SIMULTANEOUS DETERMINATION OF STAVUDINE AND LAMIVUDINE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO A BIOAVAILABILITY STUDY

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Abstract. A high performance liquid chromatographic method with UV detection was developed and validated for simultaneous determination of stavudine and lamivudine in human plasma using solid-phase extraction for sample clean-up. Zidovudine was used as an internal standard. Separation was performed on a C18 column by gradient elution with a mobile phase of 10 mM acetate buffer pH 6.5 and acetonitrile. The UV detection was set at 265 nm. The method proved to be specific, accurate, precise and linear over the concentration ranges of 50-3,000 ng/ml for stavudine and 50-5,000 ng/ml for lamivudine with correlation coefficients always >0.996 for both drugs. The intra-day and inter-day precision and accuracy were less than 9.2% for both analytes. The absolute recoveries of both compounds ranged from 93.3 to 97.5%. The method was successfully applied to a bioavailability study of a combined tablet formulation containing 30 mg of stavudine and 150 mg of lamivudine compared with each reference formulation concurrently administered in 26 healthy Thai male volunteers.

Key words: stavudine, lamivudine, HPLC-UV method, bioavailability

INTRODUCTION

Five classes of anti-retroviral drugs (ARV) have been developed for the treatment of acquired immunodeficiency syndrome (AIDS): nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry or fusion inhibitors and integrase inhibitors. A combination of these ARV drugs can reduce the risk of human immunodeficiency virus (HIV) progressing to AIDS, morbidity, and mortality (Hogg *et al*, 1998; Palella *et al*, 1998). A combination of two NRTIs with an NNRTI is the recommended first line regimen in resource-limited countries (Gurtman *et al*, 1998). The commonly used NRTIs are zidovudine, stavudine and lamivudine, while nevirapine and efavirenz are frequently used NNRTIs.

Numerous analytical methods have been developed for simultaneous determination of ARV drugs from different therapeutic classes in human plasma and biological fluids using high performance liquid chromatography (HPLC) with ultra-

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violet (UV) detection (Aymard *et al*, 2000; Rezk *et al*, 2003; Verweij-van Wissen *et al*, 2005; Notari *et al*, 2006; Tarinas *et al*, 2007) or with mass spectrometric (MS) detection (Pereira *et al*, 2000; Estrela *et al*, 2004; Jung *et al*, 2007). Although many clinical research laboratories have the ability to perform HPLC-MS analyses, UV detection is still widely used for relatively simple bioanalytical assays.

The purpose of this study was to develop and validate an HPLC-UV method for simultaneous determination of stavudine and lamivudine in human plasma and to apply it to a bioavailability study of a combined tablet formulation consisting of 30 mg of stavudine and 150 mg of lamivudine compared with each reference formulation concurrently administered in 26 healthy Thai male volunteers.

MATERIALS AND METHODS

Chemicals

Stavudine, lamivudine, and zidovudine were provided by the Government Pharmaceutical Organization (Bangkok, Thailand). Ammonium acetate and 30% ammonia solution (both from Carlo Erba, Milano, Italy) were analytical grade. HPLC-grade methanol and acetonitrile were obtained from Mallinckrodt (Paris, KY). Drug-free human plasma was purchased from the Thai Red Cross Society (Bangkok, Thailand).

Instrumentation and chromatographic conditions

The HPLC system (Dionex, Sunnyvale, CA) consisted of a pump with integrated degasser (P680A LPG), an automated sample injector (ASI-100), a column oven (TCC-100) and a UV detector (UVD 340U). The chromatographic analysis was performed at 30ºC on an Apollo C18 column (4.6 x 150 mm, Alltech Associates, Deerfield, IL) equipped with a guard column (4.0 x 3.0 mm, Phenomenex, Torrance, CA). Separation was achieved by a multi-step gradient elution employing a mobile phase of 10 mM ammonium acetate buffer pH 6.5 (phase A) and acetonitrile (phase B) delivered at a flow rate of 1.0 ml/minute. A multi-step gradient was programmed as 96% of phase A over the first 11 minutes, changing to 87% of phase A from 11 to 12 minutes and maintain at 87% of phase A from 12 to 19 minutes, followed by 96% of phase A from 19 to 20 minutes. System stabilization was established using 96% of phase A from 20 to 22 minutes. Detection was carried out at 265 nm.

Preparation of calibration curves and quality control samples

Stock solutions of stavudine, lamivudine and an internal standard (IS), zidovudine, were separately prepared in methanol (1,000 μg/ml). Working solutions for calibration standards and quality control samples (QCs) were prepared by serially diluting stock solutions with methanol-water (50:50, v/v). Calibration standards were prepared by spiking 950 μl of blank plasma with 50 μl of corresponding working solutions containing both stavudine and lamivudine to produce final concentrations of 50, 100, 250, 500, 1,000, 2,000 and 3,000 ng/ml for stavudine and 50, 100, 250, 500, 1,000, 2,500 and 5,000 ng/ml for lamivudine. QC samples were prepared at lower limit of quantification (LLOQ), low (QCL), medium (QCM), and high (QCH) concentrations of 50, 150, 1,200 and 2,500 ng/ml for stavudine and 50, 150, 2,000 and 4,500 ng/ml for lamivudine, respectively.

Sample preparation

Stavudine and lamivudine were extracted from plasma using solid-phase extraction (SPE). Briefly, a 50 μl aliquot of IS

solution (30 μg/ml) was spiked into 1 ml of plasma sample and vortex mixed for 15 seconds. A 1 ml aliquot of the resulting solution was loaded onto an SPE cartridge (Waters Oasis® HLB, 1 cc 30 mg, Waters, Milford, MA) which was preconditioned with 1 ml of methanol and 1 ml of 100 mM ammonium acetate buffer pH 7.0. After loading, each SPE cartridge was washed with 1 ml of 100 mM ammonium acetate buffer pH 7.0 and eluted with 1 ml of methanol. The eluate was evaporated to dryness under vacuum at 40ºC. The residue was reconstituted with 500 μl of a mixture of 10 mM ammonium acetate buffer pH 6.5 and acetonitrile (95:5) and a 50 μl aliquot was injected into the HPLC system.

Method validation

The proposed method was validated according to the "Guidance for Industry-Bioanalytical Method Validation" (US Department of Health and Human Services, 2001) for specificity, linearity, precision, accuracy, recovery, dilution integrity and stability. The specificity of the method was evaluated by screening at least ten different batches of blank plasma. The calibration curves for each analyte were generated by plotting the analyte to IS peak area ratios against analyte concentrations. Linearity was assessed by least-squares regression analysis with a weight factor of $1/concentration^2$. Back-calculated concentrations of calibration standards should be within 15% of their nominal values except at LLOQ which should be within 20%. The LLOQ for each analyte was defined as the lowest standard on the calibration curve measured with 20% accuracy and precision. In addition, the analyte response at LLOQ should be at least 5 times the blank response.

The intra-day accuracy and precision were determined using 5 replicates of LLOQ, QCL, QCM and QCH, while inter-day accuracy and precision were assessed on three separate days. The overall accuracy of the method was expressed as percentage relative deviation (%RD) from the nominal value whereas the precision of the method was expressed as percentage of the coefficient of variation (%CV). Recovery of each analyte was determined by comparing peak areas of extracted samples with respective unextracted standard solutions at three QC levels. Recovery of IS was also compared in a similar manner. Dilution integrity was evaluated by preparing QC samples at a concentration about 1.5-times the highest concentration of the calibration curve (ULOQ). These samples were further diluted by 2- and 10-fold with blank plasma and assayed along with calibration standards and QC samples in one of the validation runs. Accuracy and precision of dilution integrity samples should be within 15%.

Stability of stavudine and lamivudine in plasma were evaluated for short-term and long-term storage conditions, after three freeze-thaw cycles, and for post preparative stability in an auto-sampler. Short-term stability was examined by keeping replicates of QCL and QCH at room temperature (25ºC) for 6 hours while long-term stability was tested after storage at -20ºC for 3 months. Freeze-thaw stability was obtained over three freeze-thaw cycles, by thawing to room temperature and refreezing for 12-24 hours. Post-preparative stability was tested by analysis of processed and reconstituted QC samples, which were stored in an autosampler for 36 hours at room temperature. For each storage condition, three replicates of QCL and QCH were analyzed and quantified against a freshly prepared standard curve. Stability of stock solutions of both analytes and IS were also determined for short-term

Table 1
Intra-day and inter-day precision and accuracy of stavudine and lamivudine in human plasma.

Analyte	Concentrations (ng/ml)	Precision (%CV)		Accuracy (%RD)	
		Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]
Stavudine	LLOQ (50)	5.8-8.1	7.8	-1.4-9.2	4.6
	QCL (150)	1.3-3.1	2.4	-3.0-(-0.1)	-1.4
	QCM (1,200)	0.4-2.2	1.9	-1.4-1.4	0.3
	QCH (2,500)	0.9-2.5	2.6	3.0-7.4	5.8
Lamivudine	LLOQ (50)	3.2-7.7	7.3	-7.7-0.1	-3.2
	QCL (150)	1.8-4.6	3.4	-4.0-(-1.1)	-2.5
	QCM (2,000)	0.5-1.8	2.4	3.8-8.7	6.7
	QCH (4,500)	1.1-2.6	2.7	-7.2-(-2.7)	-5.2

[a]Based on $n = 5$
[b]Based on $n = 15$

storage at 25ºC, 12 hours and long-term storage at -20ºC for 3 months.

Method application

The proposed method was used to determine stavudine and lamivudine concentrations in plasma samples of 26 healthy Thai male volunteers who participated in this single dose crossover bioavailability study of a combined tablet formulation containing 30 mg of stavudine and 150 mg of lamivudine as the test formulation (manufactured by the Government Pharmaceutical Organization, Bangkok, Thailand) compared with each reference formulation (Zerit®, stavudine 30 mg capsule, manufactured by Bristol-Myers Squibb, USA and Epivir®, lamivudine 150 mg tablet, manufactured by GlaxoSmithKline, USA). The study protocol was approved by the Thai Food and Drug Administration and the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Written informed consent was obtained from each volunteer prior to study participation. Blood samples (6 ml) were collected in EDTA tubes at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 24 hours after dosing and centrifuged at 1,000*g* at 4ºC, for 10 minutes. Plasma samples were separated and kept frozen at -20ºC until analysis.

RESULTS

Specificity

Under the described chromatographic conditions, the retention times of lamivudine, stavudine and IS were approximately 7.3, 11.7 and 19.4 minutes, respectively. No peak interferences were found in the 10 different sources of human plasma or in any of the study samples. Fig 1 shows the chromatograms obtained from blank plasma, spiked plasma at LLOQ and a plasma sample obtained from a volunteer 1.75 hours after oral administration of a single dose of 30 mg stavudine and 150 mg lamivudine.

Linearity

The calibration curves for each analyte were constructed using peak area ratios of analyte to IS and were fitted by weighted ($1/\text{concentration}^2$) least-squares linear regression analysis. Excellent linearity was

Table 2
Stability of stavudine and lamivudine in human plasma.

Storage conditions	Duration	Stavudine (%)		Lamivudine (%)	
		QCL	QCH	QCL	QCH
Short-term at 25ºC	6 hours	102.6	101.1	100.4	104.0
Long-term at -20ºC	3 months	96.4	93.3	100.3	101.5
Freeze-thaw cycles	3 cycles	100.2	99.6	97.6	102.4
Post preparative	36 hours	94.0	94.6	99.0	99.9

Values are means of triplicate determinations.

Table 3
Pharmacokinetic parameters of stavudine and lamivudine in healthy Thai male volunteers after oral administration of a single dose of test and reference products containing 30 mg of stavudine and 150 mg of lamivudine (mean ± SD, *n*=26).

Parameter	Stavudine		Lamivudine	
	Test	Reference	Test	Reference
Cmax (ng/ml)	721.2 ± 156.5	685.9 ± 192.4	2,077.7 ± 616.1	1,970.4 ± 569.7
Tmax (h)	0.6 ± 0.3	0.7 ± 0.3	1.0 ± 0.5	1.0 ± 0.5
AUC_{0-t} (ng.h/ml)	1,428.3 ± 195.5	1,413.0 ± 190.0	6,669.5 ± 1,178.9	6,684.1 ± 1,368.2
$AUC_{0-\infty}$ (ng.h/ml)	1,615.6 ± 230.0	1,600.3 ± 239.7	7,010.3 ± 1,254.1	7,050.2 ± 1,387.9
Half-life (h)	1.9 ± 0.8	1.8 ± 0.4	2.8 ± 0.9	3.0 ± 1.4

observed over concentration ranges of 50-3,000 ng/ml for stavudine and 50-5,000 ng/ml for lamivudine with correlation coefficients always greater than 0.996 for both compounds from 8 calibration curves. The back-calculated concentrations of calibration standards were within the accepted range of 15% for nominal values, including the LLOQ.

Precision, accuracy and recovery

Results for the intra- and inter-day precision and accuracy are summarized in Table 1. For both analytes, the %CV and %RD were less than 9.2%, which are within the accepted limit of 15%. Mean recoveries of stavudine from plasma for QCL, QCM and QCH were 93.3, 96.1 and 97.5%, respectively, while those for lamivudine were 93.7, 93.0 and 92.1%, respectively. The mean recovery of IS from plasma was 95.6%.

Dilution integrity

The purpose of the dilution integrity experiment was to demonstrate the validity of the dilution procedure when performing routine analysis of study samples originally having concentrations above the ULOQ. Analysis of dilution integrity samples after 2- and 10-fold dilutions of QC samples containing 4,500 ng/ml of stavudine and 7,500 ng/ml of lamivudine showed a %RD of less than 10.0%, whereas the %CV was less than 2.7%. These results demonstrate the accuracy and precision were well within the accepted limit.

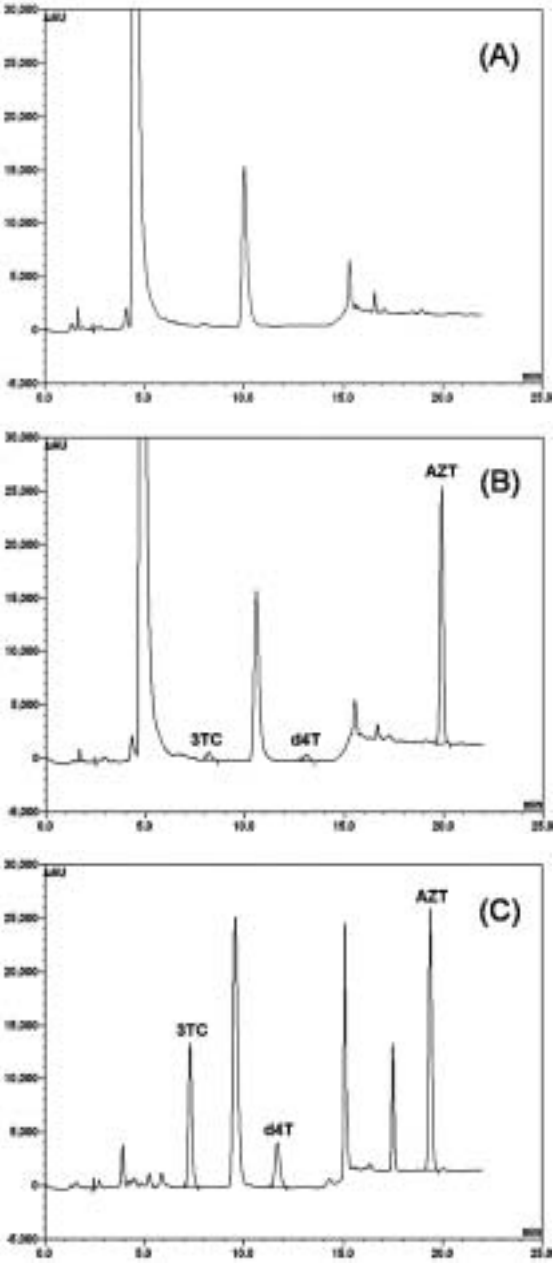

Fig 1–Chromatograms obtained from (A) human blank plasma, (B) human blank plasma spiked with stavudine (d4T) and lamivudine (3TC) at LLOQ (50 ng/ml) and zidovudine (AZT) as internal standard, and (C) plasma sample of a volunteer 1.75 hours after oral administration of a single dose of 30 mg stavudine and 150 mg lamivudine.

Stability

The stabilities of stavudine and lamivudine in human plasma were determined using triplicates of QCL and QCH under various storage conditions and compared to freshly prepared QC samples. Table 2 summarizes data for short-term stability at room temperature, long-term storage at -20ºC, freeze-thaw stability, and post-preparative stability of processed samples in an auto-sampler. The percentage of each analyte found in the stability samples ranged from 93.3 to 104.0%, these values were considered within the accepted limit. Stock solutions of stavudine, lamivudine and IS were found to be stable at room temperature for 12 hours and at -20ºC for 3 months.

Method application

To demonstrate the applicability of the method, it was used to determine stavudine and lamivudine concentrations in plasma samples from a comparative bioavailability study of a combined tablet formulation consisting of 30 mg of stavudine and 150 mg of lamivudine compared with each reference formulation concurrently administered in 26 healthy Thai male volunteers. Fig 2 illustrates the mean plasma concentration-time profiles of stavudine and lamivudine following oral administration of a single dose of test and reference products. The corresponding pharmacokinetic parameters of stavudine and lamivudine are summarized in Table 3. A total of 988 study samples were successfully analyzed for stavudine and lamivudine, demonstrating suitability of the present method for a bioavailability study.

DISCUSSION

Current clinical practice guidelines recommend a regimen of various ARV combinations for the treatment of HIV patients.

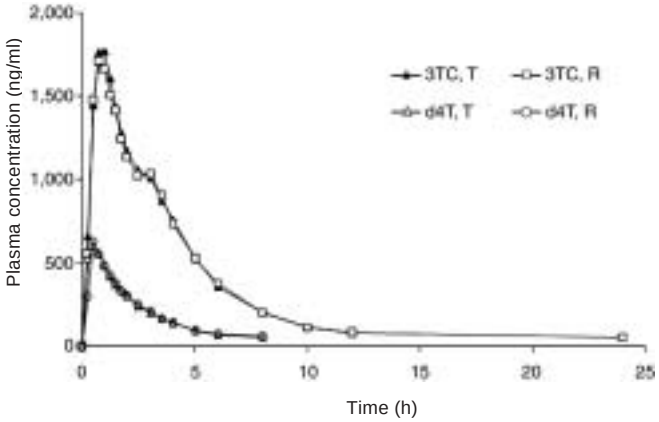

Fig 2–Mean plasma concentration-time profiles of stavudine (d4T) and lamivudine (3TC) after oral administration of a single dose of 30 mg stavudine and 150 mg lamivudine of test (T) and reference (R) products in 26 volunteers.

Several fixed-dose formulations have been developed and are available to patients. To study the pharmacokinetics of ARV drugs or to monitor therapeutic drug levels, simultaneous quantitation of these drugs is desirable. Recently, several LC-MS-MS methods with improved sensitivity and specificity have been developed to measure ARV drug concentrations in biological matrices. However, mass spectrometers are expensive and not readily available in most laboratories. HPLC-UV instruments are still widely used due to lower cost and greater robustness. Since HPLC-UV methods are susceptible to interference from endogenous and exogenous substances, it is necessary to develop a suitable and selective sample clean-up procedure. Although protein precipitation is considered a simple and rapid sample preparation technique, it usually gives dirty extracts and results in lower sensitivity. A lower limit of quantitation of 300 ng/ml for lamivudine using 1 ml plasma has been reported (Santos-Magalhaes *et al*, 2001). This sensitivity level is inadequate for our pharmacokinetic and bioavailability studies. Liquid-liquid extraction is labor-intensive and recovery is low (Mudigonda *et al*, 2008). In the present study, we used solid-phase extraction, which allows the desired limit of quantitation and clean extracts. Recovery of the two analytes and IS was greater than 90%. Most of the published LC-MS-MS methods also employed this technique for sample preparation (Narang *et al*, 2005; Marier *et al*, 2007; Mudigonda *et al*, 2008). The described chromatographic conditions provided a good separation among stavudine, lamivudine, and IS. The method proved to be specific for routine sample analyses, since no interfering peaks from endogenous substances from plasma in the 26 volunteers were observed. Calibration curves were linear over the concentration ranges found clinically. Results of dilution integrity demonstrate the ability of the method to be used for sample dilution. The stability results of both analytes in human plasma and stock solutions indicate acceptable stability under various storage conditions. The validated method was successfully applied to a comparative bioavailability study of a combined tablet formulation containing stavudine and lamivudine compared with each reference formulation concurrently administered in healthy Thai male volunteers. The pharmacokinetic parameters of stavudine and lamivudine in Thai subjects obtained from this study are comparable to previous reports (Narang *et al*, 2005; Vezina *et al*, 2006; Hosseinipour *et al*, 2007; Marier *et al*, 2007).

In conclusion, we have established an HPLC-UV method for simultaneous determination of two ARV drugs, stavudine and

lamivudine, in human plasma. The method is simple, specific and fully validated, per USFDA guidance for bioanalytical method validation.

ACKNOWLEDGEMENTS

This work was supported in part by the Government Pharmaceutical Organization, Bangkok, Thailand.

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