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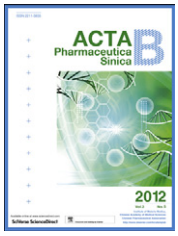

ORIGINAL ARTICLE

Simultaneous quantitation of lamivudine, zidovudine and nevirapine in human plasma by liquid chromatography–tandem mass spectrometry and application to a pharmacokinetic study

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KEY WORDS

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Pharmacokinetics

Abstract A rapid and sensitive LC–MS/MS method for the simultaneous quantitation of lamivudine, zidovudine and nevirapine in human plasma using abacavir as internal standard has been developed and validated. The analytes and IS were extracted from plasma by solid phase extraction using Oasis HLB cartridges and separated on a Hypurity Advance C18 column using a mixture of acetonitrile:0.1% formic acid (76:24, *v*/*v*) at a flow rate of 0.8 mL/min. Detection involved an API-4000 LC–MS/MS with electrospray ionization in the positive ion mode and multiple-reaction monitoring for analysis. The method was validated according to FDA guidelines and shown to provide intra- and inter-day precision and accuracy within acceptable limits in a run time of only 3.5 min. The method was successfully applied to a pharmacokinetic study involving a single oral administration of a combination tablet to human male volunteers.

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1. Introduction

Antiretroviral drugs are used to treat infections by retroviruses, primarily the human immunodeficiency virus (HIV). The aim of antiretroviral treatment is to maintain HIV at a low level in the body. Since single drug therapy rapidly becomes ineffective due to the development of HIV resistant strains, the new paradigm is to combine two to four antiretroviral drugs in what is called highly active antiretroviral therapy (HAART)[1,2]. The synergistic action of different classes of antiretroviral drugs prolongs the survival of HIV patients such that combination therapy is now considered first-line therapy.

Current treatment guidelines state that a combination antiretroviral regimen should contain at least two nucleoside analog reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) in a fixed dose combination[3]. One such combination product is Lazid-N (Emcure Pharmaceuticals, Pune, India) which is a combination of lamivudine, zidovudine and nevirapine in a single pill. Lamivudine (2′-deoxy-3′-thiacytidine) and zidovudine (3′-azido-3′-deoxythymidine) are NRTIs whereas nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6*H*- dipyrido[3, 2-b: 2′, 3′-e][1,4] diazepin-6-one) is a highly potent noncompetitive NNRTI. The combination is considered to be one of the best choices for the treatment of HIV.

According to the literatures, several LC–MS/MS[4–10] methods have been reported for the determination of lamivudine, zidovudine and nevirapine in biological samples either individually or simultaneously with other drugs. However, only one LC–MS/MS method[11] has been reported for the simultaneous determination of the three drugs in human plasma but it analyses the three drugs in combination with fourteen other antiretroviral drugs. As expected, the method suffers from various deficiencies in respect of the three drugs in question including the need for complex sample preparation, gradient elution and polarity switching. Thus the aim of the present study was to develop and validate a specific, sensitive and high throughput LC–MS/MS method for the simultaneous determination of lamivudine, zidovudine and nevirapine in human plasma. The validated method was applied to a clinical pharmacokinetic study involving oral administration of a combination of the three drugs to healthy male volunteers.

2. Material and methods

2.1. Chemicals and reagents

Reference standards of lamivudine (99.7%), zidovudine (99.1%), nevirapine (100.1%) and abacavir (99.2%, used as internal standard, IS) were obtained from Aurobindo Pharmaceuticals Ltd. (Hyderabad, India). Their chemical structures are presented in Fig. 1. Co-administration of Combivir® tablet containing lamivudine 150 mg and zidovudine 300 mg (Glaxo SmithKline, USA) and Viramune® tablet containing Nevirapine 200 mg (Boehringer Ingelheim Pharmaceuticals, Inc., USA). Water was prepared using a Milli Q water purification system from Millipore (Bangalore, India). Acetonitrile and methanol (HPLC grade) were purchased from J.T. Baker (Phillipsburg, USA). Extra pure formic acid was purchased from Fluka (Steinheim, Germany). Oasis HLB cartridges were obtained from Waters (Massachusetts, USA). Blank drug free human plasma was procured from Deccan's Pathological Laboratories (Hyderabad, India).

2.2. Instrumentation and chromatographic conditions

Chromatographic separation was carried out on a Shimadzu HPLC with a Hypurity advance C18 column (50 mm × 4.6 mm, 5 μm; Thermo Scientific Ltd., Mumbai, India) and a mobile phase consisting of acetonitrile:0.1% formic acid (74:26, *v*/*v*) delivered at a flow rate of 0.8 mL/min. The injection volume was 5 μL. Quantitation was achieved in a run time of 3.5 min by MS/MS detection in the positive ion mode using an MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray™ interface at 600 °C and ion spray voltage set at 5500 V. Source parameters *viz*. nebulizer gas (GS1), auxiliary gas (GS2), curtain gas (CUR) and collision gas (CAD) were set at 35, 35, 20 and 6 psi, respectively. Compound parameters *viz*. declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were respectively 36, 16, 10 and 6 V for lamivudine, 70, 44, 10 and 22 V for zidouvdine, 36, 11, 10 and 6 V for nevirapine and 36, 28, 10 and 6 V for IS. Detection was carried out by multiple-reaction monitoring (MRM) of the transitions (precursor ion

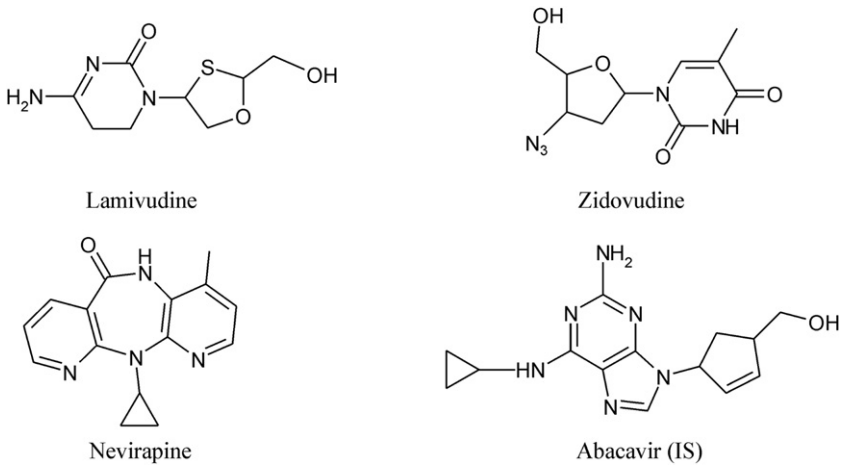

Figure 1 Chemical structures of lamivudine, zidovudine, nevirapine and abacavir (IS).

to product ion) at m/z 230.3→112.1 for lamivudine, m/z 268.1→127.1 for zidouvdine, m/z 267.2→226.2 for nevirapine and m/z 287.2→191.2 for IS. Quadrupoles Q1 and Q3 were set on unit resolution. Analytical data were processed by Analyst Software™ (version 1.4.2). Fragmentation patterns were similar to those previously reported for lamivudine[12] zidovudine[12], nevirapine[13] and IS[14].

2.3. Preparation of calibration standards and quality control samples

Stock solutions (1 mg/mL) of lamivudine, zidovudine, nevirapine and IS were prepared in methanol and diluted with 60:40 (*v*/*v*) methanol:water to produce standard solutions. Nine mixed calibration standards were then prepared by spiking blank K_2-EDTA plasma with appropriate volumes of the standard solutions to give respective final concentrations of 25, 50, 100, 301, 804, 1609, 2414, 3018 and 4024 ng/mL for lamivudine, 25, 50, 100, 301, 804, 1608, 2413, 3016 and 4022 ng/mL for zidovudine and 81, 162, 255, 506, 1013, 2026, 3242, 4559 and 6079 ng/mL for nevirapine. Four mixed quality control (QC) samples (LLOQ, LQQ, MQC and HQC) were prepared independently in the same way with respective concentrations of 25, 75, 1913 and 3223 ng/mL for lamivudine, 25, 75, 1910 and 3217 ng/mL for zidovudine and 81, 243, 3037 and 5063 ng/mL for nevirapine. All validation samples were stored at −70 °C until use.

2.4. Sample preparation

To an aliquot of human plasma (100 µL) was added 25 µL IS working solution (500 ng/mL abacavir) and 100 µL 0.5 M potassium phosphate buffer (pH 7.4) and the mixture vortexed for 10 s. The mixture was then loaded onto a HLB cartridge (30 mg/mL) pre-conditioned with 1.0 mL methanol followed by 1.0 mL water. The cartridge was washed with 1.0 mL water followed by 1.0 mL 5% methanol in water after which analytes and IS were eluted with 1.0 mL 0.5% formic acid in methanol. The eluate was subsequently injected into the LC–MS/MS system.

2.5. Assay validation

Validation parameters *viz*. selectivity, specificity, matrix effects, linearity, precision, accuracy, recovery, dilution

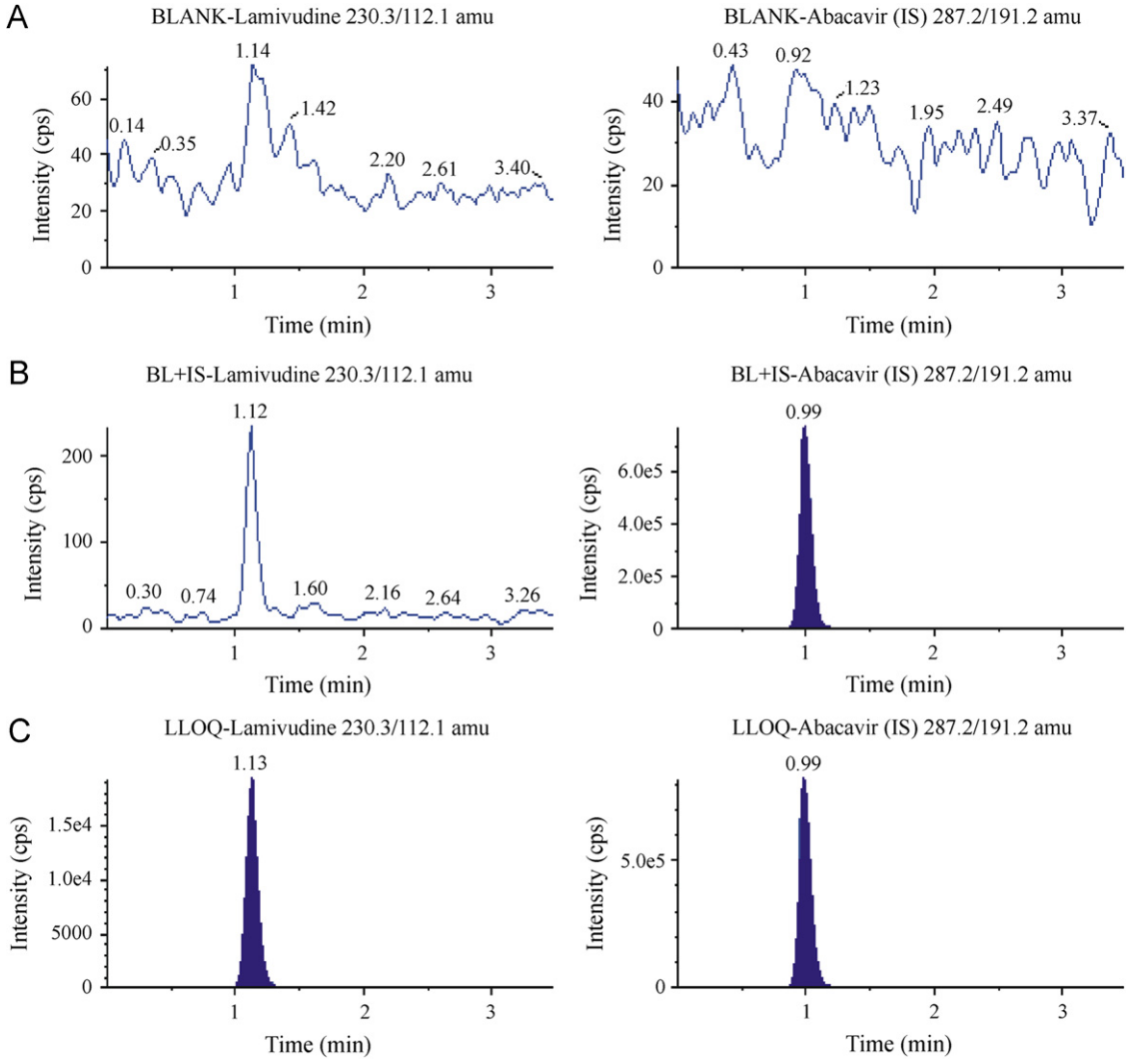

Figure 2 Typical MRM chromatograms of lamivudine (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS, and (C) an LLOQ sample along with IS.

integrity and stability were evaluated according to US FDA guidelines[15]. Selectivity was assessed by comparing the chromatograms of blank plasma samples from six different individuals analyzed alone and after spiking at the respective LLOQ concentrations. Matrix effects were checked by analysis in triplicate of LQC and HQC samples prepared from six different lots of plasma. Linearity was assessed by linear weighted least squares regression of calibration curves constructed using the nine mixed calibration standards and blank plasma samples. Intra- and inter-day precision and accuracy were determined by analyzing six replicates of QC samples on two different days and on four different days respectively. Recoveries of analytes and IS were determined by comparing peak areas of extracted QC samples (LQC, MQC and HQC) with peak areas of non-extracted standards. Recovery of IS was determined at a concentration of 500 ng/mL. Dilution integrity was assessed by analysis of six replicates of a sample containing 1.5 times the maximum calibration standard concentration diluted two- and four-fold with blank plasma.

Stability was determined by comparing peak areas of analytes with those of fresh samples for stock solutions at room temperature and under refrigerated conditions (2–8 °C) and for prepared samples in the autosampler for 30 h. Stability was also determined using LQC and HQC samples stored on the bench for 10 h, on dry ice for 10 h, subjected to four freeze–thaw cycles and at −70 °C for 60 days. In all cases six replicates were analyzed. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (85–115%) and precision (≤15% RSD).

2.6. *Pharmacokinetic study*

The protocol of a pharmacokinetic study in healthy male subjects ($n=6$) was approved by Hyderabad Independent Ethics Committee (Hyderabad, India) and the volunteers provided informed written consent. Blood samples were collected in K_2-EDTA Vacutainers (BD, Franklin, NJ, USA) pre-dose and following oral administration of a combination tablet containing lamivudine (150 mg), zidovudine (300 mg) and nevirapine (200 mg) at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 24, 36, 48 and 72 h. Plasma was collected after centrifugation at 3200 rpm for 10 min and

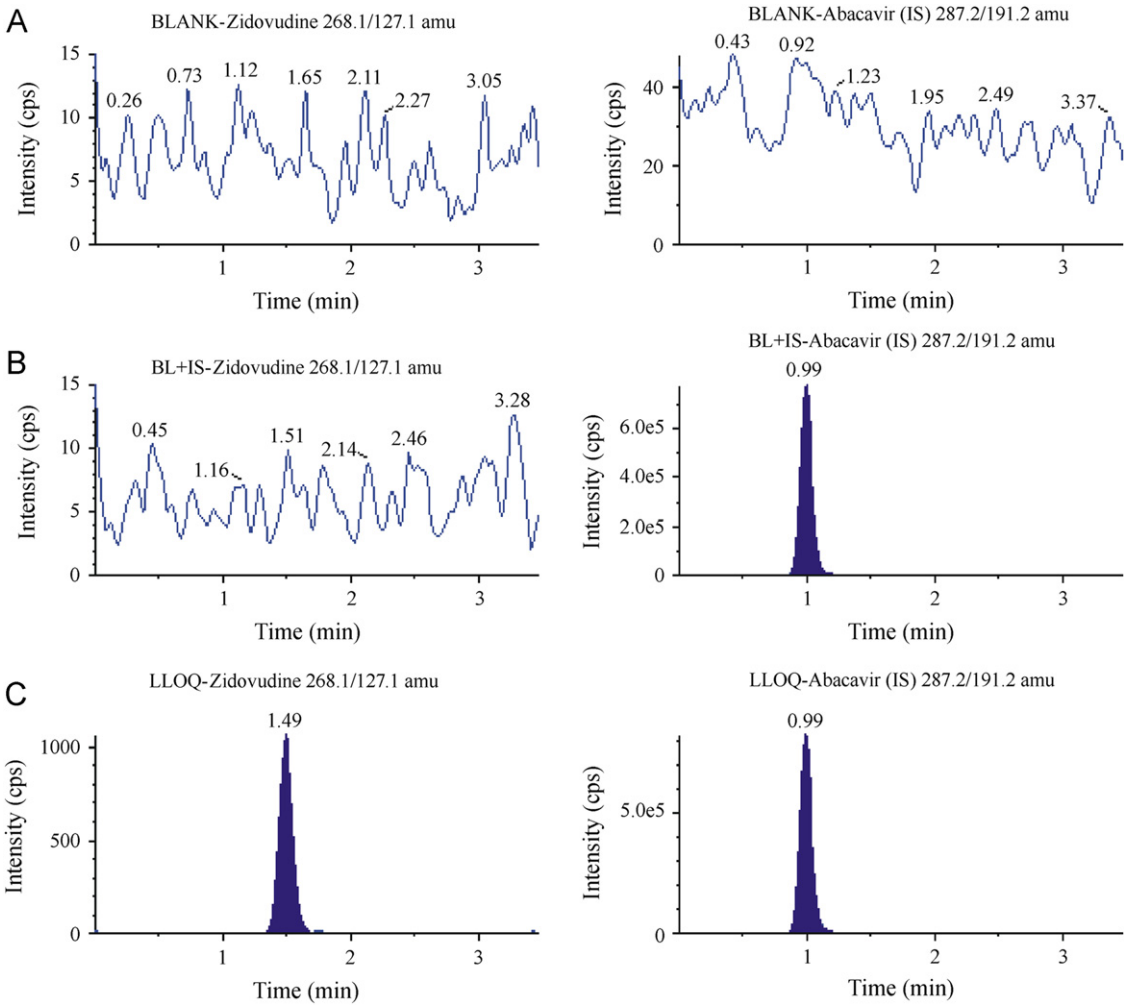

Figure 3 Typical MRM chromatograms of zidovudine (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS, and (C) an LLOQ sample along with IS.

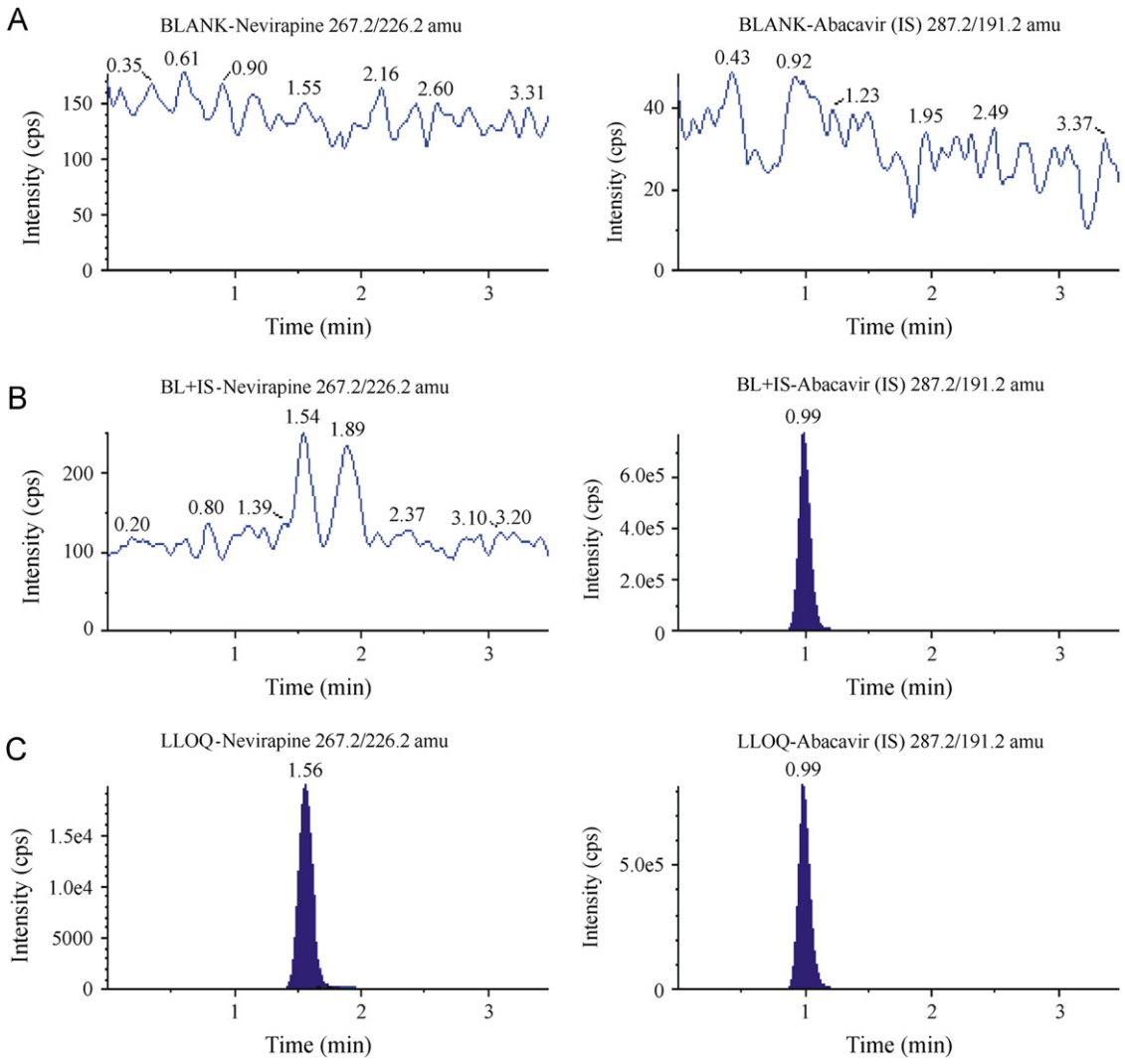

Figure 4 Typical MRM chromatograms of nevirapine (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS, and (C) an LLOQ sample along with IS (C).

Table 1 Precision and accuracy for lamivudine, zidovudine and nevirapine in human plasma samples.

Analyte	Concentration added (ng/mL)	Intra-day ($n=12$; 6 from each batch)			Inter-day ($n=24$; 6 from each batch)		
		Concentration found (mean, ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean, ng/mL)	Precision (%)	Accuracy (%)
Lamivudine	25.4	23.5	8.8	92.8	23.5	6.0	92.5
	75.8	75.9	7.9	100.2	74.9	7.0	98.9
	1913.9	1799.0	5.3	94.0	1798.9	4.8	94.0
	3223.4	3182.9	3.1	98.8	3169.7	4.2	98.3
Zidovudine	25.3	28.2	2.6	111.2	27.6	3.4	109.1
	75.6	78.8	8.1	104.2	79.4	7.8	105.0
	1910.5	1858.2	6.6	97.3	1841.7	5.8	96.4
	3217.6	3379.5	2.7	105.0	3326.6	4.1	103.4
Nevirapine	81.1	85.8	3.9	105.8	85.7	3.6	105.7
	243.0	220.3	3.3	90.7	219.1	3.2	90.2
	3037.8	2895.4	5.1	96.4	2821.3	4.5	92.9
	5063.0	4883.2	2.0	96.5	4714.4	4.5	93.1

stored at −70 °C until use. Along with clinical samples, LQC, MQC and HQC samples were assayed in triplicate. The plasma concentration–time profiles of lamivudine, zidovudine and nevirapine were analyzed by a non-compartmental method using WinNonlin Version 5.1.

3. Results and discussion

3.1. Method development

Detection using the positive ionization mode was found to produce a better response than using the negative ionization mode. Sample preparation using a simple solid-phase extraction (SPE) technique was found to provide high recoveries of all analytes. In terms of chromatographic conditions, the presence of a small amount of formic acid in the mobile phase improved the detection of analytes and the Hypurity advance C18 column gave good peak shapes and responses for all analytes and IS even at the LLOQs. Representative chromatograms obtained from blank plasma and plasma spiked with lamivudine, zidovudine and nevirapine at LLOQ are presented in Figs. 2–4, respectively. The short retention times of lamivudine, zidovudine, nevirapine and IS (1.13, 1.49, 1.56 and 0.99 min respectively) allowed a run time of only 3.5 min. The assay was free of interference at the retention time of analytes and IS. Selectivity was also evaluated in the presence of other commonly used anti-retroviral drugs such as tenofovir, lopinavir, ritonavir, stavudine, indanavir and emtricitabine. Again no interference was observed at the retention time of analytes and IS.

3.2. Assay validation

3.2.1. Sensitivity

LLOQs of analytes were set at the lowest concentrations on the standard curves. Precision and accuracy at the LLOQs

Table 2 Stability of lamivudine, zidovudine and nevirapine in plasma samples ($n=6$).

Analyte	Stability test	QC (nominal conc., ng/mL)	Mean ± SD (ng/mL)	Stability (% nominal conc.)	Precision (% CV)
Lamivudine	Autosampler[a]	75.8	76.4 ± 4.4	100.8	5.8
		3223.4	3313.7 ± 230.1	102.8	6.9
	Coolant[b]	75.8	75.3 ± 3.4	99.4	6.7
		3223.4	3289.0 ± 213.6	102.0	4.6
	Bench top[c]	75.8	65.7 ± 3.9	86.9	5.9
		3223.4	3332.1 ± 92.0	103.6	2.8
	FT[d]	75.8	71.9 ± 1.1	94.9	1.6
		3223.4	3067.4 ± 75.3	95.2	2.5
	Long-term[e]	75.8	70.3 ± 2.7	108	3.8
		3223.4	3046.4 ± 79.9	92.8	2.6
Zidovudine	Autosampler[a]	75.6	65.2 ± 1.4	86.2	2.2
		3217.6	3342.7 ± 97.9	103.9	2.9
	Coolant[b]	75.6	68.3 ± 2.2	90.3	3.2
		3217.6	3323.2 ± 61.7	103.3	1.9
	Bench top[c]	75.6	65.7 ± 3.9	86.9	5.9
		3217.6	3332.1 ± 92.0	103.6	2.8
	FT[d]	75.6	65.9 ± 4.3	87.1	6.5
		3217.6	3194.0 ± 126.7	99.3	4.0
	Long-term[e]	75.6	81.0 ± 4.9	107.1	6.0
		3217.6	3542.3 ± 117.0	110.1	3.3
Nevirapine	Autosampler[a]	243.0	233.5 ± 6.0	96.1	2.6
		5063.0	5023.6 ± 47.5	99.2	1.0
	Coolant[b]	243.0	232.2 ± 1.7	95.6	0.7
		5063.0	5000.1 ± 95.7	98.8	1.9
	Bench top[c]	243.0	233.3 ± 4.2	96.0	1.8
		5063.0	4992.4 ± 42.9	98.6	0.9
	FT[d]	243.0	226.3 ± 2.3	93.1	1.0
		5063.0	4746.3 ± 82.1	93.7	1.7
	Long-term[e]	243.0	217.5 ± 11.5	89.5	5.3
		5063.0	4599.9 ± 141.8	90.9	3.1

[a]Extracted samples in autosampler for 30 h at 10 °C;
[b]On dry ice for 10 h;
[c]At room temperature for 10 h;
[d]Four freeze–thaw cycles;
[e]At −70 °C for 60 days.

were respectively 2.50% and 94.80% for lamivudine, 4.64% and 92.49% for zidovudine, and 1.32% and 90.19% for nevirapine.

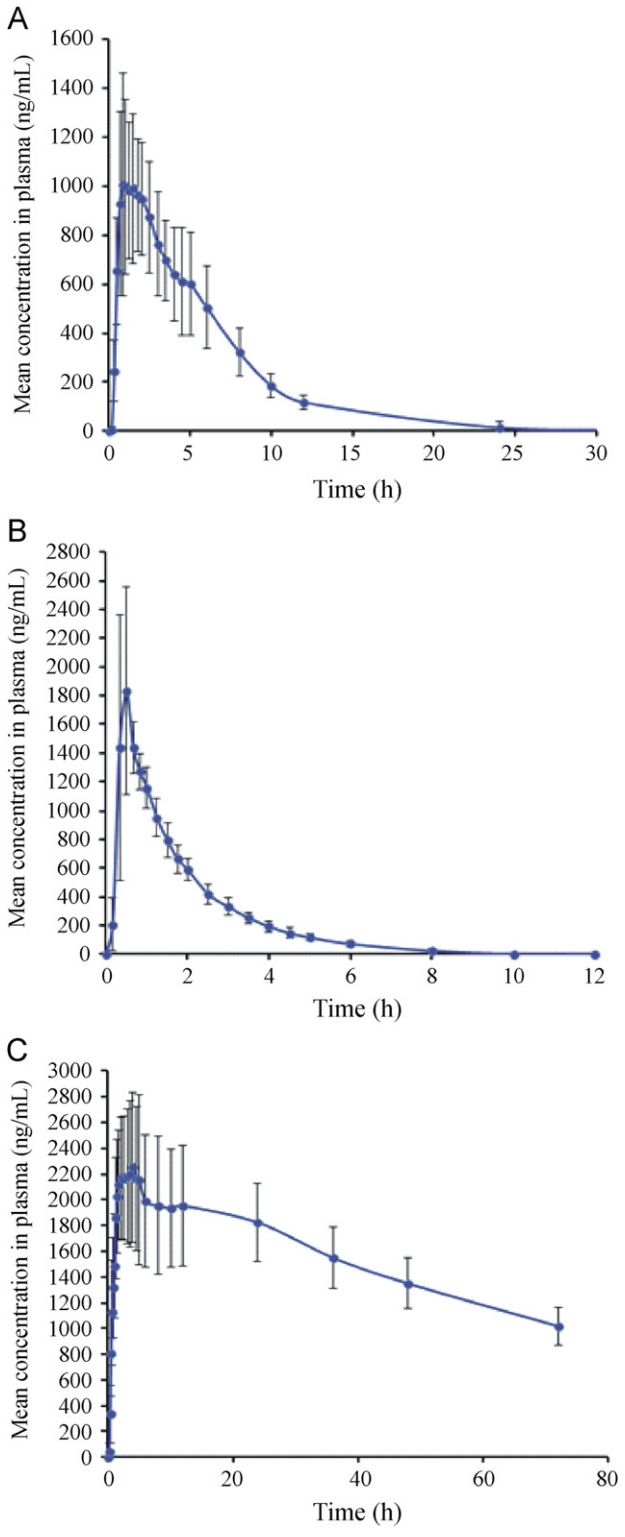

Figure 5 Mean plasma concentration–time profiles of (A) lamivudine, (B) zidovudine and (C) nevirapine in human plasma following oral administration of a combination tablet containing lamivudine (150 mg), zidovudine (300 mg) and nevirapine (200 mg) to healthy male volunteers (data are means ± SD, $n=6$).

3.2.2. *Matrix effects*

No significant matrix effects were observed in the six batches of LQC and HQC samples. Accuracy for analytes in LQC and HQC samples were respectively; 93.89% and 95.62% for lamivudine, 95.58% and 101.33% for zidovudine and 94.64% and 94.39% for nevirapine.

3.2.3. *Linearity*

Calibration curves were found to be linear for all three analytes over their respective concentration ranges. After comparing weighting by $1/x$ and $1/x^2$, a regression equation of analyte to IS concentration with a weighting factor of $1/x^2$ was found to produce the best fit for all analytes (mean correlation coefficient ≥ 0.99).

3.2.4. *Precision and accuracy*

As shown in Table 1, the intra- and inter-day precision for all analytes was in the range 85–115% with accuracy of ±15% at LQC, MQC and HQC concentrations. Precision and accuracy at the LLOQs were in the range 80–120% and ±20%, respectively.

3.2.5. *Extraction efficiency*

Solid phase extraction using HLB cartridges was shown to be robust, provide clean samples and give good and reproducible recoveries of all analytes and IS. The mean overall recoveries across QC levels (with precision) were 83.57±5.05% (2.45–4.84%) for lamivudine, 92.26±5.01% (2.55–7.19%) for zidovudine and 88.37±5.08% (1.82–2.73%) for nevirapine. The recovery of IS was 78.94±0.72% with precision of 2.82%.

3.2.6. *Dilution integrity*

The upper concentration limits were shown to be extendable up to 6000 ng/mL for lamivudine and zidovudine and up to 9000 ng/mL for nevirapine by dilution with blank plasma. The mean back-calculated concentrations for half and quarter dilutions were 85–115% of their nominal values with coefficients of variation (%CV) of <8% for all three analytes.

3.2.7. *Stability studies*

In the different stability experiments, the concentrations of analytes were found to remain at ±15% of the predicted concentrations (Table 2) indicting no stability issues for any of the analytes.

3.3. *Application to a pharmacokinetic study*

Mean plasma concentration *vs.* time profiles of lamivudine, zidovudine and nevirapine are shown in Fig. 5 with corresponding pharmacokinetic parameters listed in Table 3. The values are in close proximity to those previously reported[16].

3.4. *Incurred sample re-analysis*

The FDA now requires that incurred sample reanalysis (ISR) of dosed subject samples be carried out to demonstrate assay reproducibility[17]. ISR was performed using two plasma samples from each subject and re-assayed in a separate batch run. The differences in concentrations between the ISR and the initial values for all tested samples were <15% (Table 4) indicating good reproducibility of the method.

Table 3 Pharmacokinetic parameters of lamivudine, zidovudine and nevirapine (data are Mean ± SD, $n=6$).

Parameter	Lamivudine	Zidovudine	Nevirapine
C_{max} (ng/mL)	1195.2 ± 361.2	2020.7 ± 795.7	2472.4 ± 558.1
T_{max} (h)	1.56 ± 0.77	0.56 ± 0.25	6.33 ± 8.77
AUC_{0-t} (ng · h/mL)	6525.1 ± 1931.1	2966.3 ± 410.3	111773.3 ± 17771.5
$AUC_{0-\infty}$ (ng · h/mL)	6929.0 ± 2003.1	3051.9 ± 413.4	203579.6 ± 33637.3
$t_{1/2}$ (h)	4.70 ± 3.35	1.48 ± 0.20	61.68 ± 16.09
Ke (h^{-1})	0.19 ± 0.08	0.48 ± 0.07	0.01 ± 0.00
V_d (L)	0.14 ± 0.07	0.21 ± 0.02	0.09 ± 0.02
CL (L/h/kg)	0.02 ± 0.01	0.10 ± 0.01	0.00 ± 0.00

Table 4 Incurred samples re-analysis data of lamivudine, zidovudine and nevirapine.

Sample	Lamivudine			Zidovudine			Nevirapine		
	Initial conc. (ng/mL)	Re-assay conc. (ng/mL)	Difference[a] (%)	Initial conc. (ng/mL)	Re-assay conc. (ng/mL)	Difference[a] (%)	Initial conc. (ng/mL)	Re-assay conc. (ng/mL)	Difference[a] (%)
1	720.1	745.2	3.4	645.8	631.5	−2.2	2621.2	2354.1	−10.7
2	443.2	430.2	−3.0	68.9	60.3	−13.4	2515.6	2425.2	−3.7
3	767.9	750.7	−2.3	451.7	470.3	4.0	2338.9	2562.1	9.1
4	318.6	300.1	−6.0	68.8	65.2	−5.3	1939.5	2000.2	3.1
5	1135.5	1115.3	−1.8	735.5	726.3	−1.3	2247.6	2216.9	−1.4
6	717.0	725.3	1.1	123.3	115.5	−6.5	3267.7	3001.5	−8.5
7	1268.3	1398.2	9.7	740.8	751.5	1.4	2312.7	2384.2	3.0
8	647.5	672.1	3.7	64.7	58.2	−10.5	1855.9	1902.4	2.5
9	973.4	985.2	1.2	686.7	675.5	−1.6	1259.2	1246.7	−1.0
10	406.7	398.2	−2.1	82.8	88.5	6.6	1226.1	1219.5	−0.5
11	1190.2	1155.8	−2.9	472.9	455.3	−3.8	2157.9	2312.6	6.9
12	568.3	540.8	−5.0	96.4	90.1	−6.8	1791.1	1897.2	5.8

[a]Difference (%) = [(Initial conc. − Re-assay conc.)/Average conc.] × 100%; Average conc. = (Initial conc. − Re-assay conc.)/2.

4. Conclusions

This paper describes for the first time an LC–MS/MS method for the simultaneous determination of lamivudine, zidovudine and nevirapine in human plasma. The proposed method involves simple sample preparation and provides adequate sensitivity in a run time of only 3.5 min. The method was successfully applied to a human pharmacokinetic study and has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring.

Acknowledgments

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