Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Der Pharmacia Sinica, 2013, 4(6):32-42

Der Pharmacia Sinica
ISSN: 0976-8688
CODEN (USA): PSHIBD

A simultaneous method for quantitative determination of lamivudine and zidovudine in human plasma by using tandem mass spectrometry and its application to pharmacokinetic studies

Nagamalleswara Rao K.[a], Venkata Kumar Ch[b*], Madhuri K.[b], Anil Kumar Ch[c] and Satyanarayana P. V. V.[a]

[a]*Department of Chemistry, College of Sciences, Acharya Nagarjuna University, Nagarjuna Nagar, AP., India*
[b]*Pharmaceutical Analysis Division, Teegala Ramreddy College of Pharmacy, Hyderabad , AP., India*
[c]*Department of Microbiology, Maha Raja P. G. College, Poolbaugh, Vizianagaram, AP., India*

ABSTRACT

A method employing high performance liquid chromatography with tandem mass spectrometry has been developed and validated for the simultaneous determination of clinically significant levels of lamivudine and zidovudine in human plasma. Emtricitabine & didanosine were used as internal standards for lamivudine and zidovudine respectively. The method involves sample preparation with solid-phase extraction technique with typical mass detection. The calibration range was 1.00 - 3507.87 ng/mL for lamivudine and 1.00 – 3502.29 ng/mL for zidovudine. An aliquot of 100µL of plasma was used for solid phase extraction technique. An isocratic mobile phase consisting of 0.1% formic acid in water and methanol (20:80; v/v) was used in the method. Chromatographic separation was achieved on Discovery C_{18} column over a run time of 2.5 minutes. The molecular ion Q1 & product ion Q3 transitions were found to be 230.0 →112.1, 268.1 →127.1, 248.2 →130.1 and 237.0 →137.0 for lamivudine, zidovudine, emtricitabine and didanosine respectively. The pharmacokinetic parameters for lamivudine were T_{max} – 1.6 Hours, C_{max} – 2031.8 ng/mL, T1/2 – 5.8 Hours, AUC (0-T) – 12371.4 ng.hrs/mL and AUC (0-∞) – 12552.3 ng.hrs/mL & for zidovudine were T_{max} – 0.8 Hours, C_{max} – 1786.3 ng/mL, T1/2 – 2.2 Hours, AUC (0-T) – 3244.3 ng.hrs/mL and AUC (0-∞) – 3250.9 ng.hrs/mL. The proposed assay method was found to be acceptable to a pharmacokinetic study in human volunteers.

Keywords: Lamivudine, Zidovudine, Plasma, Tandem Mass and Pharmacokinetics.

INTRODUCTION

Lamivudine [1, 2] is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1) and hepatitis B (HBV). Lamivudine (LAM) is a synthetic nucleoside analogues are phosphorylated intracellularly to its active 5'-triphosphate metabolite and lamivudine tri phosphate (L-TP). The lack of a 3-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation and the viral DNA growth is terminated. This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination. Lamivudine was rapidly absorbed after oral administration in HIV-infected patients. Absolute bioavailability in adults is 86% ± 16%. The only detected metabolite of lamivudine is trans-sulfoxide. Protein binding was 36% and half life was about 5–7 hours.

Zidovudine [3, 4] is a structural analog of thymidine and it was inhibits the activity of HIV-1 reverse transcriptase[4] (RT) both by competing with the natural substrate GTP and by its incorporation into viral DNA. However, because of first-pass metabolism, systemic bioavailability of zidovudine (ZID) is approximately 52-75%. Bioavailability in neonates up to 14 days of age is approximately 89% and it goes on decreases with children's age. Hepatic metabolized by glucuronide conjugation to major inactive metabolite, 3′-azido-3′-deoxy-5′- O-beta-D-glucopyranuronosylthymidine. The protein binding was 30-38% and half life was about 0.5 – 3 hours. The structures of lamivudine & zidovudine were shown along with their internal standards (ISTD) emtricitabine (EMT) & didanosine (DID) in Fig.1.

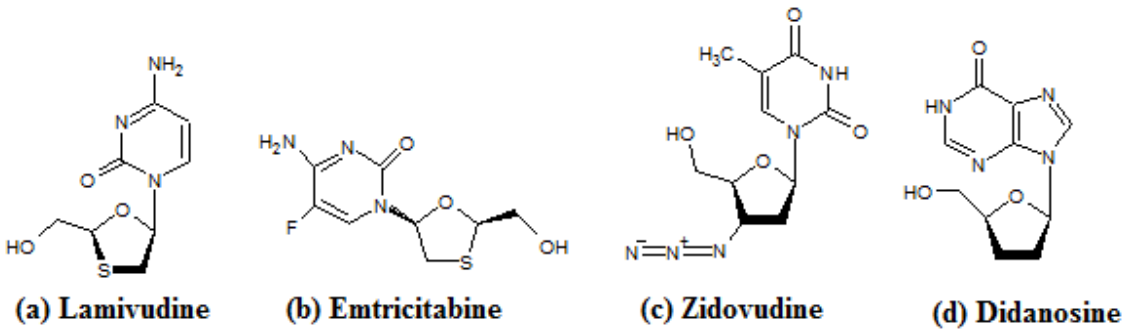

Fig.1. Chemical Structures of the analytes and their internal standards

Literature survey reveals quite a few methods have been reported for the simultaneous determination of lamivudine and zidovudine in pharmaceutical dosage forms by UV [5] & HPLC [6-11] and in plasma and other biological fluids by LC-MS/MS [12-15]. The authors now propose a fast, sensitive, accurate and precise tandem mass spectroscopic method for the determination of lamivudine and zidovudine in human plasma. The entire results obtained in the present study comply with the acceptance criteria of regulatory requirements [16-18].

MATERIALS AND METHODS

Chemicals and materials

The reference standards of Lamivudine, zidovudine, emtricitabine and didanosine were gifted by Hetero pharmaceuticals, Hyderabad, India. HPLC grade methanol and acetonitrile were obtained from J.T Baker® (Phillipsburg, USA). Analytical- grade formic acid was purchased from Merck® Ltd (Mumbai, India). The water used for the analysis was prepared by Milli-Q® water purification system (Bangalore, India). The blank (drug free) human plasmas were procured from Jeevan-dhara blood bank (Hyderabad, India).

Instrumentation and optimized chromatographic conditions

The HPLC system (Shimadzu®, Kyoto, Japan) consisting of two LC-20AD prominence pump, an auto sampler (SIL-HTc), CTO 10 ASvp column oven, a solvent degasser (DGU-20A3) and connected with a Supelco Discovery C_{18} column (50 mm X 4.6 mm, 5µm) were used for the chromatographic separation. 10µL of the sample volumes were injected into the column, which was maintained at 35°C in the column oven. The optimized isocratic mobile phase consists a mixture of 0.1% formic acid and methanol with a flow rate of 0.8 mL/min into the ionization chamber of mass spectrometer. The quantitation was achieved with daughter ion detection in positive polarity with multiple reaction monitoring (MRM) mode for both analytes and internal standards using a MDS Sciex® API-3200 mass spectrometer (Foster City, CA, USA) equipped with a Turbo Ion Spray™ interface at 500°C. The ion spray voltage was set at 5.2 Kilo Volts. The source parameters viz. the nebulizer gas, curtain gas, auxillary gas and collision gas were set at 45, 40, 50 and 6 psi respectively. The compound parameters viz. the de-clustering potential (DP), entrance potential (EP), collision energy (CE), Collision Cell Entrance Potential (CEP) and collision cell exit potential (CXP) were 35.0, 6.0, 31.0, 18.0 & 7.0 Volts for lamivudine; 30.0, 8.0, 29.0, 17.0 & 5.0 Volts for zidovudine. Detection of the ions were carried out in MRM mode, by monitoring the ion transition pairs of m/z 230.0 →112.1 for lamivudine, 268.1 →127.1 for zidovudine, 248.2 →130.1 for emtricitabine and 237.0 →137.0 for didanosine. The Positive MRM scan mass spectra of lamivudine and zidovudine for precursor and product ions were shown in Fig.2, 3. Both the quadrupoles Q1 and Q3 were set at unit resolution. The retention times obtained for the analytes LAM, ZID & ISTD EMT, DID were 0.9 ± 0.3, 1.1 ± 0.3 min & 1.5 ± 0.3, 1.2 ± 0.3 respectively over a total run time of 2.5 minutes. The analysis data obtained was processed by using Analyst Software™ (Version 1.4.2).

Preparation of calibration curve standards and quality control samples

Primary stock solutions of LAM and ZID for preparation of standards (CC) and quality control (QC) samples were prepared from separate weighing. The primary stock solutions about 1.0 mg/mL of LAM, ZID were prepared in methanol and stored below 10^{o}C; they were found to be stable for 15.25 days (data not shown). Appropriate dilutions for calibration curve were made from primary stock of CC using a 50:50 v/v mixture of methanol and water as a diluent to produce the CC spiking solutions containing concentrations (from Standard-8 to Standard-1) of 350787.18, 200650.27, 50162.57, 10032.51, 2508.13, 501.63, 200.65, 100.33 ng/mL for LAM and 350228.91, 200330.94, 50082.73, 10016.55, 2504.14, 500.83, 200.33, 100.17 ng/mL for ZID. Similarly QC spiking solutions were made by using primary stock of QC. These concentrations (HQC, MQC, LQC and LLOQ QC) were 268455.56, 146039.83, 292.08 & 99.89 ng/mL and 267502.61, 145521.42, 291.04, & 99.54 ng/mL for LAM and ZID respectively. The primary stock solutions of emtricitabine and didanisine about1.0 mg/mL were prepared in methanol. Working dilution of emtricitabine and didanosine was prepared at concentration of 1000 ng/mL by using the above diluent. These ISTD primary stock solutions were stored at below 10^{o}C. The plasma calibration curve and quality control samples were prepared by spiking 10 µL of the spiking solution of both LAM and ZID into 980µL of screened plasma. Calibration curve standard for LAM and ZID were made at concentration of 1.00, 2.01, 5.02, 25.08, 100.33, 501.63, 2006.50, 3507.87 ng/mL and 1.00, 2.00, 5.01, 25.04, 100.17, 500.83, 2003.31, 3502.29 ng/mL respectively. Similarly quality control (LLOQ QC, LQC, MQC & HQC) samples for LAM and ZID were prepared at concentration of 1.00, 2.92, 1460.40 & 2684.56 and 1.00, 2.91, 1455.21 & 2675.03 ng/mL.

Extraction process of plasma samples

One hundred micro liters of the spiked plasma calibration curve standards and the quality control samples were transferred into a set of pre-labeled polypropylene tubes containing 50 µL of internal standard dilution (approx.1000ng/mL of EMT & DID). The tubes were added 150 µL of 1.0% formic acid solution in water and vortexed for well mixing. The Strata-X 30mg/1CC cartridges placed on the solid phase extraction (SPE) chamber were conditioned with 1 mL of methanol followed by equilibrating with 1 mL of Milli-Q water. The above samples were loaded on to the cartridges and the cartridges were washed with 1 mL of Milli-Q water followed by 1 mL of 10% methanol in water. The cartridges were dried for approximately 1 min and eluted with 1 mL of acetonitrile. The eluents were evaporated in a stream of nitrogen at 50^{0}C and the residues in the dried tubes were reconstituted with 0.2 mL of the mobile phase. These tubes were vortexed and transferred into auto-sampler vials and loaded. An aliquot of 10µL of the sample was drawn each time from the loaded vials and then analyzed by LC-MS/MS.

Method Validation

A through validation of the method was carried out as per the USFDA guidelines[17]. The method was validated for selectivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stabilities. Selectivity of the method was assessed by analyzing six blank human plasma matrixes. The responses of the interfering substances or background noises at the retention time of the LAM and ZID are acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. The responses of the interfering substances or background noise at the retention time of the internal standard are acceptable if they are less than 5 % of the mean response of internal standard in LLOQ samples. Matrix effect was investigated to ensure that precision, selectivity and sensitivity were not compromised by the matrix. The matrix effect was checked with six different lots of EDTA plasma. Triplicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total). Linearity was tested for LAM and ZID in the concentration ranges 1.00-3507.87 and 1.00-3502.29 ng/mL respectively. For the determination of linearity, standard calibration curves containing at least eight points (nonzero standards) were plotted and checked. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. The acceptance limit of accuracy for each of the back-calculated concentrations was ±15% except for LLOQ, where it was ±20%. For a calibration run to be accepted at least 75% of the standards, including the LLOQ and ULOQ, were required to meet the acceptance criteria and no two consecutive standards should fail, otherwise the calibration curve was rejected. Five replicate analyses were performed on each calibration standard. The samples were run in the order from low to high concentration. Intra-assay precision and accuracy were determined by analyzing six replicates at four (H, M, L, LLOQ) different QC levels on five different runs. The acceptance criteria included accuracy within ±15% deviation from the nominal values, except the LLOQ, where it should be ±20%, and a precision of ≤15% co-efficient variance or relative standard deviation (%CV or RSD), except for LLOQ, where it should be ≤20%. Recovery of the analytes from the extraction procedure was determined by comparing the areas of the analytes in spiked plasma (six each of low, medium and high QCs) with the those of the analytes in samples prepared by spiking the extracted drug-free plasma with the same amounts of the analytes at the step immediately prior to chromatography. Similarly, recovery

of the IS was determined by comparing the mean peak areas of the extracted QC (H, M & L) samples (n=18) with those of the IS in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography. The dilution integrity exercise was performed with the aim of validating the dilution test to be carried out on higher analyte concentrations above the upper limit of quantitation (ULOQ) during real-time analysis of unknown subject samples. The dilution integrity experiment was carried out at 2.0 times the ULOQ concentration for both analytes. Six replicates each of half and quarter concentrations were prepared and their concentrations were calculated by applying the dilution factors 2 and 4. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (Below 10^oC) was determined by comparing area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability (13.47 hours), processed sample stability (auto sampler stability for 44.33 hours), freeze thaw stability (five cycles) and long term stability (49.34 days) were tested at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptance limits of accuracy (±15%) and precision (≤15% CV).

Pharmacokinetic study design

A pharmacokinetic study was performed in healthy (n=6) male subjects. Blood samples were collected following oral administration of lamivudine (150 mg) and zidovudine (300 mg) pre-dose at 0.00, 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 1.75, 2.00 2.50, 3.00, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, 20.00, 24.00, 30.00 hours in K2 EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at -70 ± 5^0C until use. Plasma samples were spiked with IS and processed as per extraction method described above. Along with clinical samples, QC samples at low, medium and high concentrations were assayed in triplicate and distributed among the unknown samples in the analytical run; not more than 33% of the QC samples were greater than ±15 of the nominal concentration and minimum 50% at each individual QC's should pass. Time profile of lamivudine and zidovudine was analyzed by non-compartmental method using WinNonlin® version 5.2 (Pharsight Corporation, Mountain View, CA, USA).

RESULTS AND DISCUSSION

Method development

Mass parameters were tuned in both positive and negative ionization modes for both the analytes. Good and reproducible responses were achieved in positive ionization mode. Data from the MRM mode were considered to obtain selectivity. The protonated form of each analyte and IS, the $[M + H]^+$ ion, was the parent ion in the Q_1 spectrum and was used as precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was monitored from m/z 230.0 → 112.1 for LAM, from m/z 268.1 → 127.1 for ZID and from m/z 248.2 → 130.1 for EMT & from m/z 237.0 → 137.0 for DID. The product ion mass spectra of LAM and ZID are presented in Fig. 2 & 3.

We aimed to develop a simple chromatographic method with a lesser run time. Separation was tried using various combinations of methanol and buffer with varying contents of each component on a variety of columns, such as C_8 and C_{18} of different makes like Kromosil, Ace, Intersil, Hypersil, Hypurity Advance, Zorbax and Discovery. The use of formic acid buffer at concentration of 0.1% formic acid helped to achieve a good response for MS detection in the positive ionization mode. To get a good chromatographic separation with the desired response, it was observed that mobile phase as well as selection of column was an important criterion. It was found that an isocratic mobile phase system consisting of 0.1% formic acid and methanol (20:80, v/v) could achieve this purpose and was finally adopted. The retention times of LAM, ZID and IS were 0.9, 1.1, 1.5 and 1.2 min respectively. A flow rate of 0.8 mL/min produced good peak shapes and permitted a run time of 2.5 min.

A good internal standard must mimic the analytes during extraction and compensate for any analyte on the column, especially with LC-MS/MS analysis, where matrix effects can lead to poor analytical results. In the initial stages of this work, several compounds were investigated to find a suitable internal standard and finally emtricitabine and didanosine were found to be best available for the present purpose. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS technique. Hence, solid phase extraction was used for the sample preparation in this work. Other extraction technique like LLE and PPT were also tried, but SPE was found to be optimal; it can produce a clean chromatogram for a blank sample and yields good, reproducible recovery for analytes from the plasma.

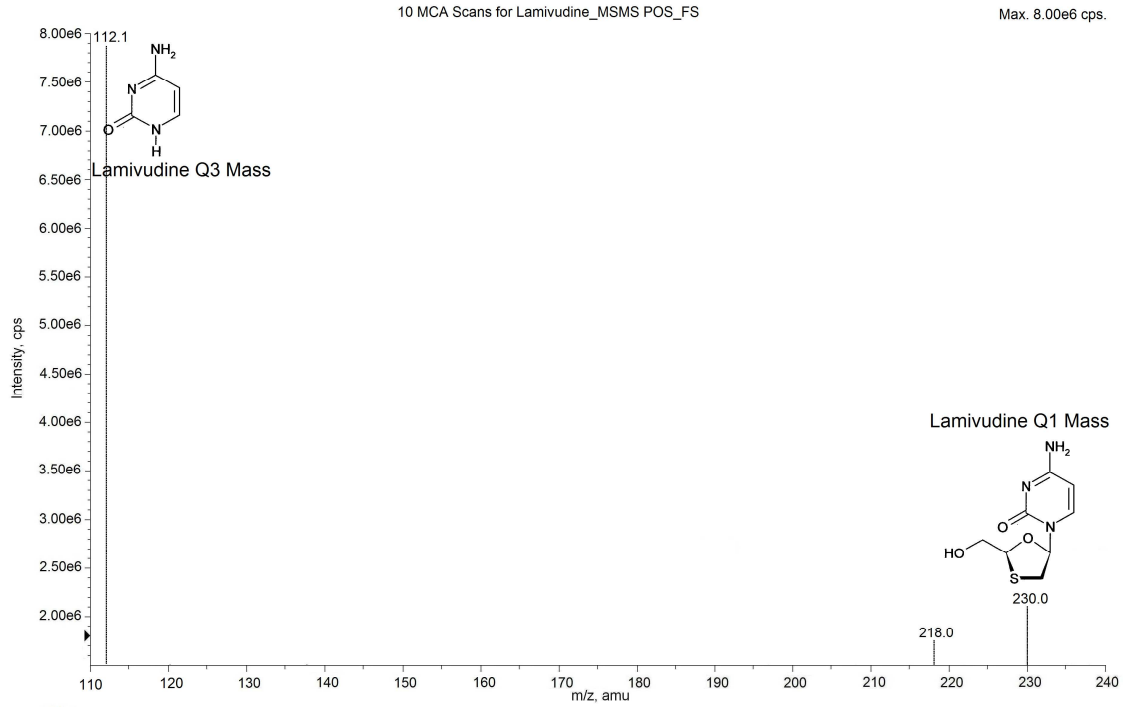

Fig.2. Positive MRM scans mass spectra of Lamivudine for precursor and product ions

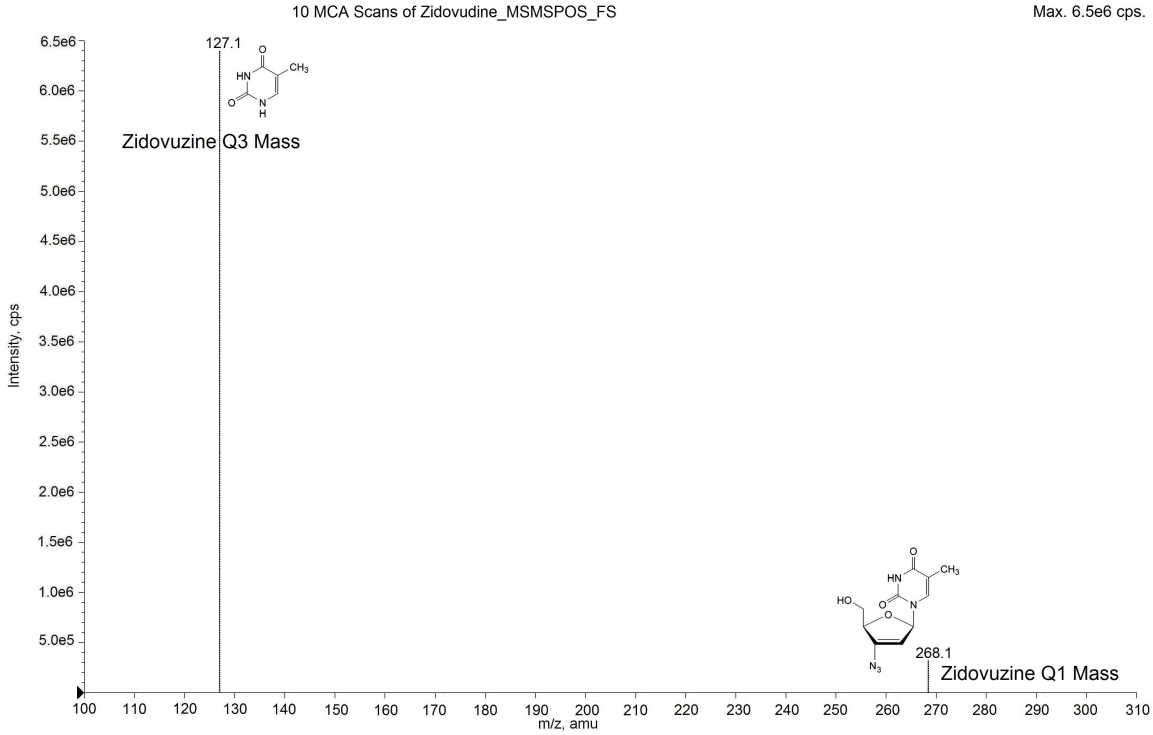

Fig.3. Positive MRM scans mass spectra of Zidovudine for precursor and product ions

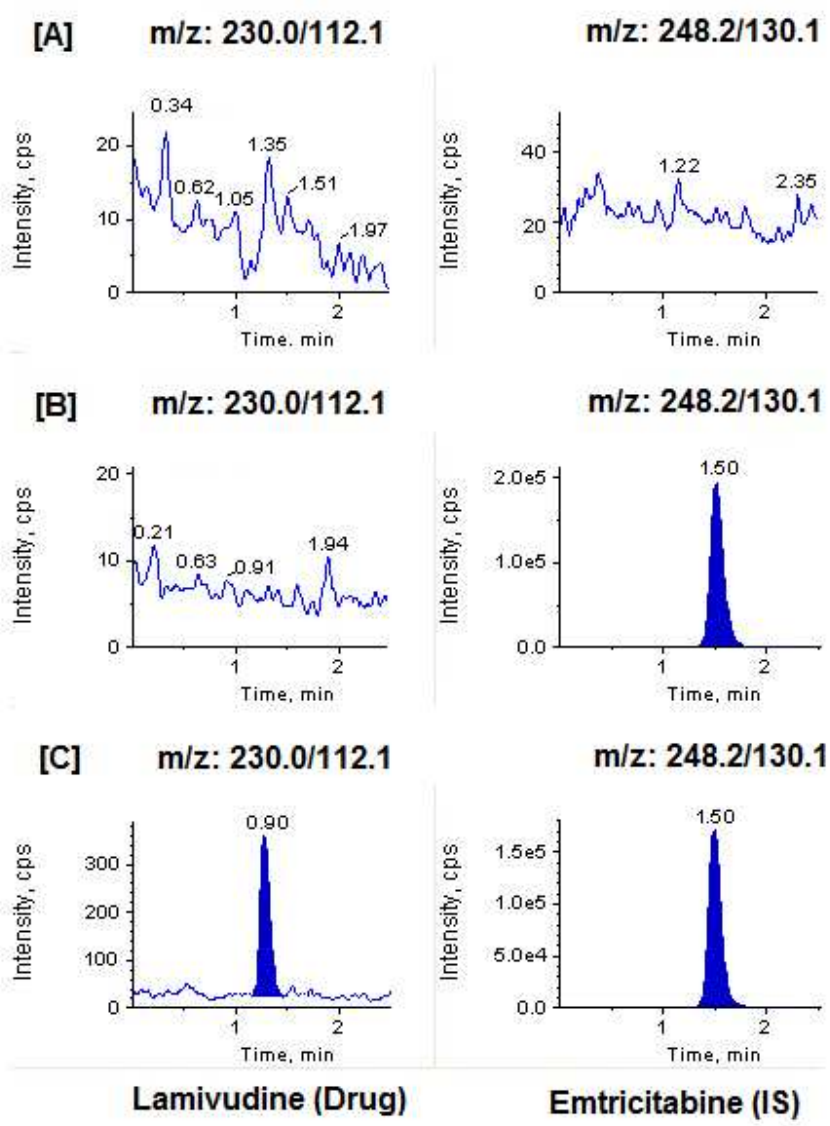

Fig.4. Typical multiple reaction monitoring mode chromatograms of lamivudine (left panel) and internal standard (right panel) in (A) human blank plasma; (B) human plasma spiked with internal standard; (C) a lower limit of quantitation sample along with internal standard

Table 1. Mean overall recoveries of lamivudine, zidovudine and emtricitabine & didanosine IS

Analyte name	Sample concentration ng/mL	Response un extracted (mean ± SD)	Response Extracted (mean ± SD)	Recovery (%)	Mean ±SD recovery
Lamivudine	2.92 (LQC)	7200.8 ± 142.2	5896.3 ± 87.7	81.9	82.5 ± 0.88 % CV 1.1
	1460.40 (MQC)	359256.8 ± 7470.7	300198.8 ± 8859.0	83.6	
	2684.56 (HQC)	654854.0 ± 12616.6	538800.2 ± 12139.6	82.3	
Zidovudine	2.91 (LQC)	7296.8 ± 113.9	5676.8 ± 126.1	77.8	76.6 ± 1.08 % CV 1.4
	1455.21 (MQC)	383281.2 ± 4832.8	289991.8 ± 10194.7	75.7	
	2675.03 (HQC)	678706.3 ± 15010.4	518749.5 ± 11971.5	76.4	
Emtricitabine (IS)	1012.26	455834.7 ± 9297.2	369104.0 ± 8838.5	81.0	
Didanosine (IS)	997.37	449317.2 ± 15218.2	340219.8 ± 16120.2	75.7	

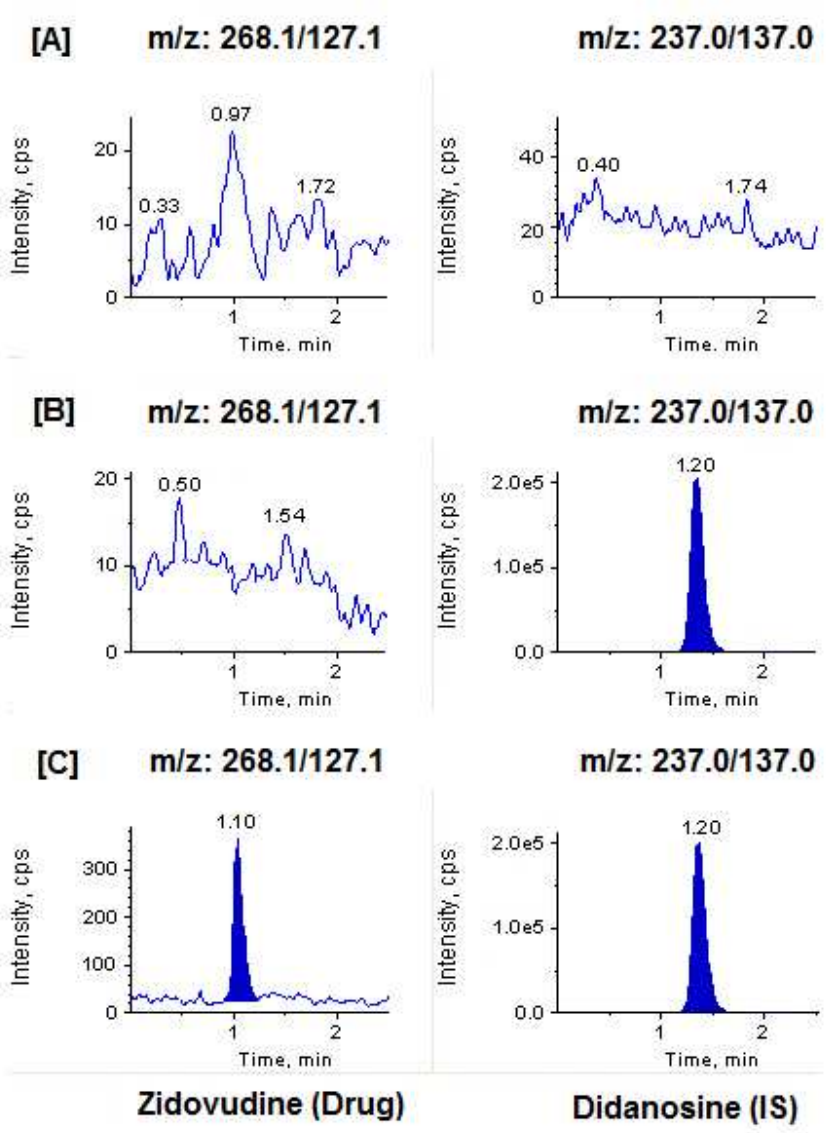

Fig.5. Typical multiple reaction monitoring mode chromatograms of zidovudine (left panel) and internal standard (right panel) in (A) human blank plasma; (B) human plasma spiked with internal standard; (C) a lower limit of quantitation sample along with internal standard

Table 2. Precision and accuracy of the method for determining Lamivudine and Zidovudine

		Intra-day precision and accuracy (n = 6) six from each batch			Inter-day precision and accuracy (n = 24) six from each batch		
Analyte	Concentration ng/mL	Concentration found (Mean; ng/mL)	Precision (% CV)	Accuracy (%)	Concentration found (Mean; ng/mL)	Precision (% CV)	Accuracy (%)
LAM	1.00 (LLOQ)	0.918	9.4	91.8	0.958	8.8	95.8
	2.92	2.982	3.0	102.1	2.904	5.1	99.4
	1460.40	1487.072	1.9	101.8	1571.280	4.5	107.6
	2684.56	2545.427	2.5	94.8	2588.452	3.2	96.4
ZID	1.00 (LLOQ)	1.030	9.7	103.0	0.963	8.7	96.3
	2.91	3.017	3.8	103.7	2.936	5.3	100.9
	1455.21	1540.172	2.4	105.8	1555.736	3.5	106.9
	2675.03	2590.647	2.0	96.8	2621.838	2.8	98.0

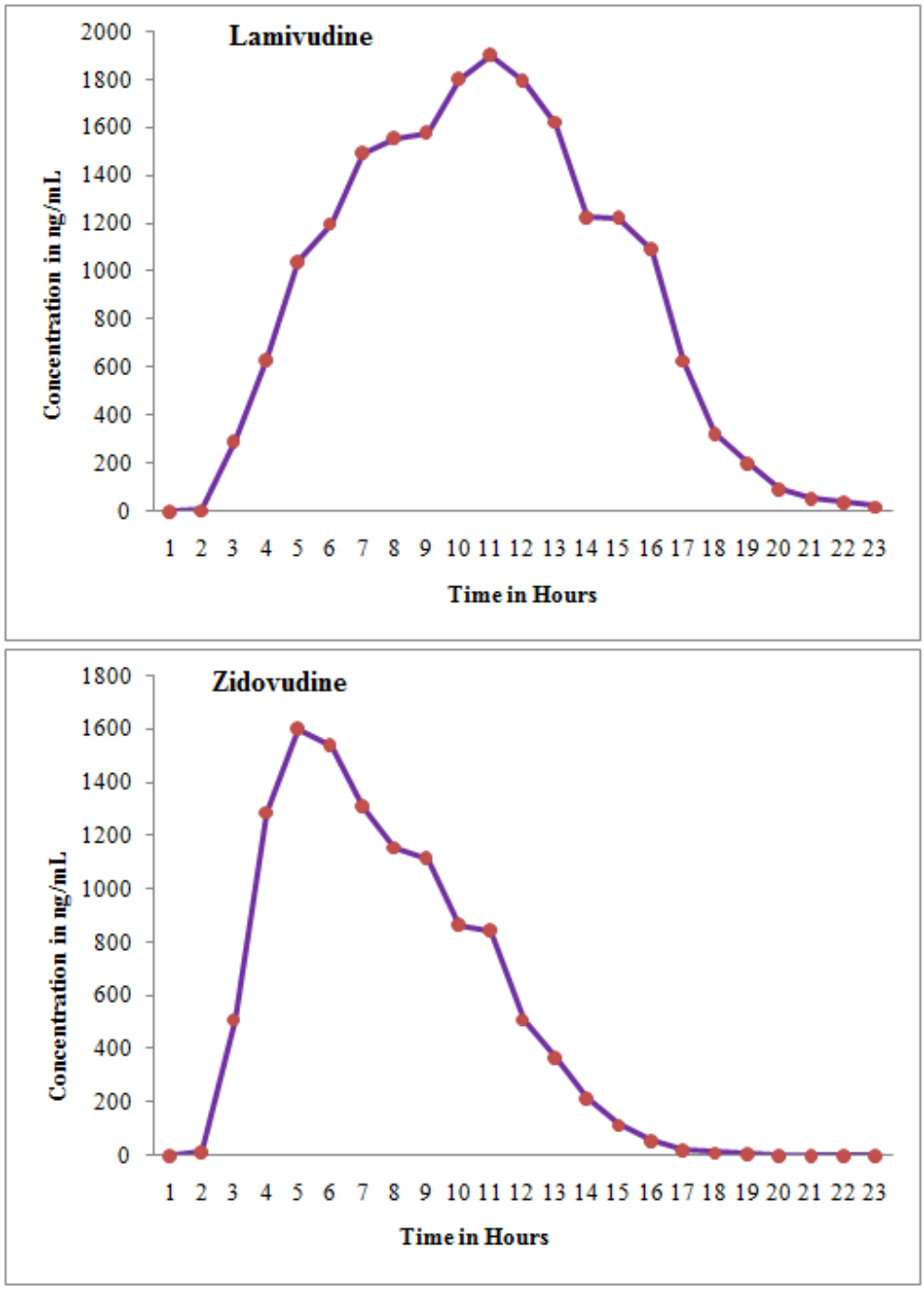

Fig.6. Mean plasma concentration Vs Time curve for 6 volunteers for Lamivudine and zidovudine

Selectivity

The degree of interference by endogenous plasma constituents with the analyte and IS was assessed by inspection of chromatograms derived from a processed blank plasma sample (pure blank & IS added blank). As shown in Fig.4 & 5 for individual analyte LAM and ZID respectively, no significant interference in the processed blank plasma samples were observed at the retention times of the analyte and internal standard.

Sensitivity

The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration was found to be 3.2 CV & 101.8% and 6.8 CV & 99.5% for LAM & ZID respectively.

Extraction efficiency

A simple solid phase extraction with strata-X cartridges were found to be rugged and provided the cleanest samples. The recoveries of the analytes and internal standards were good and reproducible. The mean over all recoveries with precision range of Lamivudine and zidovudine was presented in Table.1.

Table 3. Stability data of the lamivudine and zidovudine

Analyte	Stabilities	QC Conc. (ng/mL)	Mean ± SD	Precision (% CV)	Stability (%)
LAM	Auto-sampler (44.33Hr)	2.92 (LQC)	2.800 ± 0.21	7.5	98.4
			2734.81 ± 89.60	3.3	102.2
	Dry extract (42.44Hr)		3.033± 0.10	3.4	102.5
			2488.26 ± 194.35	7.8	105.5
	Bench top (13.47Hr)		2.96 ± 1.01	2.7	103.6
			2726.63 ± 64.14	2.4	98.0
	Freeze-thaw (5Cycle)	2684.56 (HQC)	3.02 ± 0.12	4.0	106.4
			2704.70 ± 124.68	4.6	96.2
	Re injection (42.52Hr)		3.11 ± 0.25	8.1	97.6
			2626.16 ± 55.35	2.1	94.8
	Long term (49.34Days)		2.88 ± 0.14	4.7	96.1
			2748.26 ± 88.43	3.2	103.9
ZID	Auto-sampler (44.33Hr)	2.91 (LQC)	3.06 ± 0.11	3.5	102.1
			2797.38 ± 92.18	3.3	103.6
	Dry extract (42.44Hr)		2.89 ± 0.16	5.5	98.1
			2564.9 ± 123.04	4.8	101.3
	Bench top (13.47Hr)		2.81 ± 0.08	2.8	93.6
			2751.29 ± 65.89	2.4	99.5
	Freeze-thaw (5Cycle)	2675.03 (HQC)	2.86 ± 0.16	5.7	100.5
			94.83± 47.88	1.8	106.5
	Re injection (42.52Hr)		2.83 ± 0.21	7.3	101.8
			2597.31 ± 107.50	4.1	98.8
	Long term (49.34Days)		2.93 ± 0.14	4.7	93.7
			2732.34 ± 122.88	4.5	103.5

Table 4. Pharmacokinetic data for lamivudine & zidovudine

Parameter	Lamivudine	Zidovudine
C_{max}(ng/mL)	2031.8 ± 117.2	1786.3 ± 304.1
T_{max}(Hrs)	1.6 ± 0.5	0.80 ± 0.1
AUC (0-T) (ng.h/mL)	12371.4 ± 1110.1	3244.3 ± 486.3
AUC (0-∞) (ng.h/mL)	12552.3 ± 1108.1	3250.9 ± 487.0
T1/2 in Hours	5.8 ± 0.3	2.2 ± 0.2

Matrix effect

There was no significant matrix effect was observed in all the six lots of human plasma for the analyte at low and high QC level concentrations. The precision and accuracy for LAM & ZID at LQC was 3.7, 2.9% & 106.4, 104.7. Similarly at HQC was 5.2, 6.1% & 102.1, 104.8 respectively for LAM & ZID.

Linearity

The eight-point calibration curve was found to be linear over the concentration range of 1.00-3500 ng/mL for both LAM and ZID. After comparing the weighting factor models at none, 1/X and $1/X^2$, the regression equation with weighting factor $1/X^2$ of the analytes to the internal standards concentration was found to be the best fit in plasma samples. The mean (n = 4) correlation coefficient (r) of the calibration curves generated in the validation was 0.999 for both LAM and ZID.

Precision and accuracy
Precision and accuracy data for intra, inter day samples for all the analyte were presented in Table 2. The results obtained in both the criteria were well within acceptance limits.

Stability studies
The different stability experiments carried out, viz. bench-top stability for 13.47 hours, auto-sampler stability for 44.33 hours, repeated freeze-thaw cycles for 5 cycles, re-injection stability for 41.7 hours, dry extract stability for 42.44 hours at 1-10^{o}C and long-term stability in matrix at -70 ^{o}C for 41.4 days. The mean percentage nominal values of the analyte were found to be within ± 15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table-3). Thus, the results were found to be within the acceptable limits during the entire validation.

Application to a pharmacokinetic study
In order to verify the sensitivity and selectivity of this method in a real-time analysis, the present method was used to test for Lamivudine and zidovudine in human plasma samples collected from healthy male volunteers (n=6). The mean plasma concentration verses time profiles of lamivudine and zidovudine are shown in Fig. 6. Following oral administration the maximum concentration (C_{max}) in plasma, 20318.8ng/mL for LAM and 1786.3 ng/mL for ZID was attained at 1.6 and 0.80 hrs (T_{max}) for LAM and ZID respectively. The plasma concentration verses time curve from zero hour to the last measurable concentration (AUC_{0-t}) and area under the plasma concentration-time curve from zero hour to infinity ($AUC_{0-\infty}$) for LAM were 12371.4 and 12552.3 ng.h/mL and for ZID were 3244.3 and 3250.9 ng.h/mL. These obtained results were matching with the published data [19, 22] which were shown in Table. 4.

So far published methods are available for the quantification of Lamivudine and zidovudine in the matrices. This paper describes the development and validation of a bio analytical method for the simultaneous quantitative determination of these two compounds in single chromatographic run. The challenges involved in this method include retaining both mid-polar lamivudine and polar zidovudine on the same column and extracting both analytes with reliable and consistent recoveries. The proposed method is simple, rugged and rapid owing to the utilization of a short run time of 2.5 min for each sample analysis. Here we have developed a method for the determination of Lamivudine and zidovudine in human plasma with good sensitivity (LLOQ 1.00 ng/mL).

CONCLUSION

In summary, we have developed and validated a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantify of Lamivudine and zidovudine. From the results of all the validation parameters obtained good quality results, thereby we can conclude that the developed method can be useful for BA/BE studies and routine therapeutic drug monitoring with the desired precision and accuracy.

Acknowledgements
The authors gratefully acknowledge Wellquest laboratories, for providing the necessary facilities for carrying out this study.

REFERENCES

[1] http://www.drugbank.ca/drugs/DB00709 for lamivudine.
[2] http://www.rxlist.com/epivir-drug.htm
[3] http://www.drugbank.ca/drugs/DB00495 for zidovudine.
[4] http://www.rxlist.com/retrovir-drug.htm
[5] Apparao P, Jyothi, Prabhakarreddy V, Raju J, Shashidher B, *Der Chemica Sinica*., **2011**, 3, 126.
[6] Uslu B, Ozkan S. A, *Ana. Chimica Acta*., **2002**, 466, 175.
[7] Jadhav S. D, Bhatia M. S, Thamake S. L, Pishawikar S. A, *Asian. J. Res. Chem*., **2010**, 4, 1.
[8] Venkatesh P, Smiley L, Mahesh D, *Int. J. ChemTech. Res*., **2011**, 3, 376.
[9] Manwar J.V, Sonawane B. V, Patil S. V, Takle S. P, *Der Chemica Sinica*., **2011**, 5, 152.
[10] Mahor R. P, Parcha R. Singh P, Y, Sharma R, Bhandari A, *Der Chemica Sinica*.,**2011**, 6, 12.
[11] Palavan C, Ramaprasad L. A, Varaprasad J, Rao J. V. L .N. S, *Am. J. PharmTech Res*., **2013**, 1, 3.
[12] Pereira A. S, Kenney K. B, Cohen M. S, Hall J. E, Eron J. J, Tidwell R. R, Dunn J. A, *J. Chromatogr. B*., **2000**. 1, 173.

[13] Kenney K. B, Wring S. A, Carr R. M, Wells G. N, Dunn J. A, *J. Pharm. Biomed. Anal*., **2000**, 6, 967.
[14] Alnouti Y, Lewis S. R, C. White A, Bartlett M. G, *Rapid Comm. in Mass Spec*., **2005**, 19, 503.
[15] Rower J. E, Klein B, Bushman L. R, Anderson P. L, *J. Chromatogr. B.*, **2012**. 1, 12.
[16] http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline /2009 /12/WC500018062.pdf
[17] http://bebac.at/Guidelines.htm#
[18] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/ Guidance/ucm070124.pdf
[19] http://www.accessdata.fda.gov/drugsatfda_docs/label/**2011**/020564s031020596s030lbl.pdf
[20] http://www.mhra.gov.uk/home/groups/par/documents/websiteresources/con140802.pdf
[21] http://www.mhra.gov.uk/home/groups/l-unit1/documents/websiteresources/con 025677.pdf
[22] http://www.accessdata.fda.gov/drugsatfda_docs/label/**2010**/022294s002lbl.pdf