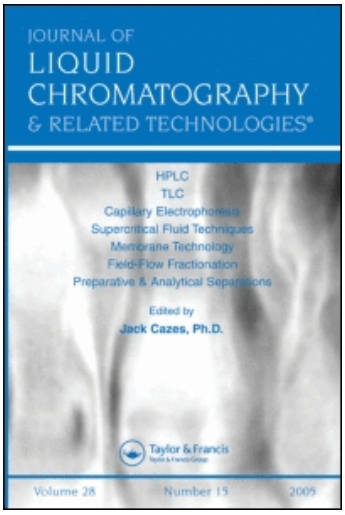

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Liberato Brum Junior[a]; Ana Maria Pugens[a]; Alexandre Dal'Maso[a]; Eduardo Gomes[a]; Maurício Bedim dos Santos[a]; Josélia Larger Manfio[a]
[a] BIOCINESE, Centro de Estudos Biofarmacêuticos, Toledo-PR, Brasil

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Validation of a Liquid Chromatographic Method for the Determination of Acyclovir in Human Plasma: Application to Bioequivalence Study

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BIOCINESE, Centro de Estudos Biofarmacêuticos, Toledo-PR, Brasil

Abstract: An analytical method based on reversed phase liquid chromatography (RP-LC) was developed and validated for the determination of acyclovir in human plasma. Acyclovir and guanine (internal standard) were extracted from the plasma by liquid-liquid extraction using acidified acetonitrile as extraction solvent, and separated on a C_{18}analytical column (150'mm × 4.6 mm I.D.) maintained at 30°C. The elution was performed by a fast gradient at a constant flow rate of 1.0 mL/min and the mobile phase A consisted of 1% formic acid, and mobile phase B consisted of acetonitrile. The fluorescence detector was set at 270 nm (excitation) and 380 nm (emission). The chromatographic separation was obtained within 16.0 min and was linear in the concentration range of 20–3000 ng/mL. The mean extraction recoveries of acyclovir and guanine from plasma were 82.2 and 76.0%, respectively. Method validation investigated parameters such as the specificity, linearity, precision, accuracy, and stability, giving results within the acceptable range. Moreover, the proposed method was successfully applied to a pharmacokinetic study in healthy human volunteers, and results showed that the two acyclovir formulations are bioequivalent in their rate and extent of absorption.

Keywords: Acyclovir, Bioequivalence, Liquid chromatography, Liquid-liquid extraction, Validation

Address correspondence to Liberato Brum Junior, BIOCINESE, Centro de Estudos Biofarmacêuticos, Av. Ministro Cirne Lima, 1541, Toledo-PR 85.903-500, Brasil. E-mail: liberatojunior@mail.ufsm.br

INTRODUCTION

Acyclovir 9-(2-hydroxyethoxy)-methylguanine is a synthetic purinic nucleosidic analogue derived from guanine. This drug is an effective agent in the treatment of herpes virus infection (refer).

The pharmacokinetics parameters of acyclovir following oral administration generally are highly variable. Peak plasma concentrations of 460–830 or 630–1210 ng/mL after a single oral dose of 200 and 400 mg, respectively, are generally obtained 1.5–2.5 h after administration.[1,2]

HPLC methods[1–9] have been published for the determination of acyclovir in human serum and plasma using UV or fluorescence detection. Since acyclovir is a polar compound and soluble in aqueous medium and practically insoluble in most organic solvents, protein precipitation with perchloric acid or solid phase extraction are applied for pretreatment of the drug in biological samples. While the sensitivity of analysis is significantly reduced due to dilution of the samples after precipitation of proteins by perchloric acid, this leads to numerous late eluting peaks and a significant reduction of the life time of the analytical column. Solid phase extraction is expensive and not available in most laboratories.

The aim of the present work was to validate a simple, fast, precise, and accurate RP-LC method to be applied to the quantitative analysis of acyclovir in human plasma using a liquid-liquid extraction, improving the current published procedures, and demonstrating the applicability of the method for the determination of acyclovir in bioequivalence studies.

EXPERIMENTAL

Chemicals and Reagents

The acyclovir reference standard was purchased from Brazilian Pharmacopeia (Brazil) and the guanine reference standard was purchased from Sigma (St Louis, MO, USA). Tablets, containing 200 mg of acyclovir were obtained from Prati, Donaduzzi & Cia Ltda and Glaxo Wellcome (Zovirax®), within their shelf life period. HPLC grade acetonitrile, formic, and acetic acid were purchased from Tedia (Fairfield, USA). All chemicals used were of pharmaceutical or special analytical grade. For all the analyses, ultrapure water (Gehaka, Brazil) filtered through a 0.22 μm membrane filter was used.

Apparatus and Analytical Conditions

The LC method was performed on a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A_{VP} system controller, LC-10

AT_{VP} pump, DGU-14A degasser, CTO-10 AS_{VP} column oven, SIL-10AF autosampler, SPD-M10A_{VP} photodiode array (PDA) detector, and RF-10A_{XL} fluorescence detector. The fluorescence detector was set at 270 nm (excitation) and 380 nm (emission). The peak areas were integrated automatically by computer using a Shimadzu Class VP®V 6.12 software program. The experiments were carried out on a reversed phase Shim-Pack (Shimadzu, Kyoto, Japan) C_{18} column (150 mm × 4.6 mm I.D.) and a C_{18} Kit Security Guard Cartridges was used to protect the analytical column. The LC system was operated at 30°C. The elution was performed by a fast gradient at a constant flow rate of 1.0 mL/min. Mobile phase A consisted of 1% formic acid, and mobile phase B consisted of acetonitrile (99:1, v/v). The applied gradient was as follows: 0.1 min 1% of B, from 7.5–8 min linear to 99% of B, from 10.5–12.5 min linear back to 1% and 12.5–16 min 1% of B. The mobile phases were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). The injection volume was 40 μL for both standard and samples.

Procedure

Preparation of Stock Solutions

The stock solution of acyclovir was prepared by weighing 10 mg of reference material into a 10 mL volumetric flask and diluting to volume with acetonitrile, obtaining a concentration of 1 mg/mL. Guanine stock solution was also made at a final concentration of 1 mg/mL using ultrapure water. The prepared stock solutions were stored at 2–8°C protected from light.

Preparation of Calibration Standards and Quality Control Samples

The stock solution of acyclovir was diluted with acetonitrile to obtain calibration standards solutions with the concentrations of 500, 1250, 2500, 5000, 10,000, 20,000, 37,500, and 75,000 ng/mL. The corresponding volumes taken of the standard solutions were diluted in blank plasma to prepare the calibration standards containing from 20 to 3000 ng/mL (20, 50, 100, 200, 400, 800, 1500, and 3000 ng/mL). The quality control (QC) samples were prepared in pooled plasma, with the concentrations of 50 (low), 400 (medium), and 1500 ng/mL (high), and then divided in aliquots that were stored at −20°C until analysis.

Plasma Extraction Procedure

A total of 500 μL of the spiked plasma was transferred to a 15 mL glass tube, followed by addition of 50 μL of the internal standard solution (40000 ng/mL of guanine in acetonitrile). All samples were mixed by

vortex agitation for 30 seconds. Then, a 4 mL aliquot of extraction solvent, acetonitrile acidified with 0.2% of acetic acid was added using Dispensette Organic (Brand GmbH, Germany). The tubes were vortex-mixed for 2 minutes, and then centrifuged for 10 minutes at 3500 rpm. The organic layer was filtered through a Millex GV 0.45 μm filter unit (Millipore, Bedford, MA, USA) into 15 mL conical glass tubes, and evaporated under nitrogen stream while immersed in a 45°C water bath. Each sample was reconstituted with 200 μL of water and vortex mixed for 30 seconds. The samples were transferred to autosampler vials and 40 μL was injected into the LC system.

Validation of the Bioanalytical Method

The method was validated by the determination of the following parameters: specificity, linearity, range, recovery, accuracy, precision, lower limit of quantitation (LLOQ), and stability studies, according to the FDA guidelines.[10]

Specificity

Randomly selected six blank human plasma samples, which were collected under controlled conditions, were carried through the extraction procedure and chromatographed to determine the extent to which endogenous plasma components could contribute to interference with the analyte or the internal standard. The results were compared with LLOQ (20 ng/mL).

Calibration Curve

The calibration curves were constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed with IS), and eight concentrations of acyclovir including the LLOQ, ranging from 20 to 3000 ng/mL. The peak area ratio of the drug to the IS against the respective standard concentrations was used for plotting the graph and the linearity evaluated by a weighted ($1/x^2$) least squares regression analysis. The acceptance criteria for each calculated standard concentration was not more than 15% deviation from the nominal value, except for the LLOQ which was set at 20%.

Recovery

The analytical recovery was calculated by comparing chromatographic peak areas from unextracted standard samples and from extracted standard samples at three different concentrations (50, 400, and 1500 ng/mL) for the acyclovir and 10000 ng/mL for the IS.

Accuracy and Precision

To evaluate the inter-day precision and accuracy, the quality control samples were analysed together with one independent calibration standard curve for 3 days, while intra-day precision and accuracy were evaluated through analysis of validation control samples at three different concentrations, in six replicates in the same day. Inter- and intra-day precision was expressed as relative standard deviation (RSD). The accuracy was expressed as the percent ratio between the experimental concentration and the nominal concentration for each sample. The evaluation of precision was based on the criteria[10] that the deviation of each concentration level should be within $\pm$15%, except for the LLOQ, for which it should be within $\pm$20%. Similarly for accuracy, the mean value should not deviate by $\pm$15% of the nominal concentration, except the LLOQ, where it should not deviate by $\pm$20% of the nominal concentration.

Lower Limit of Quantification (LLOQ) and Limit of Detection (LOD)

The lowest standard concentration on the calibration curve should be accepted as the limit of quantification if the following conditions are met: the analyte response at the LLOQ should be at least five times the response compared to blank response and analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.

Stability

The concentration of acyclovir after each storage period was related to the initial concentration as zero cycle (samples that were freshly prepared and processed immediately). The samples were considered stable if the deviation (expressed as percentage bias) from the zero cycle was within $\pm$15%.

Freeze-thaw Stability

The freeze-thaw stability of acyclovir was determined at low and high QC samples ($n = 3$) over three freeze-thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 h and refrozen for 24 h. After completion of each cycle the samples were analyzed and the results compared with that of the zero cycle.

Short-term Stability

Three aliquots each of the low and high unprocessed QC samples were kept at room temperature (25 ± 5°C) for 4 h. After 4 h the samples were analyzed and the results compared with that of the zero cycle.

Long-term Stability

Three aliquots each of the low and high QC samples were frozen at −20°C for 76 days. The samples were analyzed and the results were compared with that of the zero cycle.

Processed Sample Stability

Three aliquots each of the low and high QC samples were processed and placed into the autosampler at room temperature and were analyzed after 48 h. The results were compared with that of the zero cycle.

Bioequivalence Study

Thirty-two healthy volunteers, 16 males and 16 females, ranging in age from 19 to 43 years (mean ± S.D., 26.0 ± 5.36 years), in weight from 49.5 to 84.5 kg (mean ± S.D., 66.68 ± 9.3 kg), and in height from 150 to 184 cm (mean ± S.D., 170 ± 0.1 cm), and within 15% of their ideal body weight, were enrolled. The clinical protocol was approved by the local Ethics Committee and the volunteers gave written informed consent to participate in the study. Volunteers were healthy and had no history of heart, kidneys, neurological or metabolic diseases, no history of drug hypersensitivity, were not undergoing any pharmacological treatment, and female volunteers were not pregnant. The study was an open, randomized, two period, two group crossover trial with a 1 week washout interval. During the first period, volunteers from group A received a single 200 mg dose of Zovirax® (reference product), while volunteers from group B received a single 200 mg dose of Acyclovir (test product). During the second period, the procedure was repeated on the groups in reverse. The tablets were administered to the volunteers in the morning, after an overnight fast, with 200 mL of water. Volunteers received standard lunch and afternoon snacks, respectively, 5 and 8 h after drug administration. Volunteers did not ingest any alcoholic drink, coffee, or other xanthine containing drinks during the trial. Furthermore, they did not take any other drug, 1 week before the study and during its execution. Blood samples were collected at 0 (predose) and at 0:15, 0:30, 0:45, 1:0, 1:15, 1:30, 1:40, 1:50, 2:0, 2:10, 2:20, 2:30, 2:45, 3:00, 3:15, 3:30, 3:45, 4:0, 6:0, 8:0, 10:0, 12:0, 16:0. The blood samples were centrifuged for 10 minutes at 3000 rpm and the plasma was stored at −20°C until tetracycline quantification.

Statistical Analysis

The pharmacokinetics parameters of tetracycline were estimated by noncompartmental methods using WinNolin software version 5.0.1. The C_{max} values and the time to reach C_{max} (T_{max}) were estimated directly from the observed plasma concentration time data. The area under the plasma concentration time curve from time 0 to 48 hours ($AUC_{0\text{-}48h}$) was calculated using the linear trapezoidal rule. The AUC from time 0 to infinity ($AUC_{0\text{-}\infty}$) was calculated as:

$$AUC_{0\text{-}\infty} = AUC_{0\text{-}48h} + Ct/K_e,$$

where is C_t is the last plasma concentration measured and K_e is the elimination constant; K_e was determined using linear regression analysis of the logarithm linear part of the plasma concentration time curve. The $t_{1/2}$ of tetracycline was calculated as:

$$t_{1/2} = \ln 2/K_e$$

To determine whether the two tetracycline formulations were pharmacokinetically equivalent, we assessed the calculated individual C_{max}, $AUC_{0\text{-}48h}$, and $AUC_{0-\infty}$, and their ratios (test/reference) using logtransformed data; their means and 90% confidence intervals (CIs) were analyzed by a parametric analysis of variance method using the WinNolin software. The drugs were considered pharmacokinetically equivalent if the difference between three compared parameters was statistically nonsignificant ($P \geq 0.05$) and the 90% CI for these parameters fell within 80 to 125%.

RESULTS AND DISCUSSION

To obtain the best chromatographic conditions, different columns and mobile phases consisting of acetonitrile-water or methanol-water were tested to provide sufficient selectivity and sensitivity in a short separation time. Modifiers such as formic acid, acetic acid, and ammonium acetate were tested. The best signal was achieved using 1% formic acid/acetonitrile (99:1, v/v) with a flow rate of 1.0 mL/min in a C_{18} analytical column, using the fast gradient.

The linearity was determined by six determinations of eight concentrations in the range of 20–3000 ng/mL. The value of the determination coefficient ($r^2 = 0.9995$, $y = 0.0004x + 0.0022$) indicated significant linearity of the calibration curve for the method. The LLOQ was calculated as 20 ng/mL and LOD was found to be 10 ng/mL. Comparison of the chromatograms of the blank and spiked human plasma (20 ng/mL) indicated that no interferences were detected from endogenous substances. A typical chromatogram obtained by the proposed LC method, with the resolution of the symmetrical peak corresponding to acyclovir and guanine, is shown in Figure 1.

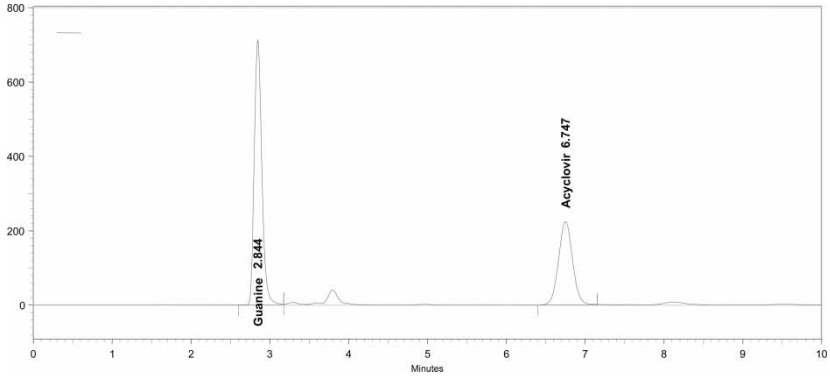

Figure 1. Representative LC chromatogram of medium QC plasma sample containing acyclovir (400 ng/mL) and IS (500 ng/mL).

The results of the liquid-liquid extraction method developed, using acetonitrile acidified with 0.2% of acetic acid as extraction solvent, allowed mean recoveries of acyclovir (82.2%) and IS (76.0%) at the specified concentration levels, confirming the suitability of the method for the plasma samples (Table 1). For the extraction, different organic solvents and mixtures were also evaluated, including ethyl acetate, diethyl ether, and dichloromethane.

Table 1. Recovery of acyclovir and guanine after the extraction procedure

Acyclovir concentration (ng/mL)	% Recovery (mean ± RSD%)	
	Acyclovir[a]	Guanine[a]
50	82.80 ± 2.02	76.86 ± 1.28
400	81.87 ± 0.98	75.57 ± 0.61
1500	82.06 ± 0.92	75.60 ± 0.96

[a]Mean of six replicates.

Table 2. Intra-day precision and accuracy for the determination of acyclovir in human plasma

Nominal concentration (ng/mL)	Mean concentration found (ng/mL)[a]	RSD (%)	Accuracy (%)
50	48.62	9.17	97.25
400	397.00	2.01	99.26
1500	1526.24	1.63	101.75

[a]Mean of six replicates.

Table 3. Inter-day precision and accuracy for the determination of acyclovir in human plasma

Nominal concentration (ng/mL)	Day	Mean concentration found (ng/mL)[a]	Mean[b]	RSD (%)	Accuracy (%)
50	1	48.62	49.53	1.97	99.07
	2	49.42			
	3	50.56			
400	1	397.0	405.09	4.15	101.27
	2	424.41			
	3	393.85			
1500	1	1526.24	1541.11	1.06	102.74
	2	1558.48			
	3	1538.61			

[a]Mean of six replicates.
[b]Mean of three days.

The intra-day accuracy of the method was between 97.25 and 101.75% with a precision of 1.63–9.17% (Table 2). The inter-day accuracy was between 99.07 and 102.74% with a RSD of 1.06–4.15% (Table 3). The data show that the method possesses adequate repeatability and reproducibility.

As shown in Table 4, the plasma samples were stable for at least 76 days at −20°C (long term) and also after three freeze-thaw cycles, demonstrating

Table 4. Summary of stability of acyclovir in human plasma

Stability	Zero cycle concentration (ng/mL)[a]	Concentration found after storage (ng/mL)[a]	RSD (%)	Bias[b] (%)
Long term	48.42	49.14	8.72	1.45
	1612.15	1525.74	0.77	5.76
Short term	48.42	49.92	7.34	2.99
	1612.15	1527.35	3.38	−5.65
Autosampler 48 h	48.42	46.56	10.89	3.72
	1612.15	1483.42	0.67	−8.59
Three freeze-thaw cycles	48.42	48.81	3.46	0.78
	1612.15	1575.57	0.75	−2.44

[a]Mean of three replicates.
[b]Bias = (measured concentration − nominal concentration/nominal concentration) × 100.

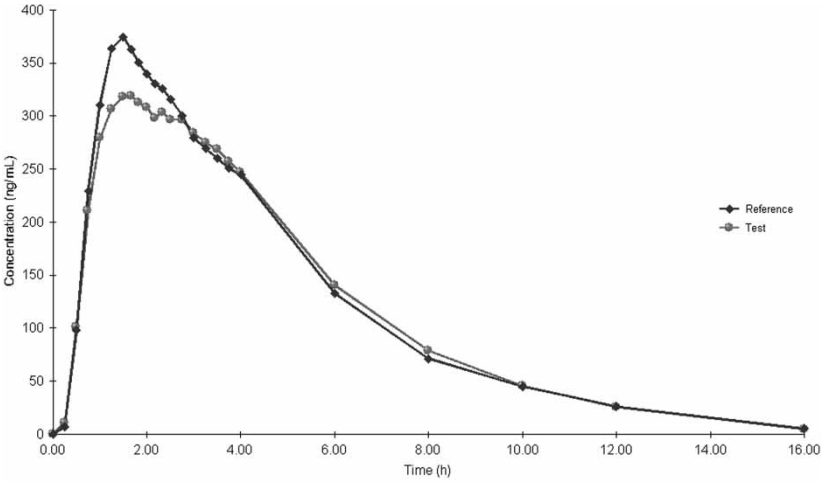

Figure 2. Average values of pharmacokinetic parameters after administration of reference (Zovirax® 200 mg, Glaxo Wellcome) and test (acyclovir 200 mg, Prati, Donaduzzi) products to 32 healthy volunteers.

that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Acyclovir was stable in neat plasma for up to 4 h at room temperature (short term). The results demonstrated that extracted samples could be analysed after being kept in the autosampler for at least 48 h with an acceptable precision and accuracy.

METHOD APPLICATION

Application to Biological Samples

The proposed method was successfully applied to the determination of acyclovir in plasma samples for the purpose of stabilizing the bioequivalence of a 200 mg formulation tablet in 32 healthy volunteers. Average concentration versus time curves after administration of reference (Zovirax® 200 mg, Glaxo Wellcome) and test (acyclovir 200 mg, Prati, Donaduzzi) products to 32 healthy volunteers are shown in Figure 2. Table 5 shows the average values of pharmacokinetic parameters after administration of reference (Zovirax® 200 mg, Glaxo Wellcome) and test (acyclovir 200 mg, Prati, Donaduzzi) products to 32 healthy volunteers. The 90% confidence intervals for the ratio of C_{max} (83.86–105.83%), $AUC_{0\text{-}t}$ (86.67–111.37%), and $AUC_{0\text{-}\infty}$ (87.76–110.87%) values for the test and reference products are within the 80–125% interval proposed by FDA requirements.

Table 5. Pharmacokinetic parameters after administration of reference (Zovirax® 200 mg, Glaxo Wellcome) and test (acyclovir 200 mg, Prati, Donaduzzi) products to 32 healthy volunteers

	Zovirax® 200 mg					Acyclovir 200 mg				
	C_{max} (ng/mL)	t_{max} (h)	$AUC_{0\text{-}t}$ (ng h/mL)	$AUC_{0\text{-}\infty}$ (ng h/mL)	$T_{(1/2)el}$ (h)	C_{max} (ng/mL)	t_{max} (h)	$AUC_{0\text{-}t}$ (ng h/mL)	$AUC_{0\text{-}\infty}$ (ng h/mL)	$T_{(1/2)el}$ (h)
Average	443.32	1.60	1815.36	1937.20	3.10	415.34	1.88	1808.45	1932.24	2.95
S.D.	153.13	0.68	1.16	583.94	1.16	132.08	0.89	638.36	641.77	0.88
C.V. (%)	34.54	42.90	31.86	30.14	37.24	47.25	47.25	35.30	33.21	29.68

CONCLUSION

A simple LC method for the determination of acyclovir in human plasma was developed and validated. This method involves a single step liquid-liquid extraction procedure, using guanine, a commercially available substance, as internal standard. The results of the validation studies show that the optimized LC method possesses specificity, sensitivity, linearity, precision, and accuracy over the entire range of significant therapeutic plasma concentrations. Moreover, the proposed method was successfully applied to a pharmacokinetic study in healthy human volunteers, and results showed that the two acyclovir formulations are bioequivalent in their rate and extent of absorption.

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