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Determination of Lamivudine and Zidovudine from Human Plasma by High Performance Liquid Chromatography-UV Detection for Bioequivalence Studies

ABHIJIT R. MORE*, A.J. VAIDYA†, V.V. VAIDYA‡ and R.G. DESHMUKH
Department of Chemistry, K.T.S.P. Mandal's K.M.C. College, Khopoli-410 203, India
Tel: (91)(22)25401935; E-mail: abhi_more@yahoo.com; vaidya_vikas@yahoo.com

A HPLC- method with UV detection at wavelength of 270 nm is described for the determination of lamivudine and zidovudine in human plasma using fluconazole as internal standard. Lamivudine and zidovudine were isolated by solid phase extraction using methanol as the solvent. Selection of mobile phase components was a critical factor in achieving good chromatographic peak shape and resolution. Good separation of the target compounds and short run time were obtained using an elution system of buffer:methanol:acetonitrile (85:10:5 % v/v). UV wavelength of 270 nm also provided better sensitivity. This assay achieved higher sensitivity and better specificity for the analysis of lamivudine and zidovudine in human plasma. The limit of quantification of ng/mL for lamivudine and zidovudine was thus attainable by HPLC-UV. The internal standard proved to be good internal standard for this assay. No significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of lamivudine and zidovudine.

Key Words: Lamivudine, Zidovudine, Fluconazole, Solid phase, Extraction, HPLC-UV, Human plasma.

INTRODUCTION

Lamivudine and zidovudine fall in a class of drugs called reverse transcriptase inhibitors. Reverse transcriptase is the enzyme that the HIV virus uses to form a new DNA. Lamivudine and zidovudine block the activity of reverse transcriptase and block the production of DNA and new viruses[1]. The effectiveness of either lamivudine or zidovudine when used alone may decrease as the HIV virus develops resistance to the effects of the individual drugs. By combining lamivudine and zidovudine, it is more difficult for the HIV virus to develop resistance to therapy since it must develop resistance to both drugs[2].

†Analytical Solutions, C-115, Ansa Industrial Estate, Saki-Vihar Road, Saki Naka, Mumbai-400 072, India.
‡S.P. Mandal's Ramnarain Ruia College, Matunga, Mumbai-400 019, India.

The chemical name of lamivudine is (2R, *cis*)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one. Lamivudine is the (-) enantiomer of a dideoxy analogue of cytidine. Lamivudine has also been referred to as (-)2',3'-dideoxy-3'-thiacytidine. Lamivudine is a white crystalline powder with m.f. $C_8H_{11}N_3O_3S$, m.w. 229.3 daltons.

The chemical name of zidovudine is 3'-azido-3'-deoxythymidine. It has a molecular formula of $C_{10}H_{13}N_5O_4$ and a molecular weight of 267.24 daltons. Zidovudine is a white to beige, crystalline solid with a solubility of 20.1 mg/mL in water at 25°C.

There is no HPLC-UV method reported in literature and therefore the goal of this experiment was to develop a simple, accurate, precise and reproducible method for the determination of lamivudine and zidovudine together in human plasma. The method employs reversed-phase HPLC using UV-detection and solid-phase extraction for sample preparation.

EXPERIMENTAL

Pooled human plasma, commercially procured and chromatographically analyzed to ensure non-interference.

Chromatographic conditions:

Mobile phase	Buffer:methanol:acetonitrile (85:10:5 % v/v)
Buffer	0.1 M Ammonium acetate in 0.1 % acetic acid
Column	Altima phenyl, 250 × 4.6 mm; 5 µ
Detector	UV detector at wavelength 270 nm
Internal standard	Fluconazole
Flow rate	1.0 mL/min
Injection volume	50 µL
Mobile phase	Buffer:methanol:acetonitrile (85:10:5 % v/v)
Buffer	0.1 M Ammonium acetate in 0.1 % acetic acid

Preparation of aqueous and plasma standards: Stock solutions of lamivudine, zidovudine and internal standard of 1 mg/mL were prepared in methanol, respectively. Standard solutions containing a mixture of lamivudine and zidovudine of concentration 0.1, 1 and 10 µg/mL were also prepared using methanol.

Calibration standards of mixture of lamivudine and zidovudine (50, 100, 500, 750, 1000, 1500, 2500, 5000 ng/mL and a LOQ sample at 50.0 ng/mL were prepared by spiking appropriate amount of the standard solutions in control plasma obtained from healthy human non-smoking volunteers. Quality samples were prepared in the blank control plasma at the concentrations of 50, 100, 1000 and 2500 ng/mL.

Sample preparation: Following extraction procedure was used for preparation of biological matrix samples *i.e.* all calibration levels, quality control samples and volunteer's plasma samples before injecting into HPLC system.

C_{18} SPE cartridge (Accubond) was conditioned with 2 mL methanol and 2 mL of water. To it 1 mL of plasma prespiked with 100 µL of internal standard (500 ppm) was added. The cartridge was washed twice with 2 mL of water. The sample was eluted with 2 × 2 mL of methanol. The eluent was collected in centrifuging tube and was evaporated to dryness in a water bath kept at 50-60°C using constant stream of nitrogen. The residue obtained was reconstituted with 250 µL of mobile phase and 50 µL of aliquots was injected into chromatographic system.

Assay validation

Specificity: 10 Bags of fresh frozen plasma obtained from different sources were analyzed to ensure non interference.

Linearity and sensitivity: A calibration curve in the range of 50-5000 ng/mL was constructed by plotting the area ratios of lamivudine and zidovudine to internal standard against lamivudine and zidovudine concentrations in plasma. Limit of quantification (LOQ) was established based on a S/N ratio of 5:1.

Precision and accuracy: The precision of the assay was determined by replicate analyses of four different concentrations LOQ (50 ng/mL), low quality control (LQC) (100 ng/mL), medium quality control (MQC) (1000 ng/mL) and high quality control (HQC) (2500 ng/mL). Intra-day precision was determined by repeated analysis of each of quality control sample on one day (n = 5) and the inter-day precision and accuracy was determined by repeated analysis on four consecutive days (n = 1 series per day). The concentration of each sample was determined using calibration standards prepared on the same day.

Stability: Analytes at low and high concentrations were tested for freeze-thaw (three cycles), bench top stability (up to 24 h), auto sampler stability (up to 24 h), long-term stability (12 weeks) and stock solution stability (up to 24 h).

Extraction recovery: The absolute recovery of lamivudine and zidovudine through extraction procedures were determined at low and high concentrations by external standard method .A known amount of lamivudine, zidovudine and internal standard was added to human plasma prior to extraction .The concentration of lamivudine and zidovudine was calculated using the calibration curves prepared on the same day and was compared to nominal concentration to estimate extraction recovery.

Pharmacokinetics and study: Each of 24 healthy male volunteers received 150 mg tablet of lamivudine and 300 mg tablet of zidovudine after overnight fasting. Blood samples were drawn at appropriate intervals centrifuged to obtain plasma samples.

Representative chromatograms: The retention times were 6.25 min for lamivudine, 7.48 min for zidovudine and 16.43 min for internal standard.

TABLE-1
RESULTS OF METHOD VALIDATION

Test	Acceptance criteria	Results for lamivudine	Results for zidovudine	Conclusion
Specificity	Non interference at the retention time of lamivudine, zidovudine and internal standard	No interfering peak at the retention time of lamivudine	No interfering peak at the retention time of zidovudine & internal standard was found	Method was found to be specific
Sensitivity	S/N more than 5:1	12.10:1	10.24:1	Method was found to be sensitive
Linearity	Regression to be more than 0.95	0.9997	0.9993	Method was found to be linear
Precision & accuracy	Intra-day	Intra-day	Intra-day	
	1. % Accuracy for			
	LOQ: 80-120 %	89.45-100.14 %	88.64-99.45 %	Method was found to be precise and accurate
	LQC: 85-115 %	93.78-100.45 %	97.85-112.63 %	
	MQC: 85-115 %	97.64-110.25 %	90.63-114.65 %	
	HQC: 85-115 %	102.34-110.65 %	97.64-104.88 %	
	2. % RSD for			
	LOQ: nmt 20 %	2.70-14.27 %	3.85-10.77 %	
	LQC: nmt 15 %	0.73-9.40 %	2.55-1.07 %	
	MQC: nmt 15 %	1.50-5.77 %	1.11-3.94 %	
	HQC: nmt 15 %	4.67-6.68 %	2.65-10.40 %	
	Inter-day			
	1. % Accuracy for	95.45 %	94.46 %	
	LOQ: 80-120 %	96.79 %	104.60 %	
	LQC: 85-115 %	103.82 %	104.10 %	
	MQC: 85-115 %	106.52 %	100.43 %	
	HQC: 85-115 %			
	2. % RSD for	9.58 %	10.82 %	
	LOQ: nmt 20 %	7.24 %	7.58 %	
	LQC: nmt 15 %	4.53 %	4.34 %	
	MQC: nmt 15 %	5.57 %.	6.58 %	
	HQC: nmt 15 %			
Recovery	Precise, consistent and Reproducible	58.27-64.74	80.39-81.60	Recovery was found to be precise consistent and reproducible

TABLE-2
PHARMACOKINETIC PARAMETERS FOR REFERENCE AND TEST FORMULATION OF LAMIVUDINE

	Tmax (h)	Cmax (ng/mL)	AUClast (h × ng/mL)	λ_z (1/h)	HL_λ_z (h)	AUCINF_obs (h × ng/mL)
Reference formulation						
N	24	24	24	24	24	24
Mean	1.05	1713.97	6525.43	0.22	3.67	6700.19
Geometric mean	0.99	1605.01	6138.73	0.21	3.37	6310.12
95 % CI Lower mean	0.89	1431.06	5485.85	0.18	3.01	5637.24
95 % CI Upper mean	1.21	1996.87	7565.01	0.26	4.34	7763.14
Test formulation						
N	24	24	24	24	24	24
Mean	1.06	1630.58	6318.29	0.22	3.80	6506.78
Geometric mean	1.00	1513.49	5840.20	0.20	3.43	6035.22
95 % CI Lower mean	0.90	1346.95	5330.18	0.18	3.07	5507.28
95 % CI Upper mean	1.23	1914.20	7306.41	0.27	4.53	7506.27

TABLE-3
PHARMACOKINETIC PARAMETERS FOR REFERENCE AND TEST FORMULATION OF ZIDOVUDINE

Zido	Tmax (h)	Cmax (ng/mL)	AUClast (h × ng/mL)	λ_z (1/h)	HL_λ_z (h)	AUCINF_obs (h × ng/mL)
Reference formulation						
N	24	24	24	24	24	24
Mean	0.53	2956.41	3274.55	0.60	1.25	3340.62
Geometric mean	0.49	2755.44	3099.47	0.58	1.20	3164.73
95 % CI Lower mean	0.45	2498.58	2799.76	0.53	1.10	2858.69
95 % CI Upper mean	0.62	3414.23	3749.35	0.67	1.39	3822.55
Test formulation						
N	24	24	24	24	24	24
Mean	0.55	3112.17	3441.94	0.57	1.29	3505.47
Geometric mean	0.51	2955.81	3288.82	0.55	1.26	3351.72
95 % CI Lower mean	0.46	2688.13	3000.17	0.51	1.16	3057.53
95 % CI Upper mean	0.64	3536.22	3883.72	0.62	1.43	3953.42

RESULTS AND DISCUSSION

The method described above was successfully applied to the pharmacokinetic study in which plasma concentrations of lamivudine and zidovudine in 24 healthy volunteers were determined up to 24 h after the

administration of 150 and 300 mg tablet of lamivudine and zidovudine tablet .The mean plasma concentration is shown in Fig. 1.

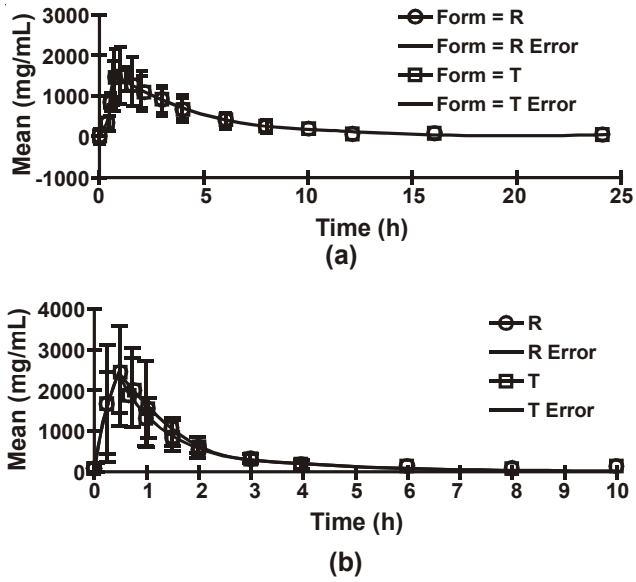

Fig. 1. Bioequivalence of (a) Lamivudine and (b) Zidovudine

Conclusion

This assay achieved higher sensitivity and better specificity for the analysis of lamivudine and zidovudine in human plasma. The limit of quantification of 50.0 ng/mL for lamivudine and zidovudine was thus attainable by HPLC-fluorescence. The internal standard proved to be good internal standard and no significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of lamivudine and zidovudine.

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