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High throughput LC–MS/MS method for simultaneous quantification of lamivudine, stavudine and nevirapine in human plasma

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Abstract

A selective and high throughput liquid chromatography/tandem mass spectrometry (LC–MS/MS) method has been developed and validated to separate, detect and simultaneously quantify lamivudine (3TC), stavudine (d4T) and nevirapine (NVP) in human plasma using metaxalone as internal standard (IS). After solid phase extraction (SPE), the analytes and the IS were chromatographed on a Symmetry C18 (150 mm × 3.9 mm i.d., 5 μm particle size) column using 5 μL injection volume with a run time of 4.5 min. An isocratic mobile phase consisting of 0.5% glacial acetic acid in water:acetonitrile (20:80, v/v) was used to separate all these drugs. The precursor and product ions of these drugs were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring mode (MRM) without polarity switch. The method was validated over the range of 25–3000 ng/mL for 3TC, 20–2000 ng/mL for d4T and 50–5000 ng/mL for NVP. The absolute recoveries for analytes (≥86%) and IS (98.12%) achieved from spiked plasma samples were consistent and reproducible. Inter-batch and intra-batch precision (%CV) across four validation runs (LLOQ, LQC, MQC and HQC) was less than 10. The accuracy determined at these levels was within ±8% in terms of relative error. The method was successfully applied to a pivotal bioequivalence study of [60 (3TC) + 12 (d4T) + 100 (NVP)] mg dispersible tablets in 60 healthy human subjects under fasting condition.

Keywords: Lamivudine (3TC); Stavudine (d4T); Nevirapine (NVP); LC–MS/MS; Multiple reaction monitoring (MRM); Pivotal bioequivalence

1. Introduction

Combination therapy has now become the standard line of treatment to manage acquired immunodeficiency syndrome (AIDS) [1]. The need for such a therapy has arisen due to development of resistance by the causative human immunodeficiency virus (HIV), to single anti-HIV drugs and to minimize potential dose-dependent side effects [2]. The US Department of Health and Human Services has recommended highly effective combination regimens of antiretroviral drugs to combat mortality and morbidity in humans [3]. The current regimen for treating HIV infection is to use a combination of at least three drugs, a practice sometimes called highly active antiretroviral therapy (HAART) [4]. This potent combination generally includes two nucleoside reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase (NNRTI) or protease inhibitor. Nucleoside reverse transcriptase inhibitors are intracellularly phosphorylated to their corresponding triphosphorylated derivatives, which compete with the corresponding natural nucleotide for binding to HIV reverse transcriptase. While non-nucleoside reverse transcriptase inhibitors act as non-competitive inhibitors of the HIV reverse transcriptase. The protease inhibitors are a class of anti-HIV drugs which prevent T cells that have been infected from HIV from producing new copies of the virus and in preventing maturation of the HIV virus [5–10]. This complimentary/synergistic action of different classes of anti-HIV drugs in combination therapy has increased survival rate and improved quality of life for AIDS patients [4]. These

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encouraging results are a result of extensive and through understanding of the pharmacokinetic, pharmacological and toxicological profiles of single drug therapy in the HIV infected individuals.

Lamivudine (3TC), a nucleoside reverse transcriptase inhibitor is a synthetic dideoxynucleoside derivative with potent activity against human immunodeficiency virus (HIV) and hepatitis B virus (HBV) [11]. Treatment of HIV infections with an antiviral regimen that includes 3TC is highly desirable since it shows lowers toxicity than other nucleoside derivatives [12]. It is administered orally and is rapidly absorbed with a bioavailability of more than 80% in humans [13]. Stavudine is an analog of thymidine which is phosphorylated by cellular kinases into active triphosphate. Stavudine triphosphate inhibits the HIV reverse transcriptase by competing with natural substrate, thymidine triphosphate. The oral absorption rate is over 80%. Its main adverse effect is peripheral neuropathy, which can be corrected by reducing dosage [14]. Nevirapine (NVP) a highly potent inhibitor was the first NNRTI to be approved by the FDA. It displays activity in several different cell lines in the immune system, including macrophages and T cells. It is readily absorbed after oral administration (90%) and has a half-life of approximately 45 h. Nevirapine in triple combination therapy has been shown to suppress viral load effectively when used as initial antiretroviral therapy. It has shown in vitro to block an early event in the viral replication cycle, completely inhibiting replication if administered within 24 h of infection, but having no effect on protein production or viral release [15].

Several bioanalytical methods are reported to determine 3TC, d4T and NVP individually in different biological matrices like plasma [16–24], tissues and amniotic fluids [16], saliva [17], urine [18,20], cerebrospinal fluids [17,21] and serum [21,25]. Sensitive and selective methods [26–36] based on HPLC-UV and LC–MS/MS methodologies have also been developed for simultaneous analysis of these three drugs in combination with other NRTI, NNRTI or protease inhibitors as they have higher efficacy, acceptable toxicity and broader action range. Fan and Stewart [37] have determined zidovudine, lamivudine and nevirapine in human plasma by ion pair HPLC using solid phase extraction. The LLOQ achieved was 59 and 53.2 ng/mL for lamivudine and nevirapine, respectively, with a run time of 15 min. In another study by the same authors [38], a sensitive method was developed and validated to simultaneously quantify lamivudine, stavudine and efavirenz in human serum. Here, the LLOQ's obtained were 1.1 ng/mL for lamivudine and 12.5 ng/mL for stavudine by LC–MS/MS with ionization polarity switch. The total run time between injections was high (18 min) under gradient conditions.

Very few methods are available to determine 3TC, d4T and NVP combination in biological matrices. Two methods based on HPLC-UV and HPTLC have been reported for simultaneous quantification of these three antiretroviral drugs in tablet formulations [39,40]. Both these methods require a total run time of 10 min or more on C-18 reverse phase columns. Recently Narang et al. [41] have developed a high throughput LC–MS/MS method to quantify 3TC, d4T and NVP in human plasma with special reference to bioequivalence study. Extraction of plasma was done by SPE involving a reconstitution step to get a LLOQ of 50, 51 and 47 ng/mL for 3TC, d4T and NVP, respectively. But the absolute recoveries obtained were very low (47–65%), which exhibits a poor extraction efficiency of the method.

Thus, the aim of this study was to develop a selective and high throughput method for simultaneous quantification of 3TC, d4T and NVP in human plasma for therapeutic drug monitoring and pharmacokinetic studies. As a part of our ongoing research in this area, we have developed and validated a LC–MS/MS assay for this highly potent combination in human plasma without polarity switch. Special emphasis was given to optimize the extraction step in order to get quantitative and reproducible recovery for all the analytes. The method presents a simple and clean SPE procedure without drying and reconstitution steps. The positive ion ESI mode selected for this study, gave high response for all three analytes as they possess amino groups which can be readily protonated by the acidic mobile phase consisting of 0.5% glacial acetic acid in water:acetonitrile (20:80, v/v). All the analytes and IS were well separated with minimum matrix interference in a run time of 4.5 min under isocratic conditions. The LLOQ of 25, 20 and 50 ng/mL for 3TC, d4T and NVP, respectively, achieved was adequate to study the pharmacokinetics of [60 (3TC) + 12 (d4T) + 100 (NVP)] mg dispersible tablets in 60 healthy human subjects under fasting condition.

2. Experimental

2.1. Chemicals and materials

Working standards of lamivudine (3TC), stavudine (d4T), nevirapine (NVP) and metaxalone (IS) were provided by Cipla Ltd. (Mumbai, India) having purity greater than 99%. HPLC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade ammonium acetate and glacial acetic acid were procured from Qualigens Ltd. (Mumbai, India). Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Bangalore, India). Blank human blood was collected with heparin from healthy and drug free volunteers. After centrifugation at 4000 rpm at room temperature, plasma was collected and stored at −20 °C. Strata X, 30 mg; 1 mL solid phase extraction (SPE) cartridges were procured from Phenomenex (Torrance, CA, USA).

2.2. Liquid chromatography and mass spectrometric conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-20AD prominence pump, autosampler (SIL-HTc), solvent degasser (DGU-20A_3 prominence) and a temperature-controlled compartment for column (CTO 10AVP) were used for all the analyses. The chromatographic system consisted of Symmetry C18 (150 mm × 3.9 mm i.d., 5 μm particle size) analytical column from Waters (India) Pvt. Ltd. (Bangalore, India). The flow rate of the mobile phase under isocratic condition was kept at 0.4 mL/min. The autosampler temperature was set at 10 °C and the injection volume was 5 μL. The mobile phase consisted

of 0.5% glacial acetic acid in water:acetonitrile (20:80, v/v). The column oven temperature was maintained at 25 °C. The pressure of the system was 70 bar. The total LC run time was 4.5 min.

Detection of analyte and IS was performed on a triple quadrupole mass spectrometer, API-3000 (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray ionization source and operating in the positive ion mode. Analyst software version 1.4 was used to control all parameters of LC and MS. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion (*m/z*) transitions for 3TC (230.0 → 112.1) (Fig. 1); d4T (225.3 → 127.1) (Fig. 2); NVP (267.2 → 226.2) (Fig. 3); and IS (222.1 → 161.0) (Fig. 4), respectively. Source-dependent parameters optimized were—gas 1 (nebuliser gas): 12 psi; gas 2 (heater gas flow): 6000 cm^3/min; ion spray voltage (ISV): 5000 V; temperature (TEM): 300 °C. Compound-dependent parameters like declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) were 10, 37, 51 V; 8, 7, 9 V; 80, 220, 160 V; 15, 15, 38 eV; and 10, 15, 15 V for 3TC, d4T and NVP, respectively. Nitrogen was used as collision activated dissociation (CAD) gas and was set at 6 psi. Quadrupoles 1 and 3 were maintained at unit and low resolution, respectively, and dwell time was set at 0.2 s.

2.3. Analytical data processing

Chromatographic data were collected and integrated using Analyst software version 1.4. Peak area ratio of the analyte to IS was utilized for the construction of calibration curve. A weighing of $1/x^2$ (least-squares linear regression analysis, where x is the analyte concentration) was used for curve fitting. Concentration in unknown samples was calculated from the best-fit equation ($y = mx + c$), where y is the peak area ratio. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level.

2.4. Standard and quality control preparation

Standard stock solutions of 3TC, d4T and IS were prepared by dissolving their accurately weighted amounts in methanol while NVP was dissolved in acetonitrile to give a final concentration of 1000 μg/mL. Combined working solutions in the

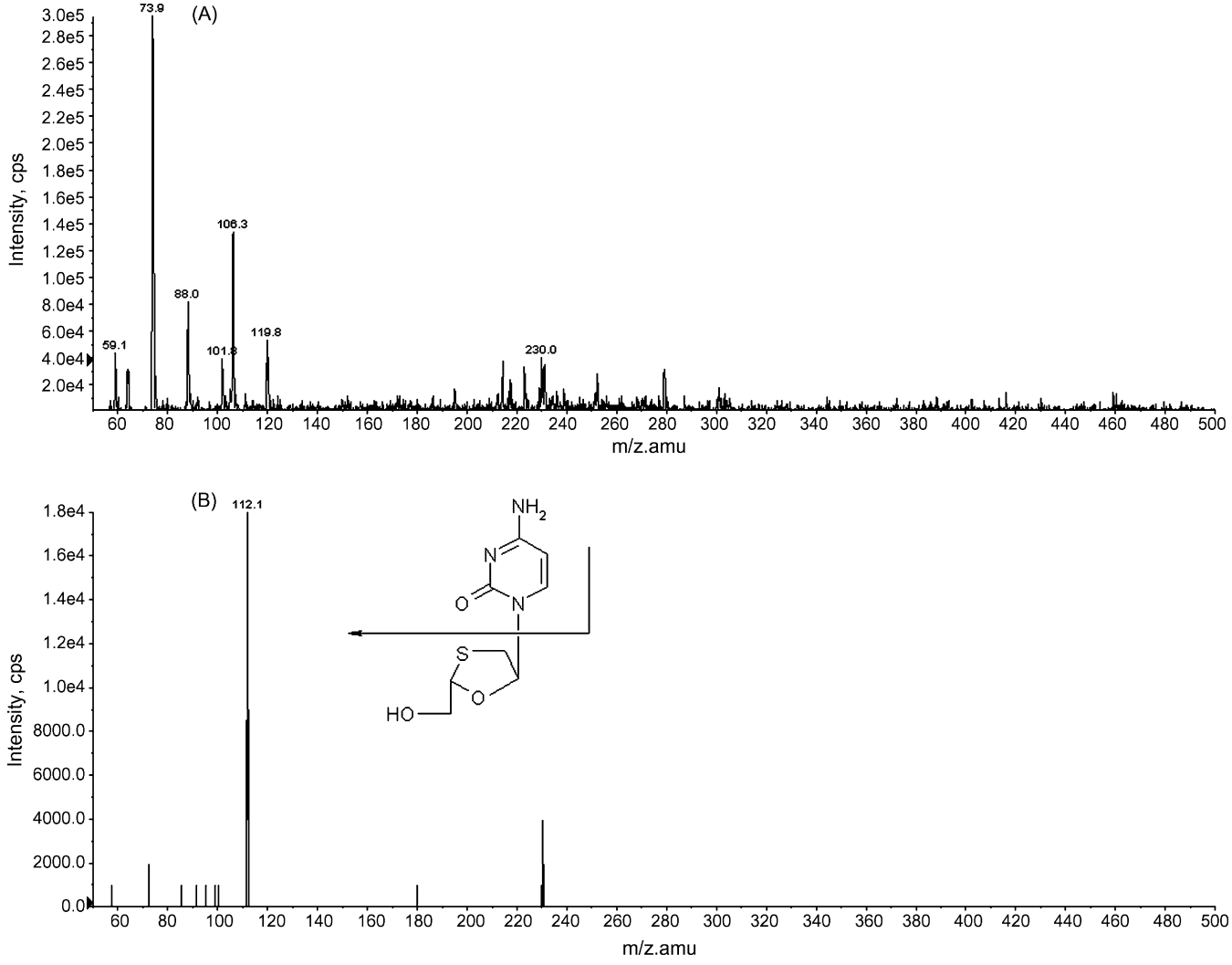

Fig. 1. Parent (A) and product ion (B) mass spectra of lamivudine (3TC).

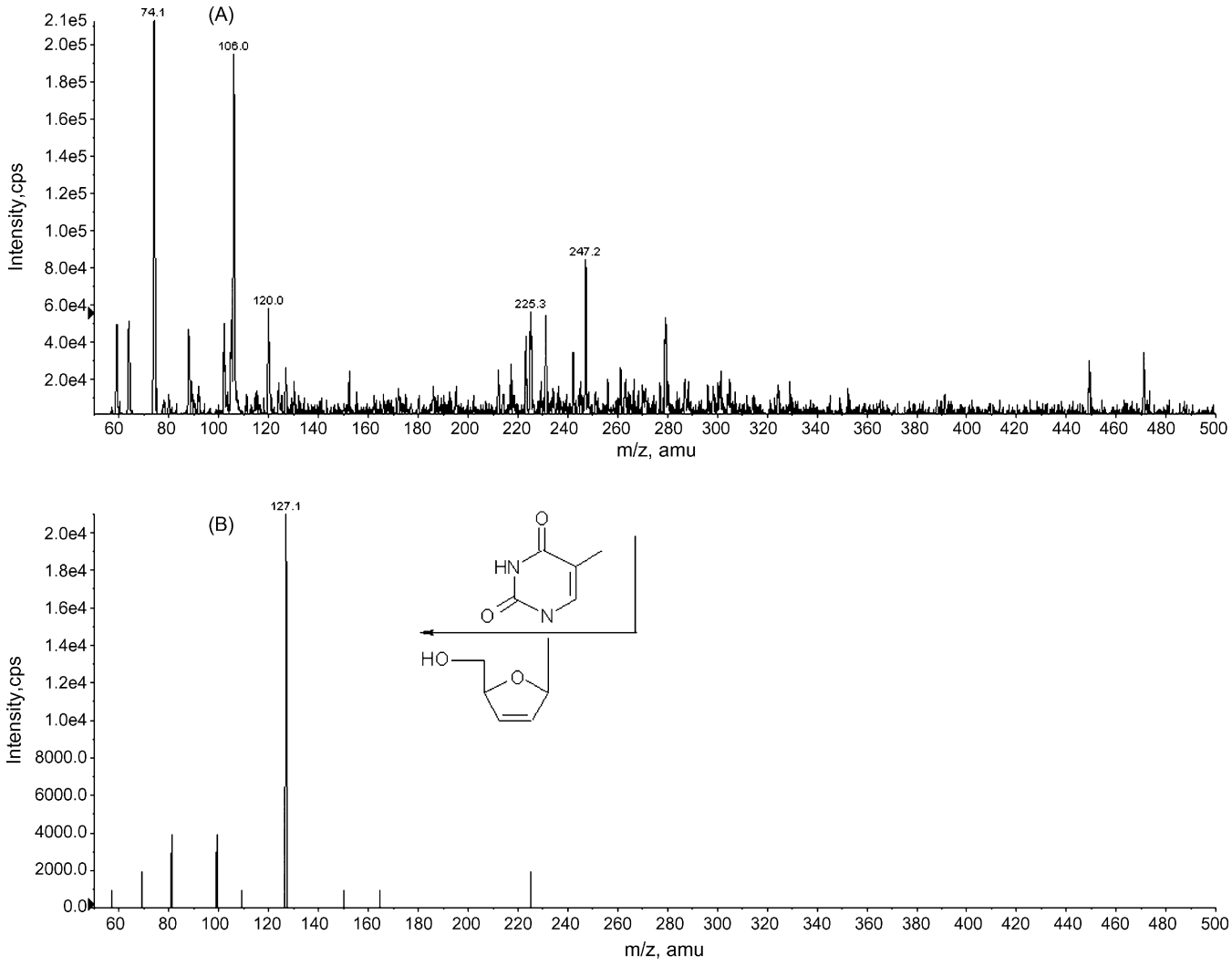

Fig. 2. Parent (A) and product ion (B) mass spectra of stavudine (d4T).

required concentration range were prepared by appropriate dilution of their stock solutions in methanol:water (60:40, v/v). All the solutions were stored at 2–8 °C and were brought to room temperature before use.

The calibration standards and quality control (QC) samples were prepared by spiking (5%) blank plasma with working solutions. Calibration samples were made at concentrations of 25, 50, 100, 200, 500, 1000, 2000 and 3000 ng/mL for 3TC; 20, 50, 100, 200, 500, 1000, 1500 and 2000 ng/mL for d4T; and 50, 100, 500, 1000, 2000, 3000, 4000 and 5000 ng/mL for NVP. Quality control samples were prepared at 75 ng/mL (LQC), 1500 ng/mL (MQC) and 2650 ng/mL (HQC) for 3TC; 60 ng/mL (LQC), 750 ng/mL (MQC) and 1800 ng/mL for d4T; and 150 ng/mL (LQC), 2500 ng/mL (MQC) and 4350 ng/mL (HQC) for NVP. Aliquots of spiked plasma samples were taken in micro-centrifuge tubes and stored at −20 °C.

2.5. Sample preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. Samples of 0.5 mL of plasma were dispensed into micro-centrifuge tubes and 50 μL of 5.0 μg/mL of internal standard were added. The resulting samples were vortex to mix for 10 s and centrifuged at 3200 rpm at 10 °C for 5 min to remove any solid present that may block the cartridge. The samples were then extracted by SPE using DVB-HLB cartridges, which exhibit both hydrophilic and lipophilic retention characteristics. Samples were applied to the cartridges previously conditioned with 1 mL methanol followed by 1 mL water. Thus, water-soluble compounds like 3TC and 4dT, and acetonitrile-soluble NVP were retained on the cartridges. Further, plasma was drained out under vacuum and cartridges were washed with 1 mL each of 2 mM ammonium acetate and water to clean up the samples. After proper drying of the cartridge, elution was carried out using 1 mL mobile phase to obtain all three analytes and IS quantitatively. The eluates were transferred into 1.5 mL glass vials and 5 μL was injected into the chromatographic system.

2.6. Method validation

A thorough and complete method validation of 3TC, d4T and NVP in human plasma was done following the USFDA

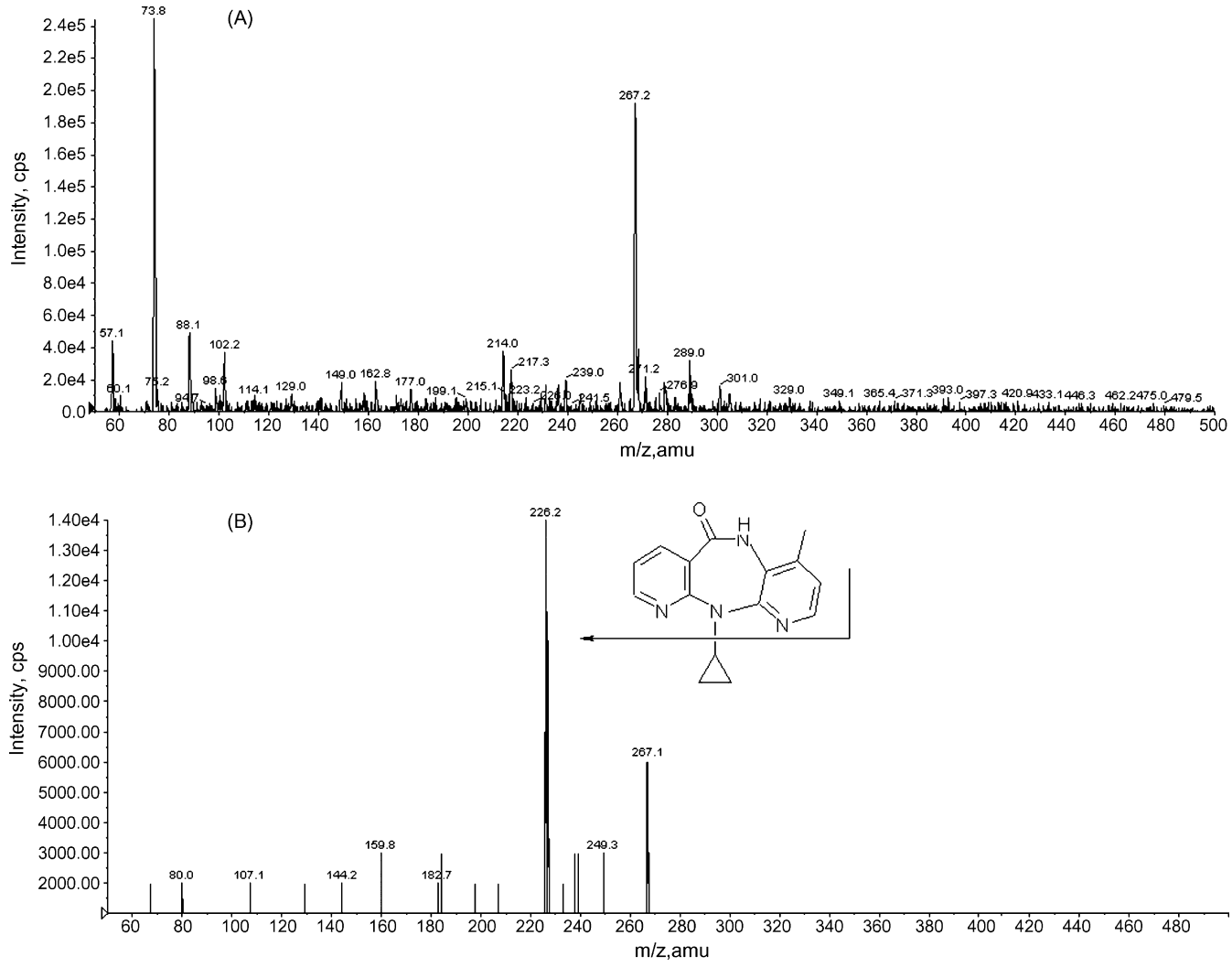

Fig. 3. Parent (A) and product ion (B) mass spectra of nevirapine (NVP).

guidelines [42]. The method was validated for selectivity, sensitivity, linearity, accuracy and precision, recovery, stability, matrix effect and dilution integrity.

The selectivity towards endogenous and exogenous plasma matrix components was assessed in 10 different batches (8 normal, 1 hemolyzed and 1 lipemic) of human plasma samples by analyzing blank and spiked samples at LLOQ level. It was performed in two sets, in the first set, plasmas were extracted and directly injected for LC–MS/MS detection and in the second set, blank plasmas spiked with LLOQ working solution of 3TC, d4T and NVP were extracted and analyzed. The second set was also used for sensitivity determination.

The linearity of the method was determined by analysis of standard plots associated with an eight-point standard calibration curve. Eleven linearity curves containing eight non-zero concentrations were analyzed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes was calculated from the simple linear equation using regression analysis of spiked plasma calibration standard with reciprocate of the drug concentration as a weighting factor ($1/\text{concentration}^2$, i.e. $1/x^2$). The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the range tested.

Inter-batch and intra-batch accuracy and precision was evaluated at four different concentrations (LLOQ, LQC, MQC and HQC) for each analyte. Mean and standard deviation (S.D.) were obtained for calculated drug concentration at each level. Accuracy and precision were evaluated in terms of relative error (RE) and %CV, respectively.

Recovery presents the extraction efficiency of a method. It was performed at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing peak area of extracted samples (spiked before extraction) to that of unextracted samples (quality control working solutions spiked in extracted plasma). The absolute recoveries were calculated by comparing the peak area of extracted samples to that of aqueous standards at equivalent concentrations.

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample handling and analysis. Stock solution stability was performed by comparing area response of stability

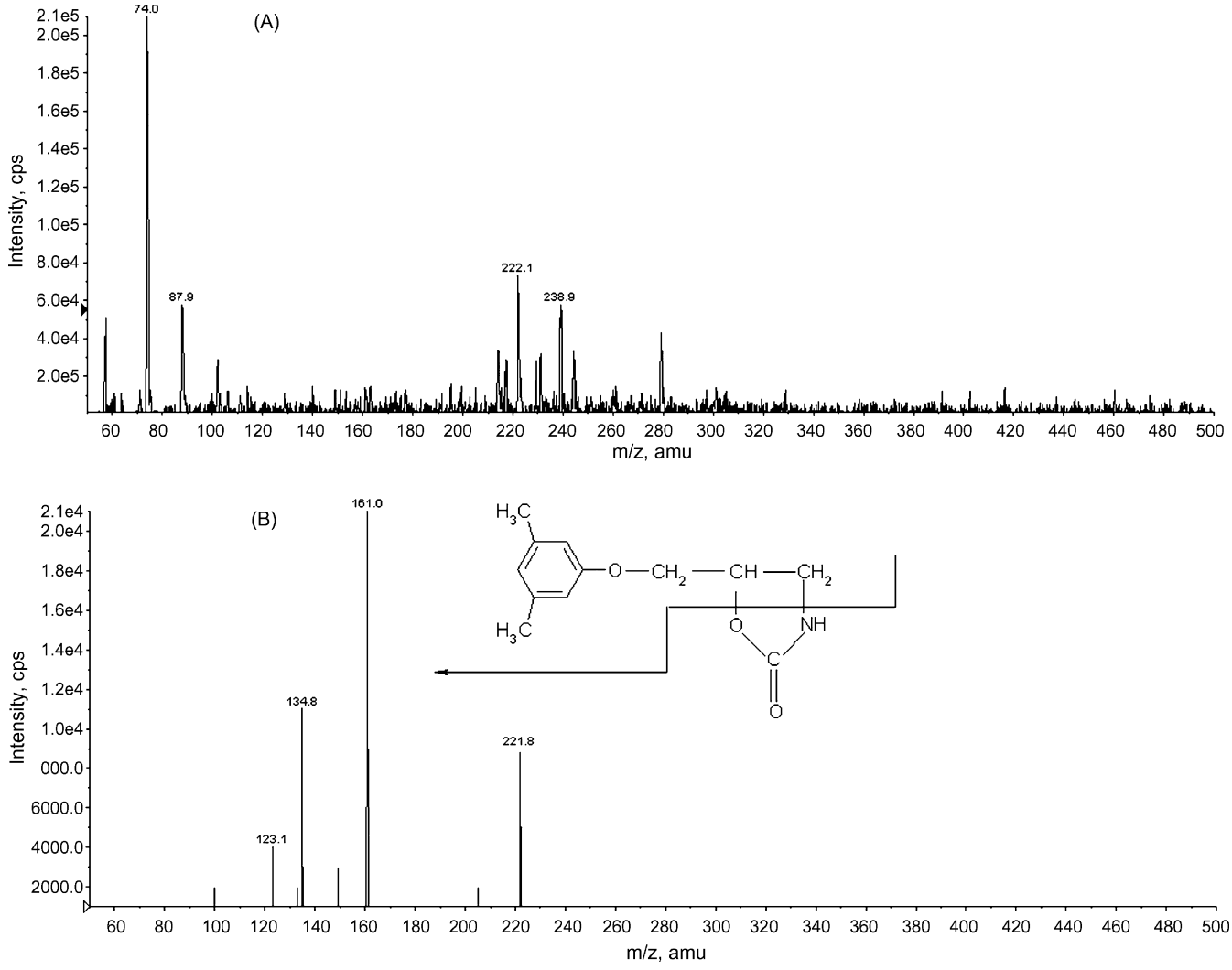

Fig. 4. Parent (A) and product ion (B) mass spectra of metaxalone (IS).

sample of analytes and internal standard with the area response of sample prepared from fresh stock solutions. Bench top stability, extracted sample stability (process stability), freeze–thaw stability and long-term stability were performed at LQC and HQC level using six replicates at each level.

Ion suppression/enhancement due to matrix was checked using three different lots of blank plasma. Area response of post-extraction spiked samples to that of aqueous standards at equivalent concentrations were compared. To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with six different lots of plasma. These lots of heparinised plasma comprised of: four lots of normal control plasma, one lot of lipemic control plasma and one lot of hemolyzed control plasma. Three replicates each of LQC and HQC were prepared from different lots of plasma (total 36 QC samples) and checked for accuracy in terms of relative error in all the QC samples.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above upper limit of quantification (ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 1.5 times the ULOQ concentration for all the analytes. Six replicates each of 1/2 and 1/4 concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 against the freshly prepared calibration curve.

2.7. Bioequivalence study

The design of study comprised of "A randomized, open label, single dose, two treatment, parallel design bio equivalence study of 3TC (60 mg), d4T (12 mg) and NVP (100 mg) dispersible tablet under fasting condition". All subjects were informed of the aim and risk involved in the study and informed consent were obtained. Ethics committee approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [43]. Health check up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. All subjects were negative for HIV, HBSAg and HSV tests. Subjects were

orally administered a single dose with 240 mL of water. Drinking water was not allowed and supine position was restricted 2 h post-dose. Standardize meals were provided as per schedule. Blood samples were collected in tubes containing heparin before and after 00.20, 00.40, 00.60, 00.80, 01.00, 01.25, 01.50, 01.75, 02.00, 02.50, 03.00, 03.50, 04.00, 04.50, 05.00, 06.00, 08.00, 12.00, 16.00, 24.00, 48.00, 72.00, 96.00, 120.00, 168.00, 216.00, 264.00 and 312.00 h of administration of drug. Blood samples were centrifuged at 3200 rpm for 10 min and plasma was separated, stored at −20 °C until use.

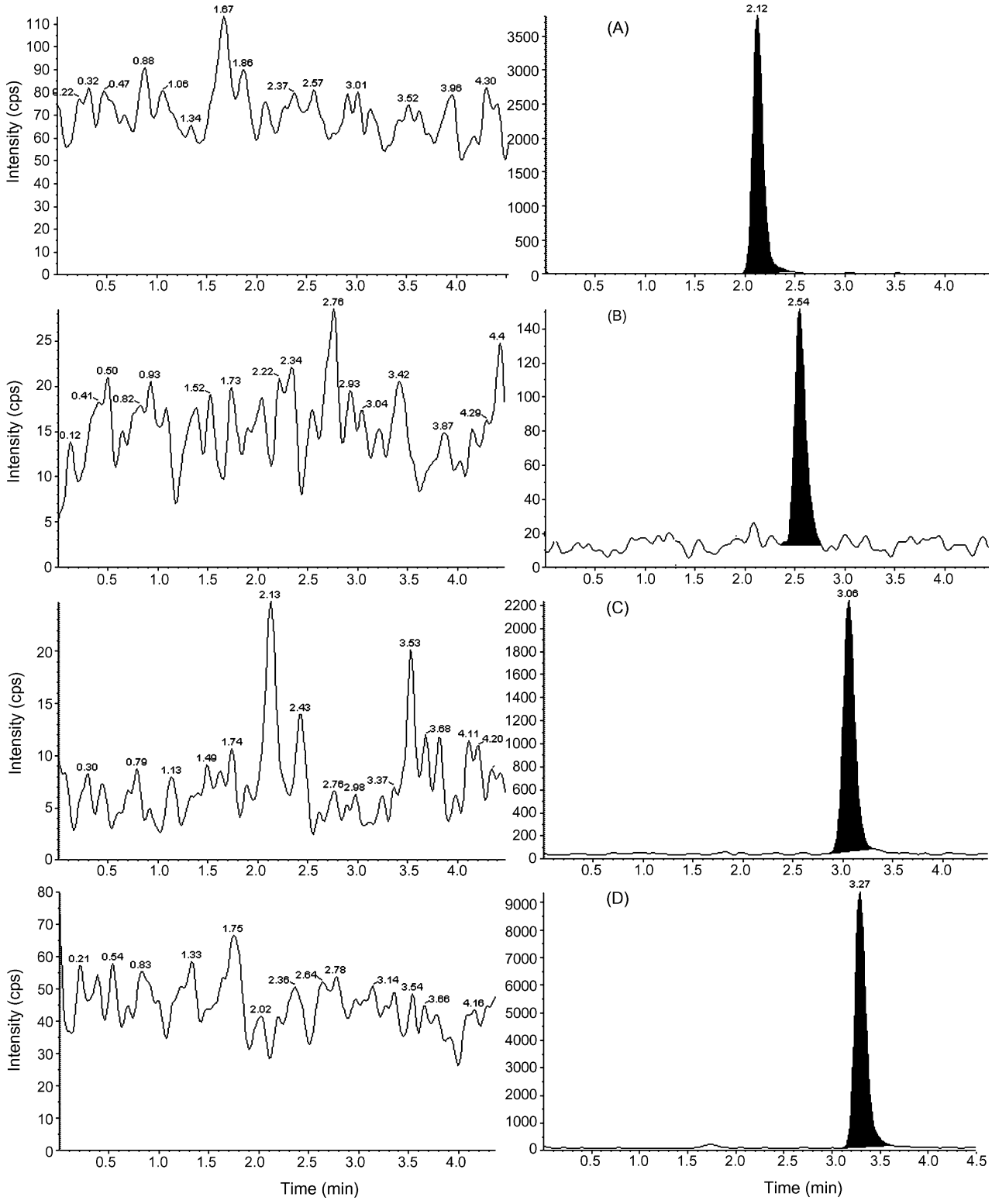

Fig. 5. Representative chromatograms of 3TC (A), d4T (B), NVP (C) and IS (D) at LLOQ with their respective blank plasma.

3. Results and discussion

3.1. Method development

For optimum detection and simultaneous quantification of nevirapine, lamivudine and stavudine with IS in human plasma, it was necessary to adjust not only the chromatographic conditions and mass parameters but also to develop an efficient extraction method that gives consistent and reproducible recovery of analytes from plasma. Parent ions and product ions were optimized by infusing 500 ng/mL solutions into mass spectrometer in 5–500 *m/z* range, both in positive and negative polarity modes using electro spray ionization technique. Best intensity for $[M+H]^+$ ions was found in positive mode for all three analytes including IS as they have an ability to accept protons. Addition of acid further enhanced the intensity and gave $[M+H]^+$ peaks at *m/z* 230.0 for 3TC, 225.3 for d4T and 267.2 for NVP. Most abundant product ions were obtained at *m/z* 112.1, 127.1 and 226.2 for 3TC, d4T and NVP, respectively, by applying sufficient collision activated dissociation gas and collision energy (CE). Optimization of source temperature and heater gas flow is important as they play an important role in minimizing ion suppression and altering the sensitivity. Increase in source temperature and heater gas above 300 °C and 6000 cm^3/mL, respectively, augmented the intensity for all the analytes except stavudine as it decomposes at high temperature. Thus, APCI source was deliberately avoided as it requires high temperature and voltage. With proper setting of CAD gas, curtain gas and CE, a high signal to noise ratio was obtained for all the analytes even at unit and low resolution for Q1 and Q3, respectively. Minor changes in ion spray voltage and nebuliser gas did not have a marked effect on the signal intensity and were maintained at 5000 V and 12 psi, respectively. A dwell time of 0.2 s was sufficient and no cross-talk was found between all MRMs.

Chromatographic analysis of the analytes and IS was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Initially, it was difficult to achieve a good separation of all three analytes under isocratic conditions as lamivudine and stavudine are highly water-soluble compounds while nevirapine is insoluble in water but soluble in acetonitrile. Separation was tried using various combinations of acetonitrile and acidic buffer with varying contents of each component on variety of columns like C8 and C18 Hypersil, hypurity; C18 advance high purity and Symmetry shield RP18 to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. Use of acidic buffers helped in achieving good response for MS detection operating in the positive mode. To get a good chromatographic separation with desired response it was observed that, adjusting the pH of mobile phase as well as selection of column is an important criterion. Thus, a mobile phase consisting of 0.5% acetic acid:acetonitrile (20:80, v/v) and having pH around 4.1 was found suitable as all the analytes were protonated and well separated at this pH. High content of acetonitrile (80%) in the mobile phase helped in eluting all the analytes and IS within 4 min at a flow rate of 0.4 mL/min. Use of formic acid, formate or acetate buffers in place of acetic acid gave good response for nevirapine and lamivudine but not for stavudine. Symmetry shield RP18

Table 1
Summary of calibration curve standards

Analyte	Nominal concentration (ng/mL)	Mean back-calculated concentration (ng/mL)	%CV	RE (%)
Lamivudine	25.0	24.04	1.50	−3.84
	50.0	51.77	2.90	3.53
	100.0	107.06	3.11	7.06
	200.0	206.61	4.82	3.30
	500.0	517.04	4.10	3.41
	1000.0	1033.15	5.52	3.31
	2000.0	1856.94	2.94	−7.15
	3000.0	2735.17	4.03	−8.83
Stavudine	20.0	19.99	1.69	−0.05
	50.0	47.96	9.79	−4.09
	100.0	98.12	3.74	−1.88
	200.0	201.51	3.56	0.76
	500.0	512.37	3.05	2.47
	1000.0	1040.86	3.29	4.09
	1500.0	1466.51	1.85	−2.23
	2000.0	1917.86	4.27	−4.11
Nevirapine	50.0	50.76	2.24	1.52
	100.0	100.41	4.85	0.41
	500.0	510.79	2.30	2.16
	1000.0	999.42	4.26	−0.06
	2000.0	2036.02	2.93	1.80
	3000.0	3158.57	2.49	5.29
	4000.0	3955.30	2.65	−1.12
	5000.0	4979.74	2.49	−0.41

CV: coefficient of variation; RE: relative error.

Table 2
Intra-batch and inter-batch precision and accuracy

Analyte	Level	Concentration added (ng/mL)	Intra-batch			Inter-batch		
			Mean concentration found[a] (ng/mL)	RE (%)	%CV	Mean concentration found[b] (ng/mL)	RE (%)	%CV
Lamivudine	LLOQ	25	24.68	−1.3	2.28	24.71	−1.15	3.82
	LQC	75	78.48	4.64	2.96	79.98	6.64	4.60
	MQC	1500	1531.40	2.09	2.19	1465.35	−2.31	5.21
	HQC	2650	2453.29	−7.42	2.24	2426.46	−8.44	6.62
Stavudine	LLOQ	20	18.86	−5.7	3.48	20.24	1.19	9.23
	LQC	60	56.55	−5.76	6.27	61.12	1.86	9.14
	MQC	750	783.24	4.43	2.87	765.73	2.10	4.62
	HQC	1800	1684.50	−6.42	3.52	1731.87	−3.74	7.60
Nevirapine	LLOQ	50	50.97	1.93	4.15	49.77	−0.45	6.88
	LQC	150	147.60	−1.60	3.21	146.94	−2.04	6.75
	MQC	2500	2694.59	7.78	2.72	2605.43	4.22	4.84
	HQC	4400	4279.51	−2.74	2.35	4449.71	1.13	6.67

RE: relative error; CV: coefficient of variance.
[a] Mean of six replicates at each concentration.
[b] Mean of 24 replicates over 3 different analytical runs.

(150 mm × 3.9 mm, 5 μm particle size) column gave good peak shape and response even at LLOQ level for all the analytes including IS. During run, droplets were observed on curtain plate due to low source temperature (300 °C) as it was not sufficient to evaporate solvent molecules. Moreover, raising the temperature or increasing the heater gas resulted in loss of stavudine peak. Thus, to compensate for this effect, a post-column splitter was attached to divert 50% of the eluate to waste. Low injection volume of 5 μL reduced overloading of column with analytes, thereby ensuring more number of analyses on the same column.

For simultaneous extraction of 3TC, d4T, NVP and IS with quantitative recovery and negligible matrix effect from plasma samples, an efficient extraction method was mandatory. All three extraction methodologies were tried namely, liquid–liquid extraction (LLE), protein precipitation (PP) and solid phase extraction (SPE). Due to lower values of distribution coefficient (log *P*) for 3TC and d4T owing to their high water solubility, it was difficult to obtain sufficient recovery for all the analytes using LLE. Liquid–liquid extraction was tried using different solvents like dichloromethane (DCM), diethyl ether (DEE), hexane, methyl *tert*-butyl ether (MTBE) and isopropyl alcohol (IPA). Relative recovery found in DCM and DEE was less than 25% for 3TC and 4dT and 40% for NVP. Recovery was higher in hexane for nevirapine (65%) but less than 15% for 3TC and 4dT. Combination of DCM and DEE was tried with vary proportion of IPA (5–10%, v/v) but the recovery was not quantitative. In addition, ion suppression found was higher which resulted in low absolute recovery.

Protein precipitation method was not found suitable as it gave very low recoveries with frequent clogging of column. It was tried with mobile phase, acetonitrile and methanol. Though the recovery was better compared to LLE, but was not consistent even for six consecutive injections of same concentration for all the analytes. Relative recovery found was 72% for 3TC, 65% for 4dT and 80% for NVP. Moreover, ion suppression was found at the retention time of analytes.

Table 3
Absolute and relative recovery for lamivudine, stavudine and nevirapine

Analyte	*A*	*B*	*C*	Absolute (%*D*)	Relative (%*E*)
LQC					
Lamivudine	225,865	218,076	197,948	87.64	90.85
Stavudine	4,913	4,731	4,608	93.80	97.90
Nevirapine	124,773	120,998	120,344	96.45	99.50
MQC					
Lamivudine	4,212,070	4,065,346	3,388,189	80.44	83.39
Stavudine	64,863	62,736	54,388	83.85	86.94
Nevirapine	2,246,063	2,175,301	2,052,452	91.38	94.36
HQC					
Lamivudine	7,122,582	6,855,708	5,871,857	82.44	85.64
Stavudine	151,431	145,793	126,899	83.80	87.24
Nevirapine	3,807,209	3,673,605	3,673,195	96.48	100.01

A: area response of the samples prepared in mobile phase (aqueous sample); *B*: area response of the samples prepared by spiking externally in extracted blank plasma; *C*: area response of the extracted samples; *D*: (*C*/*A*) × 100; *E*: (*C*/*B*) × 100.

Thus, a simple and rapid solid phase extraction method was developed using Strata X, 30 mg; 1 mL solid phase extraction (SPE) cartridges. Extraction of analytes was carried out with 1 mL of mobile phase and the eluate was directly used for injection without drying and reconstitution. SPE method gave quantitative recovery and higher selectivity compared to LLE and PP. Ion suppression was minor (3–4%) and no interference was observed at the retention time of analytes and IS. It was possible to increase the sensitivity (lower LLOQ) with improved response by drying and reconstitution but was deliberately avoided as it would make the method time consuming and cumbersome.

It was difficult to find a compound which could ideally mirror the analytes to serve as a good IS. Several compounds were investigated to find a suitable IS, and finally metaxalone though belonging to a different class of compounds was found most appropriate for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. The results of method validation using metaxalone as the IS were acceptable in this study based on FDA guidelines.

3.2. Selectivity and sensitivity (LLOQ)

The selectivity of the method towards endogenous plasma matrix was evaluated in 10 different batches of human plasma by analyzing blanks and spiked samples at LLOQ levels. Endogenous peaks at the retention time of the analytes were not observed for any of the plasma batches. Fig. 5 demonstrates the selectivity results with the chromatograms of blank plasma and the peak response of analytes with IS at LLOQ level. The response was calculated in terms of signal to noise (S/N) ratio for spiked and unspiked plasmas. The mean S/N ratio for 10 plasma samples found was 87.15, 1115.31 and 15.18 for NVP, 3TC and d4T, respectively. The mean accuracy (%) for back-calculated concentration of all three analytes was within 98–105 with %CV between 6.14 and 12.38. The retention times for NVP, 3TC, d4T and IS were 2.12, 2.54, 3.06 and 3.27, respectively.

3.3. Linearity, accuracy and precision, recovery

Calibration curves were linear from 25 to 3000 ng/mL with correlation coefficient, $r \geq 0.9968$ for 3TC, from 20 to 2000 ng/mL with $r \geq 0.9986$ for d4T and from 50 to 5000 ng/mL with $r \geq 0.9992$ for NVP. The r values, slopes and intercepts were calculated using linear regression ($1/x^2$) weighing analysis. The observed mean back-calculated concentration with accuracy (%) and precision (%CV) of 11 linearities are given in Table 1.

For inter-batch, four runs and for intra-batch, a single run was assayed. Each run consisted of six replicates at four concentration levels (LLOQ, LQC, MQC and HQC). Intra-batch and inter-batch precision was less than 7% for NVP and 3TC while it was less than 10% for d4T. Accuracy was within 92–108%

Table 4
Stability results for lamivudine, stavudine and nevirapine

Stability	Analyte	Level	*A*	%CV	*B*	%CV	%Change
Bench top (21 h at room temperature)	Lamivudine	LQC	81.36	2.85	79.64	3.97	−2.12
		HQC	2565.97	7.27	2612.55	3.53	1.82
	Stavudine	LQC	66.65	8.17	65.71	5.91	−1.42
		HQC	1826.27	6.97	1872.73	2.71	2.54
	Nevirapine	LQC	154.78	0.89	151.20	3.40	−2.31
		HQC	4801.49	4.95	4882.10	2.31	1.68
Autosampler (39 h, 10 °C)	Lamivudine	LQC	76.68	6.76	80.51	5.10	4.99
		HQC	2284.32	4.22	2269.68	1.70	−0.64
	Stavudine	LQC	58.31	5.43	57.20	7.79	−1.90
		HQC	1582.94	5.43	1647.73	2.15	4.09
	Nevirapine	LQC	135.04	4.63	141.76	3.69	4.98
		HQC	4134.16	4.72	4200.20	3.03	1.60
5th freeze–thaw cycle	Lamivudine	LQC	79.02	1.09	86.50	4.48	9.47
		HQC	2501.20	1.97	2551.70	3.05	2.02
	Stavudine	LQC	61.90	10.05	55.65	7.21	−10.09
		HQC	1785.09	1.82	1637.96	4.03	−8.24
	Nevirapine	LQC	148.53	3.11	146.24	3.78	−1.55
		HQC	4538.66	3.53	4464.05	3.16	−1.64
Long-term (98 days, −20 °C)	Lamivudine	LQC	78.48	2.96	78.19	3.72	−0.37
		HQC	2453.29	2.24	2457.73	3.07	0.18
	Stavudine	LQC	56.55	6.27	58.19	5.73	2.90
		HQC	1684.50	3.52	1514.83	4.61	−10.01
	Nevirapine	LQC	147.60	6.27	163.15	3.53	10.53
		HQC	4279.51	3.52	4081.92	4.63	−4.62

A: comparison sample concentration (ng/mL); *B*: stability sample concentration (ng/mL); CV: coefficient of variation.

Table 5a
Matrix effect in different lots of plasma

Analyte	Lot-1		Lot-2		Lot-3		Lot-4		Lot-5		Lot-6	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Lamivudine												
LQC	80.19	6.92	77.98	3.97	79.21	5.61	81.64	8.85	77.96	3.95	73.98	−1.36
HQC	2513.45	−5.15	2421.93	−8.61	2448.47	−7.60	2643.31	−0.25	2411.48	−9.00	2505.06	−5.47
Stavudine												
LQC	53.99	−10.02	59.26	−1.33	56.36	−6.07	52.27	−12.89	57.08	−4.86	56.33	−6.12
HQC	1734.73	−3.63	1690.92	−6.06	1703.78	−5.35	1872.87	4.05	1777.82	−1.33	1847.39	2.63
Nevirapine												
LQC	149.82	−0.12	151.64	1.09	151.32	0.88	152.04	1.36	145.26	−3.16	145.79	−2.81
HQC	4483.39	1.90	4377.40	−0.51	4403.16	0.07	4649.95	5.68	4371.39	−0.65	4508.01	2.45

A: mean calculated concentration (ng/mL); *B*: relative error (%).

Table 5b
Evaluation of matrix effect for ion suppression

Analyte	*A*	*B*			*%C*		
		Lot-1	Lot-2	Lot-3	Lot-1	Lot-2	Lot-3
Lamivudine	213,490	206,225	207,007	208,204	96.60	96.96	97.52
Stavudine	5,022	4,894	4,781	4,825	97.45	95.20	96.08
Nevirapine	6,987,597	6,809,127	6,789,927	6,866,612	97.44	97.17	98.27

A: area response of the sample prepared in mobile phase (aqueous sample); *B*: area response of the samples prepared by spiking externally into extracted blank plasma; *C*: $(B/A) \times 100$.

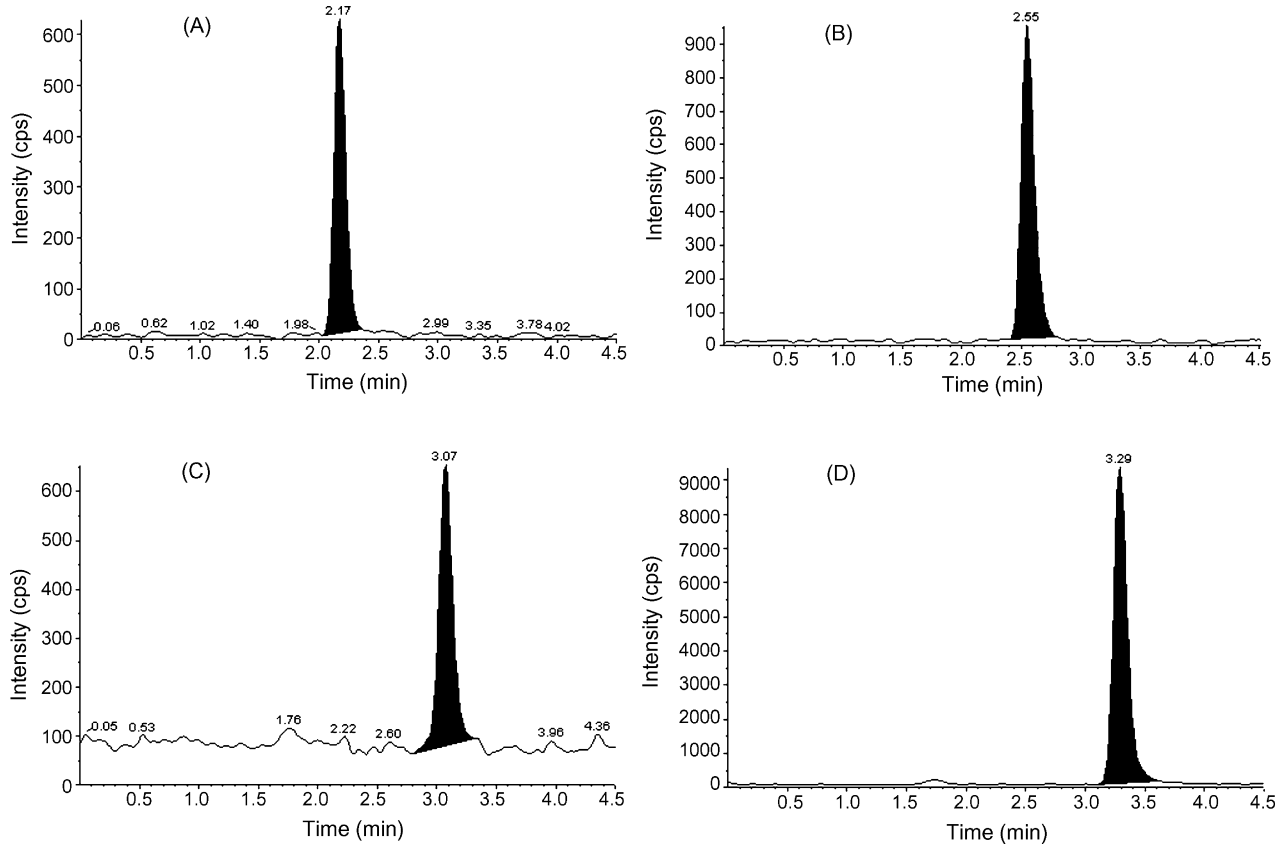

Fig. 6. Chromatograms for 3TC (A), d4T (B), NVP (C) and IS (D) of real subject sample.

for all analytes of their nominal concentration, respectively, as given in Table 2.

Six replicates at LQC, MQC and HQC level were prepared for recovery determination. Mean relative recovery found was 86.63, 90.69 and 97.95% with a precision (%CV) of 4.42, 6.88 and 3.19 for 3TC, d4T and NVP, respectively. Recovery of IS was 98.12% with %CV of 4.80. The data for absolute and relative recovery are given in Table 3. This indicates that the extraction efficiency for all the analytes as well as IS was consistent and reproducible.

3.4. Stability, matrix effect and dilution integrity

Stock solution of all three analytes and IS were stable at room temperature for 24 h and at 2–8 °C for 17 days. 3TC, d4T and NVP in control human plasma at room temperature were stable at least for 21 h and for minimum of five freeze and thaw cycles. Process stability was of 39 h at 10 °C. Spiked plasma samples stored at −20 °C for long-term stability experiment were stable for minimum 98 days. Different stability experiments in plasma and the values for the precision and percent change are shown in Table 4. There was no significant degradation observed since the deviations in concentration was within 15% of their nominal values.

Matrix effect is due to co elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS/MS method for supporting pharmacokinetics studies. It was performed with the aim to see the matrix effect of different lots of plasma on the back-calculated value of QC's nominal concentration. To assess the suppression or enhancement of analyte signals due to matrix, three different lots of blank plasma were extracted and then spiked with each analyte and IS. The corresponding peak areas were then compared to those of the aqueous standards at equivalent concentrations. Peak area comparison showed that there was a slight decrease in the area of these analytes in post-extraction spiked plasma samples. This indicates the matrix effect on the ionization of these analytes resulting in minor suppression. Moreover, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analytes and IS peak. The results found were well within the acceptable limits as shown in Tables 5a and 5b. Thus, the extraction method was rugged enough and gave accurate and consistent results when applied to real patient samples.

The mean back-calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal. The coefficient of variation (%CV) for 1/2 and 1/4 dilution samples of 3TC, d4T and NVP were less than 7.0%.

3.5. Application of the method on healthy human volunteers

The proposed validated method was successfully applied to a pivotal bioequivalence study in 60 healthy human male subject samples for reference and test formulations of 3TC (60 mg), d4T (12 mg) and NVP (100 mg) dispersible tablet under fasted condition. All 1740 samples including the calibration, QC and volunteer samples were run and analyzed in only 16 days and precision and accuracy for calibration and QC samples were within acceptable limits. The chromatograms for 3TC, 4dT and NVP in real subject samples are presented in Fig. 6. The 90% confidence interval of the individual ratio geometric mean for test/reference was within 80–125% for $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ and C_{max} (AUC: area under curve, C_{max}: peak plasma concentration). Mean plasma concentration versus time profiles for 3TC, d4T and NVP under fasted condition are presented in Fig. 7.

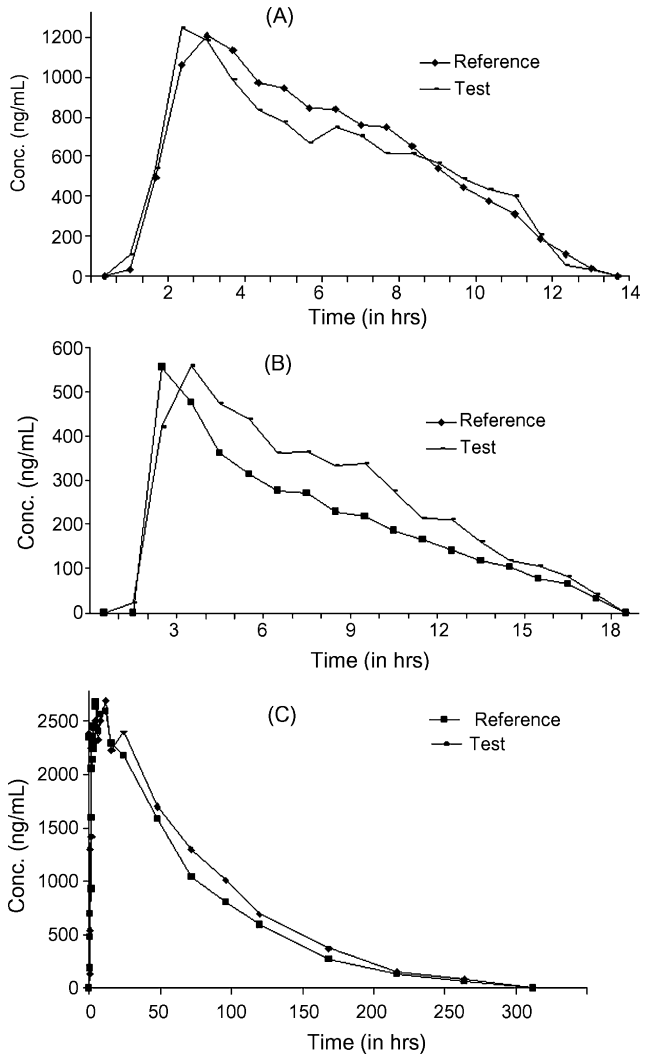

Fig. 7. Mean plasma concentration-time profile combined dosage of 3TC (60 mg) (A), d4T (12 mg) (B) and NVP (100 mg) (C) after oral administration to 60 healthy Indian male volunteers under fasting condition.

4. Conclusion

The developed LC–MS/MS assay for NVP, 3TC and d4T is selective, rugged and suitable for routine measurement of subject samples. This method has significant advantages in terms of clean and reproducible SPE extraction procedure and a short

chromatographic run time of 4.5 min. The extraction method gave consistent and reproducible recoveries for analytes and IS from plasma, with minimum matrix interference and ion suppression. The SPE eluate (5 μL) is directly submitted for LC–MS analysis without drying and reconstitution to give high throughput. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with test formulation of 3TC, d4T and NVP.

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