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Determination of lamivudine in human plasma by HPLC and its use in bioequivalence studies

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Abstract

A simple, accurate, precise and sensitive high-performance liquid chromatographic (HPLC) method with ultraviolet detection was developed to quantificate lamivudine (3-TC) in human plasma samples from bioequivalence studies. 3-TC and stavudine (internal standard, I.S.) were extracted from 0.5 ml of human plasma by acetonitrile protein precipitation. The method was validated over a concentration range of 0.05–3.00 μg/ml and used in a bioequivalence trial between two lamivudine formulations, to assess its usefulness in this kind of study. FURP–lamivudine (Fundação para o Remédio Popular, Brazil, as test formulation) and Epivir® (GlaxoSmithKline, Brazil, as reference formulation) were evaluated following a single 150 mg oral dose to 24 healthy volunteers of both genders. The dose was administered after an overnight fast according to a two-way crossover design. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and AUC_{0-inf} values for the test and reference products, using logarithmic transformed data. The 90% confidence intervals for the ratio of C_{max} (0.86–1.06), AUC_{0-t} (0.96–1.04) and AUC_{0-inf} (0.97–1.05) values for the test and reference products are within the 0.80–1.25 interval proposed by FDA and EMEA. It was concluded that the two 3-TC formulations are bioequivalent in their rate and extent of absorption, and thus, may be used interchangeably.

Keywords: Lamivudine; 3-TC; Bioequivalence; HPLC; Plasma

1. Introduction

Acquired immunodeficiency syndrome (AIDS) was first recognized in 1981, and the human immunodeficiency virus (HIV), that causes AIDS, was identified in 1983 (Balint, 2001). Since the emergence of the HIV epidemic, approximately 90% of all HIV infections

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have occurred in developing countries. A similar or even greater percentage of all new infections also take place in these nations, where over 5 million new HIV infections occur each year, or about 14,000 each day. Worldwide, it is estimated that over 40 million persons are now living with HIV infections, and in the absence of effective treatment the overwhelming majority of them will die of AIDS (Crotty and Andino, 2004).

There are five groups of drugs available for the treatment of patients with HIV infection: nucleoside reverse transcriptase inhibitors (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine); nucleotide reverse transcriptase inhibitors (tenofovir disoproxil fumarate); non-nucleoside reverse transcriptase inhibitors (nevirapine, delavirdine and efavirenz); protease inhibitors (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir and atazanavir) and viral entry inhibitors (enfuvirtide) (De Clercq, 2004). Since mono-therapy with nucleoside reverse transcriptase inhibitors (NRTIs) for treatment against HIV-1 results in rapid development of resistant HIV strains, co-administration of other anti-retroviral drugs is necessary (Zheng et al., 2001).

Lamivudine (3-TC), 2′-deoxy-3′-thiacytidine, is less potent than zidovudine and zalcitabine, but an antiviral regimen that includes 3-TC is desirable, since 3-TC had been shown to be somewhat less toxic than other NRTIs (Zheng et al., 2001). Moreover, lamivudine is active against zidovudine-resistant HIV (Balint, 2001). The US Department of Health and Human Services' current guideline for the treatment of established HIV infection strongly recommends 3-TC in combination with another NRTI and either a protease inhibitor or efavirenz (Soudeyns et al., 1991; Zheng et al., 2001). The mechanism of action of lamivudine seems to be similar to that of zidovudine (Hart et al., 1992). 3-TC has approximately 80% oral bioavailability in human and its usual dosage is 150 mg twice daily or 300 mg once daily in combination with other antiretroviral agents (Piliero, 2004). In such doses, the compound is well tolerated (Balint, 2001).

Several high-performance liquid chromatographic (HPLC) methods with ultraviolet detection for the determination of 3-TC in biological fluids have been published. Zhou and Sommadossi (1997) used protein precipitation with trichloroacetic acid for sample cleanup for 3-TC analysis in human serum. Solid-phase extraction (SPE) methods have been developed by different authors (Harker et al., 1994; Hoetelmans et al., 1998; Zheng et al., 2001). Morris and Selinger (1994) incorporated a column switching scheme into the procedure for measuring 3-TC in urine. Other methods for 3-TC quantification include radioimmunoassay and mass spectrometry (Pereira and Tidwell, 2001).

This paper describes the development and validation of a sensitive, specific, rapid, simple and economic HPLC bioanalytical method for 3-TC quantification in human plasma, and its application in a bioequivalence study between two pharmaceutical products containing lamivudine available in the Brazilian market.

2. Materials and methods

2.1. Materials

Lamivudine (3-TC) was kindly provided by Fundação para o Remédio Popular (FURP) (São Paulo, Brazil) and stavudine (internal standard, I.S.) was supplied by Labogen (São Paulo, Brazil). Acetonitrile and methanol HPLC grade and sodium dihydrogen phosphate monohydrate and ammonium acetate analytical grade were provided by Merck (São Paulo, Brazil). Water (18.2 MΩ) used in the mobile phase was freshly prepared from Milli-Q Academic (Millipore, Bedford, USA).

Pharmaceutical products used in the bioequivalence study were FURP–lamivudine (tablets containing 150 mg of lamivudine, lot no. 9007, produced by Fundação para o Remédio Popular, Brazil), as the test medication, and Epivir® (tablets containing 150 mg of lamivudine, lot no. B063679, produced by GlaxoSmithKline, Brazil), as the reference medication.

2.2. Instrumentation

Analyses were performed on a Shimadzu Scientific Instruments (Kyoto, Japan) liquid chromatographic system composed of a LC-10ADVP pump, a SPD-10ADVP variable wavelength detector, a CTO-10ADVP column oven and a SIL-10ADVP auto-sampler fitted with a 50 μl loop. The analytical column was a Shim-pack® CLC-C8(M) column

(150 mm × 4.6 mm i.d., 5 μm particle size) protected with a Phenomenex® AJO-4287 C18 guard cartridge (5 mm × 4.6 mm i.d., 5 μm) (Torrance, CA, USA).

2.3. Chromatographic conditions

The mobile phase consisted of sodium dihydrogen phosphate monohydrate (10 mM), methanol and acetonitrile (94:3:3, v/v/v, pH 4.8). Before analyses, the mobile phase was filtered through a 0.22-μm filter (Sartorius, USA), and then degassed ultrasonically for 15 min. The analyses were conducted at 40 °C, flow-rate of 1.2 ml/min and detection wavelength at 270 nm.

2.4. Extraction procedure of lamivudine (3-TC) and stavudine (I.S.) from plasma

Calibration standards, validation control samples and volunteers plasma samples were prepared by aliquoting 500 μl of plasma into 8 ml glass tubes followed by addition of 50 μl of internal standard solution (10 μg/ml stavudine in methanol) and 25 μl of ammonium acetate 0.2 M, which increases peak resolution. All samples were mixed by vortex agitation for 30 s. After these procedures, a 2 ml volume of acetonitrile was added for protein precipitation. The tubes were vortex-mixed for 60 s, and then centrifuged for 10 min at 3500 rpm. The supernatant layers were filtered through a Millex GV 0.45-μm filter unit into 8 ml conical glass tubes and evaporated under nitrogen stream while immersed in a 40 °C water bath. Each sample was reconstituted with 500 μl of mobile phase and vortexed for 30 s. The samples were transferred to auto-sampler vials and 25 μl were injected into the HPLC system.

2.5. Method validation

Calibration standards and quality control samples were prepared by spiking blank human plasma with standard solutions of 3-TC.

The specificity of the method was verified using six different blank plasma samples obtained from healthy human volunteers who did not take lamivudine. The anticoagulant (heparin) interference was also verified during this stage. The linearity was tested by the calibration curve ranging from 0.05 to 3.00 μg/ml.

The analytical recovery was calculated by comparing chromatographic peak areas from unextracted standard samples and from extracted standard samples at three different concentrations (0.15, 1.50 and 2.50 μg/ml).

The lower limit of quantification (LLOQ) was the smallest analytical concentration which could be measured with accuracy and precision still better than 20%.

To evaluate the inter-assay precision and accuracy, validation control samples with drug concentrations of 0.15, 1.50 and 2.50 μg/ml were analyzed together with one independent calibration standard curve for 3 days, while intra-assay precision and accuracy were evaluated through analysis of validation control samples at three different concentrations in replicate of six in the same day. Inter- and intra-assay precision were expressed as relative standard deviation (R.S.D.). The accuracy was expressed as the percent ratio between the experimental concentration and the nominal concentration for each sample.

Stability of 3-TC in spiked plasma control samples was determined in triplicate at three concentrations (0.15, 1.50 and 2.50 μg/ml) after three freezing-thaw cycles. Long-term stability at −20 °C was determined after 50 days. Additionally, stability of spiked processed plasma samples during storage in the auto sampler for 24 and 48 h at room temperature was determined.

2.6. Bioequivalence study

This study was performed according to the revised Declaration of Helsinki for Biomedical Research involving human subjects and the rules of Good Clinical Practice. The protocol of this study was approved by the Ethical Committee of Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. A total of 24 healthy volunteers, 12 females and 12 males, participated in the study after signing a consent form. The subjects had mean age of 28 years, mean body weight of 64 kg, and mean height of 1.67 m. Subjects with history of drug allergies or idiosyncrasies, renal or hepatic impairment, history of any illness of cardiovascular system, or alcohol and drug abuse were excluded. Subjects were selected after passing a clinical screening procedure including a physical examination and lab-

oratory tests. All subjects avoided using other drugs for at least 1-week prior to the study and until after its completion. They also abstained from alcoholic beverages, and xanthine-containing foods and beverages 48 h prior to each dosing and until the collection of the last blood sample.

The study was an open, randomized, two-period crossover trial with a 1-week washout period.

Subjects were admitted into hospital at 9:00 p.m. the day before the study and fasted 10 h before each drug administration. A single dose (150 mg) consisting of one FURP–lamivudine or Epivir® tablet according to the randomization plan was given to each subject in a fasting state for each treatment period. Fasting continued for a further 4 h after drug administration. The drug was administered with 200 ml of water. Subjects were provided with standard meals 4 h (lunch), 7 h (snack) and 10 h (supper) after drug administration in each treatment.

Heparinized venous blood samples, 8–10 ml, were collected by means of an indwelling venous cannula of the cubital vein on profile days according to the time schedule, which included a blank before-drug sample just prior to dosing and then at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 8, 12, 24 and 36 h after drug administration. Any deviation from the stated sampling times was recorded. Plasma was immediately separated by centrifugation at 3000–4000 rpm for 10 min, then was transferred to properly labeled tubes and stored at −20 °C until the high-performance liquid chromatographic analysis.

2.7. Pharmacokinetics and statistical analyses

The following pharmacokinetic parameters were calculated using non-compartmental methods: area under the plasma concentration–time curve from zero to the last measurable 3-TC concentration sample time (AUC_{0-t}), area under the plasma concentration–time curve from zero extrapolated to infinite time (AUC_{0-inf}), maximum plasmatic drug concentration (C_{max}) and time to reach C_{max} (T_{max}). C_{max} and T_{max} were obtained directly from the concentration–time curve. AUC_{0-t} was calculated using the linear trapezoidal method. The terminal rate constant, K_{el}, was calculated by applying a log-linear regression analysis to at least the last three quantifiable concentrations of 3-TC. The terminal half-life ($T_{1/2}$) was calculated as $0.693/K_{el}$ (Ritschel, 1992).

For the purpose of bioequivalence analysis AUC_{0-t}, AUC_{0-inf} and C_{max} were considered as primary variables. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and AUC_{0-inf} values for the test and reference products, using logarithmic transformed data. Analysis of variance (ANOVA) was used to assess product, group and period effects. The products were considered bioequivalent if the 90% CI for AUC_{0-t} and C_{max} fell within 0.80–1.25.

3. Results

3.1. Validation of HPLC method

Retention time for lamivudine was 5.1 min and it was well resolved from stavudine (I.S.) (Fig. 1).

The lower limit of quantification was 0.05 μg/ml, with relative standard deviation lower than 10%.

Linearity was observed within the range of 0.05–3.00 μg/ml ($y = 0.0259 + 1.0774x$, $r^2 = 0.999$) (Fig. 2).

As expected, excellent recovery of 3-TC and I.S. was accomplished (Table 1).

The method showed to be precise and accurate. Intra-assay precision is between 1.68 and 3.83% and inter-assay precision is between 3.78 and 8.11%. Accuracy ranged from 85.80 to 94.09% (intra-assay) and from 95.10 to 97.10% (inter-assay).

Organic extracts were stable at room temperature for at least 48 h. Plasma samples were stable for at least 50 days at −20 °C and also after three freeze-thaw cycles. These results allow stockpiling of plasma samples obtained in the bioequivalence study for subsequent bath analysis.

3.2. Bioequivalence evaluation

Safety was evaluated by monitoring adverse events and vital signs and through physical examination, clinical laboratory tests and electrocardiograms. Each

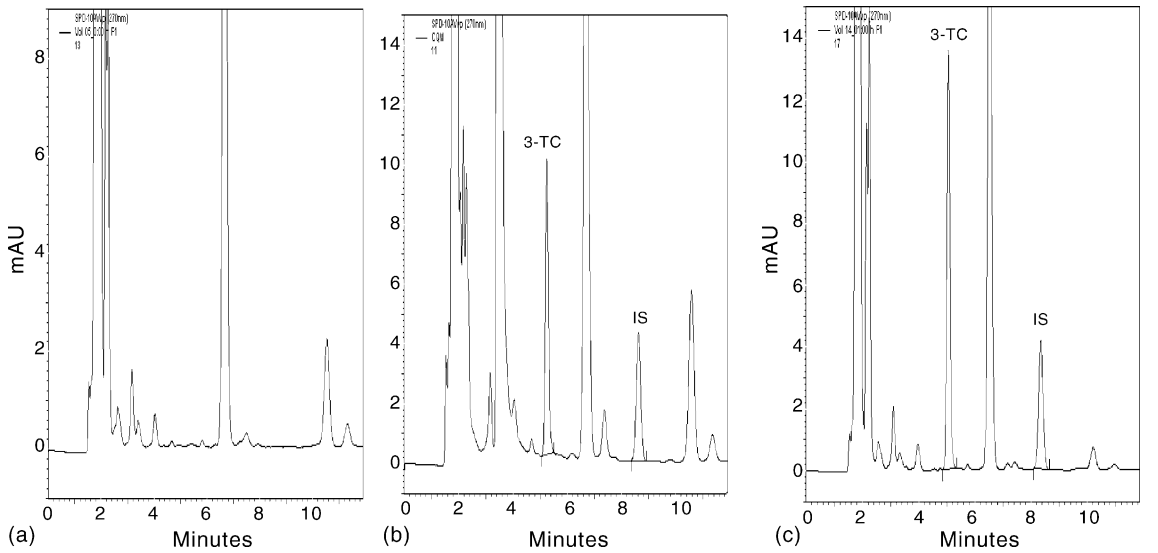

Fig. 1. Typical chromatogram of (a) blank human plasma, (b) blank human plasma spiked with 3-TC (1.50 μg/ml) and stavudine (0.50 μg/ml) and (c) plasma from healthy volunteer 60 min after receiving 150 mg of 3-TC, spiked with stavudine (0.50 μg/ml).

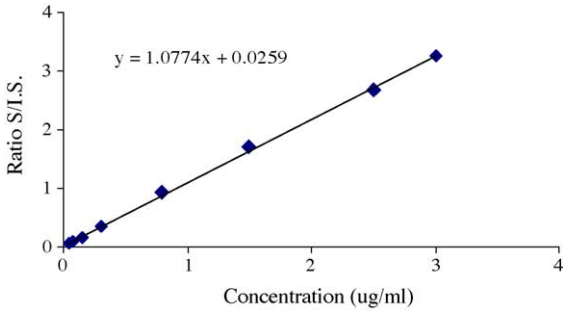

Fig. 2. Standard calibration curve for lamivudine quantification in human plasma by HPLC method with UV detection.

subject was questioned periodically throughout the study regarding adverse effects.

Average lamivudine plasma concentration–time profiles and average values of pharmacokinetic parameters after test and reference products administration to 24 healthy human volunteers are shown in Fig. 3 and Table 2, respectively.

The results of the analysis of variance for assessment of product, group and period effects and 90% confidence intervals for the ratio of C_{max}, AUC_{0-t} and AUC_{0-inf} values for test and reference products are shown in Table 3.

Table 1
Recovery of lamivudine (3-TC) and stavudine (I.S.) after the extraction procedure ($n = 6$)

3-TC concentration (μg/ml)	% Recovery (mean ± R.S.D.)	
	3-TC	I.S.
0.15	102.62 ± 1.60	106.78 ± 1.14
1.50	101.52 ± 1.17	104.08 ± 0.97
2.50	104.06 ± 0.46	106.77 ± 0.69

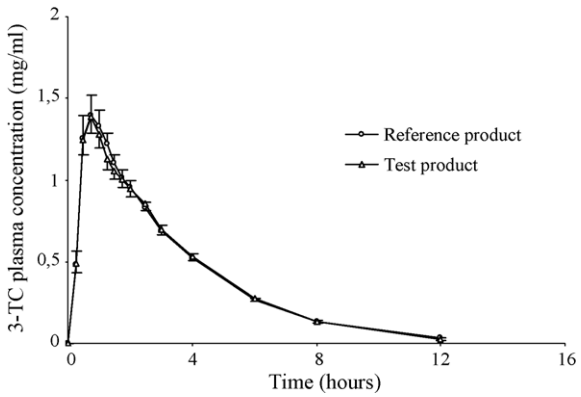

Fig. 3. Average 3-TC plasma concentration–time profiles after test and reference products administration to 24 healthy human volunteers. Bars indicate mean standard error (upper bars for reference product and lower bars for test product).

Table 2
Pharmacokinetic parameters of 3-TC after administration of test and reference products to 24 healthy volunteers (mean ± standard deviation)

Pharmacokinetic parameter	FURP–lamivudine (test)	Epivir® (reference)
AUC_{0-t} (μg h/ml)	4.85 ± 0.72	4.87 ± 0.88
AUC_{0-inf} (μg h/ml)	5.19 ± 0.72	5.17 ± 0.88
C_{max} (μg/ml)	1.54 ± 0.37	1.62 ± 0.48
T_{max} (h)	0.96 ± 0.51	0.96 ± 0.48
$T_{1/2}$ (h)	2.37 ± 0.73	2.34 ± 0.70

Table 3
Analyses of variance (ANOVA) for the assessment of the product, period and group effects and 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and AUC_{0-inf} values for the test and reference products, using logarithmic transformed data, after administration of reference (Epivir® 150 mg, GlaxoSmithKline) and test (FURP–lamivudine 150 mg, Fundação para o Remédio Popular) products to 24 healthy volunteers

Pharmacokinetic parameter	ANOVA (*p*-value) variation source			90% CI
	Product	Period	Group	
C_{max}	0.4874	0.5065	0.4943	0.87–1.06
AUC_{0-t}	0.9534	0.7867	0.5098	0.96–1.04
AUC_{0-inf}	0.7626	0.9428	0.5420	0.97–1.05

$\alpha = 0.05$.

Power of statistical test was 100% for AUC_{0-t} and AUC_{0-inf} and 95% for C_{max}.

4. Discussion

The analytical method developed for 3-TC quantification in plasma samples showed good specificity, sensitivity, linearity, precision and accuracy over the entire range of clinically significant and therapeutically achievable plasma concentrations, thereby enabling its use in bioequivalence trials.

The method showed some advantages over other published methods. The method proposed by Zhou and Sommadossi does not employ internal standard and requires 25 min for each chromatographic analysis. The method developed in this study uses stavudine, a commercially available substance, as internal standard and requires only 15 min for each chromatographic analysis.

Expensive solid-phase extraction procedures are reported by several authors (Zheng et al., 2001; Hoetelmans et al., 1998; Harker et al., 1994). Protein precipitation with acetonitrile is more economic and allows high recoveries of 3-TC and I.S. Liquid–liquid extraction is described by Alnouti et al. (2004), but the recovery is lower than reported herein. In a recent study, Estrela et al. (2004) reported the determination of 3-TC and zidovudine using LC–MS–MS detection but, in developing countries, UV detection is more available. The lower limit of quantification and linearity obtained using this method were adequate for 3-TC quantification in human plasma following administration of therapeutic doses of lamivudine.

Average plasma decay curves (Fig. 3) and pharmacokinetic parameters (Table 2) obtained for the test product (FURP–lamivudine) were similar to those obtained for the reference product (Epivir®).

The multivariate analysis accomplished through analysis of variance revealed the absence of period, group and product effects and the power of statistical test indicates that the sample size ($n = 24$) was adequate.

The 90% confidence intervals for AUC_{0-t} (0.96–1.04), AUC_{0-inf} (0.97–1.05) and C_{max} (0.87–1.06) are within the 0.80–1.25 interval proposed by most regulatory agencies.

It was concluded that the test medication, FURP–lamivudine from Fundação para o Remédio Popular, Brazil, and reference medication, Epivir® from GlaxoSmithKline, Brazil, are bioequivalent and, thus, may be used interchangeably, without any prejudice of therapeutic effect.

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