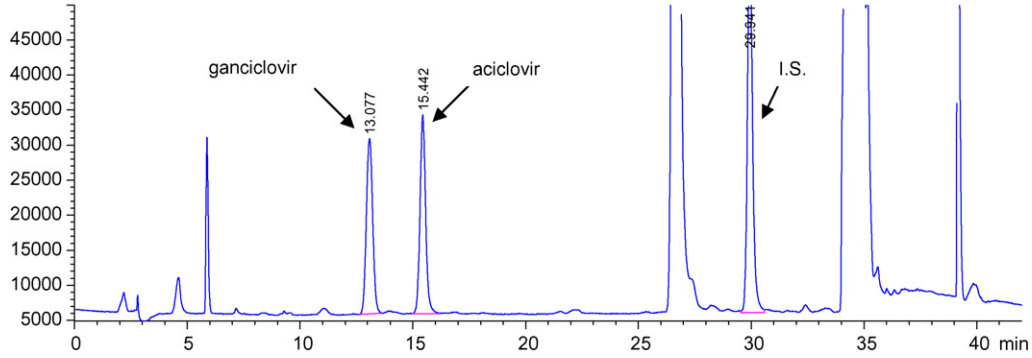

Fig. 3. Chromatographic profile of a plasma sample of aciclovir and ganciclovir (3000 ng/ml) spiked with 9-ethyl-guanine (I.S.).

3.2. Mobile phase composition

For prolonged routine analyses, it is particularly important to control the elution conditions, especially the pH of solution A (at 2.60) and gradient program, to ensure consistent peak shape and retention time of aciclovir and ganciclovir and for satisfactory separation from matrix peaks. Solvent was found stable at room temperature up to 48 h: the pH remained identical (pH ±0.04) during this period of time and there were no signs of microbiological contamination.

3.3. Calibration curves

The calibrations curves have been calculated and fitted by least-squares linear regression either unweighted, or using 1/concentration ($1/x$), and 1/concentration^2 ($1/x^2$) as weighting factor. To establish the best weighting factor, back-calculated concentrations were determined. The model with the lowest total bias and the most constant bias across the concentration range was considered to be the best fit. Visual inspection of the plot of residuals of the $1/x$-weighted regression indicates that there is no trend in variability throughout the delineated range of concentrations. Moreover, the homogeneity of variances of the residuals have been statistically verified according to Levene's test [24] yielding Pr values $> F = 0.08$ for aciclovir and $> F = 0.4$ for ganciclovir, verifying the hypothesis of homoscedasticity, and indicating that the chosen $1/x$ model was indeed adequate.

The slope of the calibration curves appeared stable, with values averaging 0.89 (±4.6%) ($n = 6$) and 0.90 (±1.7%) ($n = 6$) for ganciclovir and aciclovir, respectively. Over the concentration range 0.1–10.0 μg/ml for aciclovir and ganciclovir, the regression coefficient r^2 of the calibration curves remained excellent, always greater than 0.999.

The calibration samples are prepared with citrate plasma whereas patients' plasma samples are collected on EDTA Monovettes®. For the sake of validation, a cross-comparison has been performed between three series of three levels of QC determined against calibration curves established on both matrices (citrate *versus* EDTA). The results of the head-to-head comparison reveal a small albeit significant difference between both anticoagulants ($p < 0.05$, two-way ANOVA), with mean relative differences of 0.7 ± 3.2% and of 2.5 ± 3.4%, for ganciclovir and aciclovir, respectively, if EDTA samples are read against citrate curve. Considering these values and the intra- and inter-assay accuracy (bias) (Table 1), no correction of the results deserves to be applied when the analysis of EDTA samples rather than citrate samples is performed against citrate calibrators.

3.4. Validation of the HPLC method: precision, accuracy and LLOQ/LOD

Precision and accuracy achieved with control samples are given in Table 1. The concentration levels of control samples

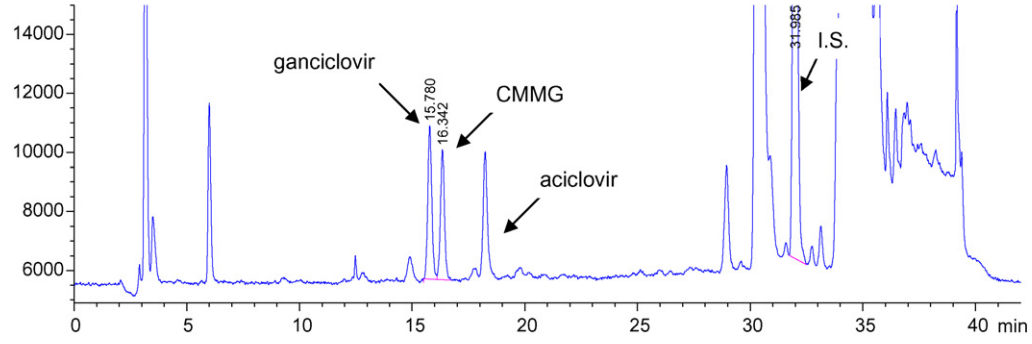

Fig. 4. Chromatographic profile of a plasma quality control sample (20 μl) of aciclovir, ganciclovir (750 ng/ml) spiked with 10 μl of CMMG (1500 ng/ml) and with 9-ethyl-guanine (I.S.). (Gradient program at pH 2.90, see text 3.1.)

Table 1
Precision and accuracy of the assay for aciclovir and ganciclovir in plasma (750, 3000 and 8000 ng/ml)

Nominal concentration (ng/ml)	Concentration found (ng/ml)	S.D. (±)	Precision C.V. (%)	Accuracy[a] bias (%)
Aciclovir				
A. Intra-assay ($n=6$)				
750	747	1	0.2	−0.4
3000	3024	52	1.7	0.8
8000	7979	63	0.8	−0.3
B. Inter-assay ($n=6$)				
750	762	13	1.6	1.6
3000	2995	45	1.5	−0.2
8000	7869	80	1.0	−1.6
Ganciclovir				
A. Intra-assay ($n=6$)				
750	747	4	0.6	−0.4
3000	3042	49	1.6	1.4
8000	7984	53	0.7	−0.2
B. Inter-assay ($n=6$)				
750	746	25	3.5	−0.4
3000	2957	48	1.6	−1.4
8000	7885	91	1.2	−1.4

[a] (Found − nominal)/nominal × 100.

of aciclovir and ganciclovir (750, 3000 and 8000 ng/ml) were selected to encompass the clinically relevant range of concentrations expected in plasma samples.

Throughout these concentration ranges, the mean intra-assay precision was similar, always lower than 2%. Overall, the mean inter-day precision for aciclovir and ganciclovir was good with mean C.V.s within 1.0–1.6 and 1.2–3.5%, respectively. The intra-assay deviation (bias) from the nominal concentrations of aciclovir and of ganciclovir was comprised between −0.4 to +0.8 and −0.4 to +1.4%, respectively, and the range of inter-day deviation was always <1.6 and <−1.4%, respectively.

By analysing plasma from outdated transfusion bags spiked with decreasing concentrations of aciclovir and ganciclovir (50–12.5 ng/ml), the limit of detection was experimentally found to be 25 ng/ml. The lower limit of quantification of aciclovir and ganciclovir, independently determined by back-calculation, is 100 ng/ml. The precision (C.V.) is 4.2 and 5.8%, respectively, and the accuracy (i.e. bias, calculated by back-calculation) at this level is −1.6 and −2.1%, respectively. As both values are thus comprised well within the ±20% limit recommended by the Arlington Workshop [16], 100 ng/ml was therefore chosen as the lower level of calibration.

3.5. Recovery

The mean absolute recovery of aciclovir and ganciclovir measured with the high, medium and low QC controls were 99.2 ± 2.5 and 100.3 ± 2.5%, respectively. The protein precipitation with TCA 20% was found to be a reliable way of eliminating plasma protein with a high absolute recovery and low recovery variability. The internal standard is fully recovered at the concentration spiked (5000 ng/ml) with a low variability: 98.6 ± 0.1%.

3.6. Samples stability

3.6.1. Stability of plasma samples at −20 °C

No evidence of aciclovir and ganciclovir decomposition was found during plasma samples storage in the freezer at −20 °C for at least 4 months. In fact, for aciclovir and ganciclovir, the mean slope of calibration curves ($n=3$) established with samples let 4 months at −20 °C ($m=0.81$ and 0.85, respectively) was not different than the slope of calibration curves calculated with samples analysed immediately ($m=0.80$ and 0.85; $n=3$), ($p=0.2$ and 0.5, Student's t-test) indicating good aciclovir and ganciclovir stability in plasma at −20 °C for at least 4 months.

3.6.2. Stability at 4 °C and at room temperature

The stability of *plasma* samples left at 4 °C and at room temperature was ascertained up to 24 h. For aciclovir, the mean slope of calibration curves ($n=2$) established with plasma samples left 24 h at 4 °C and at RT ($m=0.84$ and 0.83, respectively) was not different than the slope of calibration curves calculated with samples stored during the same time at −20 °C ($m=0.80$; $n=2$), ($p=0.1$ and 0.3, at 4 °C and at RT, respectively; Student's t-test), indicating good stability of aciclovir in plasma at RT. For ganciclovir, the mean slope of calibration curves ($n=2$) established with samples left 24 h at 4 °C and at RT ($m=0.84$ and 0.83, respectively) did not differ from the slope of calibration curves calculated with samples stored during the same time at −20 °C ($m=0.82$; $n=2$), ($p=0.2$ and 0.3, at 4 °C and at RT, respectively; Student's t-test), also indicating good stability of ganciclovir in plasma at RT.

The stability of aciclovir and ganciclovir spiked to *blood* samples left at room temperature and at 4 °C was also checked. After 48 h, there was a significant decrease in plasma levels collected from blood left both at RT and at 4 °C (−17 and −15%, −28 and −24%, for aciclovir and ganciclovir, respectively, $p<0.05$,

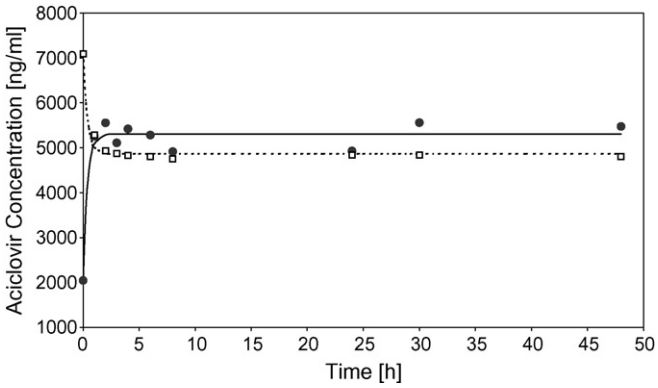

Fig. 5. Aciclovir concentrations profile after the addition of aciclovir at 7000 ng/ml (in 0.9% NaCl) in whole anticoagulated blood. Concentration in plasma (□) and in haemolysed erythrocyte (●). The equilibrium is reached according to an exponential curve, fitted by least-square regression.

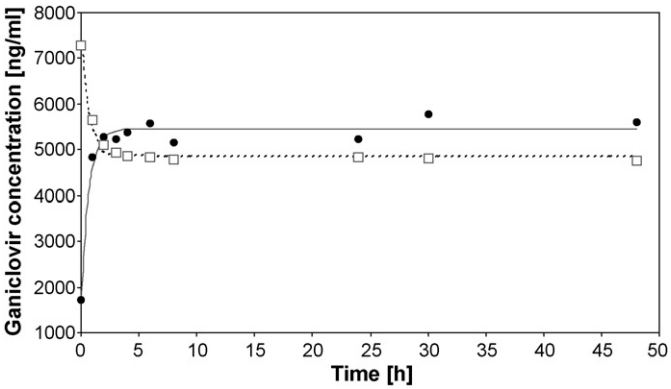

Fig. 6. Ganciclovir concentrations profile after the addition of ganciclovir at 7000 ng/ml (in 0.9% NaCl) in whole anticoagulated blood. Concentration in plasma (□) and in haemolysed erythrocyte (●). The equilibrium is reached according to an exponential curve, fitted by least-square regression.

Student's *t*-test) in comparison to plasma collected immediately after the addition of aciclovir and ganciclovir to blood. A substantial decrease in aciclovir and ganciclovir plasma concentrations observed *in vitro* after the addition of aciclovir and ganciclovir into blood has been previously reported [13] and is most probably due to the cellular uptake by erythrocytes *via* purine nucleobase carriers and nucleoside transporters, of which aciclovir and ganciclovir are known to be substrates [14]. The distribution of aciclovir and ganciclovir in plasma and in red blood cells *in vitro* after their addition into *whole blood* left at room temperature was therefore studied in more details. Figs. 5 and 6 show that there is a pronounced initial drop in aciclovir and ganciclovir concentration in plasma, −26 and −22% after 1 h, followed by a less marked decrease, −32 and −35% after 48 h, for aciclovir and ganciclovir, respectively. This is accompanied by a corresponding increase in aciclovir and ganciclovir levels in haemolysed erythrocytes, supporting the hypothesis that the observed decrease in aciclovir and ganciclovir concentrations in plasma *in vitro* is indeed due to drug uptake by erythrocytes until erythrocyte/plasma equilibrium is reached (in our case, the erythrocyte/plasma ratio was 1.11 ± 0.06). *In vivo* however, this phenomenon is unlikely to affect the accuracy of drug measurements in blood from patients treated for few days with ganciclovir or valganciclovir, as the drug distribution in cell and plasma has already reached an equilibrium in circulating blood at the time of sampling. Indeed, the stability of ganciclovir in plasma was assessed in three patients whose anticoagulated blood samples were left at room temperature for 24 or for 48 h, showing a bias of −1.5 and +3.5%, respectively, demonstrating the good stability of ganciclovir in plasma from blood samples left at least for 48 h at RT after collection from patients. This is of particular interest for samples shipment in the perspective of multicentric studies. Our observations contrast to those of Boulieu and Bleyzac [13] who have previously found a limited stability of ganciclovir, necessitating, according to these authors, stringent conditions of samples collection.

3.6.3. Stability of plasma samples after one, two and three freeze-thaw cycles

The variations of aciclovir and ganciclovir concentrations when submitting control plasma to three successive freeze-thaw cycles are reported in Table 2. This indicates that no significant loss of aciclovir and ganciclovir is to be expected after up to three freeze-thaw cycles.

3.6.4. Stability of extracts samples into HPLC vials (i.e. ready for HPLC analysis) at room temperature

The stability of plasma extracts (i.e. reconstituted in solution A, in HPLC vials) submitted to HPLC analysis was checked at RT for 24 and 48 h and is reported in Table 3. The variations of drug concentration, for aciclovir and ganciclovir, respectively, over time in samples left at RT, expressed in percentage of the starting levels (i.e. after immediate analysis), were less than −8.1 ± 2.8 and −2.6 ± 1.6% after 24 h, and less than −11.4 ± 0.8 and −9.1 ± 2.2% after 48 h. This indicates that even though there is a slight decrease in aciclovir concentra-

Table 2
Stability of aciclovir and ganciclovir plasma samples after one, two and three freeze-thaw cycles for the QC samples at nominal concentration of 750, 3000 and 8000 ng/ml, respectively (concentration change expressed in % of the initial concentration)

No. of thaw-freeze cycles	Aciclovir			Ganciclovir		
	Nominal concentration (ng/ml)			Nominal concentration (ng/ml)		
	750	3000	8000	750	3000	8000
1	97.9 ± 1.3	96.3 ± 1.0	99.0 ± 0.3	99.4 ± 0.8	100.3 ± 0.3	100.3 ± 0.4
2	93.9 ± 2.5	97.6 ± 0.2	105.6 ± 2.1	95.5 ± 2.2	97.4 ± 0.5	99.0 ± 1.0
3	100.2 ± 0.2	102.6 ± 1.9	105.7 ± 0.8	98.0 ± 0.9	97.8 ± 0.1	97.8 ± 0.4

Table 3
Stability of aciclovir and ganciclovir in extract samples left at room temperature (RT) for 24 and 48 h

Duration (h)	Nominal concentration (ng/ml)		
	750	3000	8000
Aciclovir[a]			
24	−8.1 ± 2.8	−6.8 ± 0.3	−5.1 ± 0.4
48	−11.4 ± 0.8	−9.0 ± 0.4	−9.5 ± 0.1
Ganciclovir[a]			
24	−2.6 ± 1.6	−1.4 ± 1.3	−1.1 ± 0.3
48	−9.1 ± 2.2	−8.9 ± 0.9	−8.6 ± 0.9

[a] Mean percent change of the initial concentration ± S.D.; $n = 2$.

tion in plasma extracts left after 24 h at room temperature this difference is comprised within 10%, which is still acceptable if the HPLC run does not exceed one day. However, the extracted plasma in HPLC vials should not be left more than 24 h at room temperature because of the apparent reduced stability after 48 h in this condition.

3.6.5. Stability during thermisation (HIV inactivation)

The slope of the calibration curves of aciclovir and ganciclovir established in samples submitted to the thermisation procedure (60 °C for 60 min) was slightly lower (variation of −6.2 ± 0.2%) than that obtained with non-heated samples for aciclovir, and not different for ganciclovir (0.5 ± 0.5%), as shown in Table 4. This difference was however not significant for both compounds ($p = 0.2$ and 0.5, for aciclovir and ganciclovir, respectively, Student's t-test), indicating that such a procedure does not affect to a significant extent aciclovir and ganciclovir concentrations, within the considered concentrations range. Thus, thermisation can be considered in case HIV inactivation of samples is required.

3.7. Selectivity

Among the 25 different blank plasma tested, none showed the presence of significant interfering endogenous peaks at the retention time of aciclovir, ganciclovir and the internal standard. The method selectivity was confirmed by analysing the various immunosuppressive treatment regimens and more than 40 different other drugs possibly prescribed to transplant patients at our hospital, and 10 anti-HIV drugs. Few drugs were detected by spectofluorimetry at 380 nm, and all were eluted at times not perturbing aciclovir and ganciclovir analysis.

3.8. Clinical applications

This HPLC assay is currently applied to the analysis of samples collected as part of an ongoing clinical research study

Table 4
Parameters of the calibration curves for aciclovir and ganciclovir before and after plasma thermisation at 60 °C for 60 min ($n = 2$)

Sample treatment ($n = 2$)	m	r^2	b	Variation (%)
Aciclovir				
1/Thermisation 60 min at 60 °C	0.8616	0.99984	1.073E−2	
1/No thermisation	0.9200	0.99980	1.087E−2	−6.4
2/Thermisation 60 min at 60 °C	0.7903	0.99997	1.073E−2	
2/No thermisation	0.8409	0.99994	1.089E−2	−6.0
Mean ± S.D.				−6.2 ± 0.2
Ganciclovir				
1/Thermisation 60 min at 60 °C	0.8625	0.99997	0.588E−3	
1/No thermisation	0.8704	0.99999	1.508E−3	0.9
2/Thermisation 60 min at 60 °C	0.8102	0.99960	1.546E−3	
2/No thermisation	0.8114	0.99992	1.714E−3	0.1
Mean ± S.D.				0.5 ± 0.5

m, slope; r^2, coefficient of determination; b, y-axis intercept.

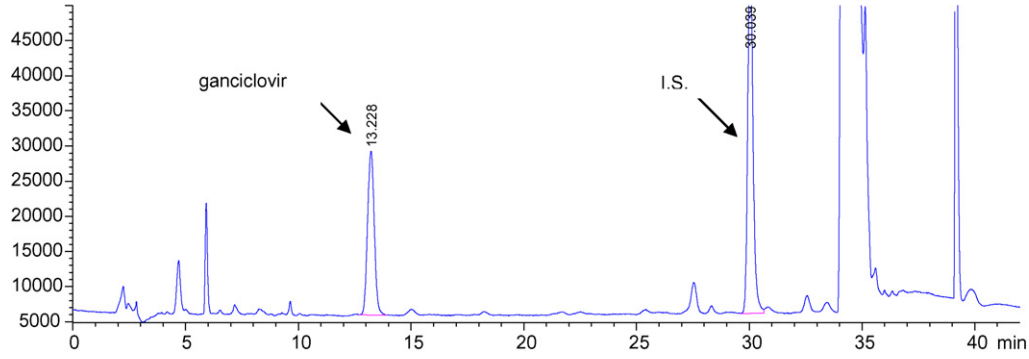

Fig. 7. Chromatographic profile of a plasma from a solid organ transplant patient receiving valganciclovir (explanation in the text).

on the pharmacokinetics and pharmacodynamics of ganciclovir after administration of valganciclovir in solid organ transplant patients. For example, Fig. 7 shows the chromatographic profile of one plasma obtained from a SOT patient receiving prednisone, tacrolimus, and a prophylactive regimen of valganciclovir 450 mg once a day (OD). The plasma level of ganciclovir measured 2 h 30 min after the Valcyte® intake is 3.1 μg/ml (IC 95% = 3.0–3.2 μg/ml).

Of note, ganciclovir and aciclovir drug levels measurements were in some instances asked for neonates and for pediatric patients for whom the volume of blood collected must be limited. In these cases, aliquots as low as 100 μl of plasma have been successfully analysed using the proposed method, using accordingly corresponding volumes of calibration and QC samples, with satisfactory quantification limits.

4. Conclusion

This HPLC method provides a simple and robust procedure for determining aciclovir or ganciclovir in patients' plasma. It has been developed using instruments available in conventional hospital laboratories, including a spectrofluorimetric detector. This procedure, through relatively time consuming, represents a practicable, cheap and robust method providing the required level of sensitivity for measuring clinically relevant ranges of concentrations of aciclovir and ganciclovir.

During this study, we have pointed out the potential interference of the metabolite of aciclovir, 9-carboxymethoxymethylguanine with ganciclovir, a problem that has been up to now only limitedly addressed. This interference potential does not represent a strong limitation of our analytical method since it can be easily circumvented by a slight pH change of the aqueous mobile phase to pH in the exceptional cases (i.e. during treatment switch) where ganciclovir and aciclovir would be present simultaneously in a same patient.

In addition, we have demonstrated the good stability of ganciclovir in patients' blood samples at room temperature up to 48 h. Thus, according to our observations, patients' samples do not require the stringent sample collection conditions recommended by Boulieu and Bleyzac [13]. Finally, since plasma extract samples are stable at room temperature in the autosampler rack over 24 h, the duration of one analytical run does not represent a limitation of our method. Using a devoted HPLC apparatus, it is possible to analyze 20 patients' samples per analytical series. The method is currently applied for the monitoring of ganciclovir and aciclovir in SOT patients.

Acknowledgment

Support for this work was provided in part by an unrestricted research grant from Roche (Switzerland).

References

[1] J.A. Fishman, R.H. Rubin, N. Engl. J. Med. 338 (1998) 1741.
[2] C.V. Paya, Transpl. Infect. Dis. 1 (Suppl. 1) (1999) 8.
[3] D.F. Martin, J. Sierra-Madero, S. Walmsley, R.A. Wolitz, K. Macey, P. Georgiou, C.A. Robinson, M.J. Stempien, N. Engl. J. Med. 346 (2002) 1119.
[4] C. Paya, A. Humar, E. Dominguez, K. Washburn, F. Blumberg, B. Alexander, R. Freeman, N. Heaton, M.D. Pescovitz, Am. J. Transplant. 4 (2004) 611.
[5] J.C. Scott, N. Partovi, M.H. Ensom, Ther. Drug. Monit. 26 (2004) 68.
[6] M.D. Pescovitz, J. Rabkin, R.M. Merion, C.V. Paya, J. Pirsch, R.B. Freeman, J. O'Grady, C. Robinson, Z. To, K. Wren, L. Banken, W. Buhles, F. Brown, Antimicrob. Agents Chemother. 44 (2000) 2811.
[7] H. Wiltshire, S. Hirankarn, C. Farrell, C. Paya, M.D. Pescovitz, A. Humar, E. Dominguez, K. Washburn, E. Blumberg, B. Alexander, R. Freeman, N. Heaton, Clin. Pharmacokinet. 44 (2005) 495.
[8] H. Miyazaki, T. Sekine, H. Endou, Trends Pharmacol. Sci. 25 (2004) 654.
[9] C.M. McMullin, B. Kirk, J. Sunderland, L.O. White, D.S. Reeves, A.P. MacGowan, J. Antimicrob. Chemother. 38 (1996) 739.
[10] S. Kishino, Y. Takekuma, M. Sugawara, T. Shimamura, H. Furukaw, S. Todo, K. Miyazaki, J. Chromatogr. B: Anal. Tech. Biomed. Life Sci. 780 (2002) 89.
[11] D. Teshima, K. Otsubo, T. Yoshida, Y. Itoh, R. Oishi, Biomed. Chromatogr. 17 (2003) 500.
[12] J.O. Svensson, L. Barkholt, J. Sawe, J. Chromatogr. B, Biomed. Sci. Appl. 690 (1997) 363.
[13] R. Boulieu, N. Bleyzac, J. Pharm. Biomed. Anal. 12 (1994) 1205.
[14] W.B. Mahony, B.A. Domin, T.P. Zimmerman, Biochem. Pharmacol. 41 (1991) 263.
[15] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.
[16] 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. Viswanathn, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[17] FDA Home page, Guidance for Industry: Bioanalytical Method Validation, May 2001. http://fda.gov/cder/guidance/index.htm.
[18] J.S. McDougal, L.S. Martin, S.P. Cort, M. Mozen, C.M. Heldebrant, B.L. Evatt, J. Clin. Invest. 76 (1985) 875.
[19] S.S. Good, D.J. Reynolds, P. De Miranda, J. Chromatogr. 431 (1988) 123.
[20] G.V. Quinnan Jr., M.A. Wells, A.E. Wittek, M.A. Phelan, R.E. Mayner, S. Feinstone, R.H. Purcell, J.S. Epstein, Transfusion 26 (1986) 481.
[21] C.H.W. Koks, H. Rosing, P.L. Meenhorst, A. Bult, J.H. Beijnen, J. Chromatogr. B 663 (1995) 345.
[22] J. Alcorn, P.J. McNamara, Antimicrob. Agents Chemother. 46 (2002) 1831.
[23] S. Brown, C.A. White, C.K. Chu, M.G. Bartlett, J. Chromatogr. B 772 (2002) 327.
[24] G.W. Snedecor, W.G. Cochran, Statistical Methods, eight edition, Iowa-State University Press, 1989, p. 252.