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A LC–MS/MS method with column coupling technique for simultaneous estimation of lamivudine, zidovudine, and nevirapine in human plasma

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Abstract

Background: A new LC–MS/MS method for the simultaneous determination of lamivudine, zidovudine, and nevirapine in human plasma is developed using column-coupling technique.

Method: Labeled compound of respective analyte was used as an internal standard. After extraction from 100 μL plasma by solid phase extraction method, analytes were separated on a C18 column coupled with a cation exchange column. Total run time was 4.5 min. A tandem mass spectrometric detection was conducted using multiple reaction monitoring under positive ionization mode with an electrospray ionization interface. The method was validated as per the FDA guidelines over the concentration range of 9.47–1466.67 ng/mL for lamivudine, 10. 32–1600.00 ng/mL for zidovudine, and 15.05–2426.67 ng/mL for nevirapine.

Results: Precision was in the range 0.86–5.77 (intraday) and 1.92–8.19 (interday) while accuracy was 93.25–104.36 % (intraday) and 96.83–103.28 % (interday). Stabilities of stock in aqueous solutions and in plasma were also determined.

Conclusion: The method can be applied to the pharmacokinetic study of a combination treatment.

Background

Acquired immunodeficiency syndrome (AIDS) due to infection of human immunodeficiency virus type 1 (HIV-1) is one of the deadliest diseases in recent years causing death of millions of people across the world. Due to rapid development of resistance of this virus against the single anti-HIV drugs and also the dose-dependent side effects produced by these drugs leads to the treatment failure within 1 year after the initiation of antiretroviral therapy (Ledergerber et al. 1999). In recent years, combination therapy has become the standard line of treatment to manage acquired immunodeficiency syndrome (AIDS) (De Clercq 2002). Effective combination therapy generally used for treatment of AIDS contains two nucleoside reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a single protease inhibitor (PI) (Gallant 2002). The NRTIs like lamivudine [(2R, cis)-4- amino-l-(2-hydroxymethyl-l, 3-oxathiolan-5-yl)-(lH)-pyrimidin-2-one)] and zidovudine (3-azido- 3-deoxythymidine) can inhibit the replication of HIV-1 and HIV-2 in different kinds of cells. However, lamivudine has very low cellular cytotoxicity and is generally less potent than zidovudine in inhibiting HIV-1 and HIV-2 replication in vitro (Richman et al. 1987; Goodman and Gillman's 2001; Lai et al. 1998; Van Leth et al. 2004). Nevirapine (11-cyclo-propyl-5, 11-dihydro-4-methyl-6H-dipyrido- [3, 2-b: 2, 3 e] (Ledergerber et al. 1999; Richman et al. 1987) diazepin-6-one), on the other hand, belongs to a class of NNRTI and can inhibit reverse transcriptase of HIV-1 (HIV-1 RT) (Van Leth et al. 2004). Due to the synergistic action of different classes of antiretroviral drugs, the survival of HIV patients is prolonged to a great extent and thus such combination therapy, called highly active antiretroviral therapy (HAART), is now considered as a first-line therapy.

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Several LC–MS/MS methods have been reported for the determination of lamivudine, zidovudine, and nevirapine in biological samples either alone or in combination with other drugs (Font et al. 1999; Kenney et al. 2000; Pereira et al. 2000; Chi et al. 2003; Mistri et al. 2007; Elase et al. 2010; Gehrig et al. 2007; Murali Krishna et al. 2012; Zhou et al. 2010). However, these methods had their own problems in respect to the sample preparation, gradient elution, run time, polarity switching, etc. A LC–MS/MS method for the simultaneous determination of lamivudine, zidovudine, and nevirapine in human plasma was recently reported (Valluru Rajani et al. 2013). This method was validated as per FDA regulations and successfully applied to a clinical pharmacokinetic study involving oral administration of a combination of the three drugs to healthy male volunteers. However, this method requires larger plasma sample. Moreover, in this method, both lamivudine and zidovudine were eluting in the void volume. We developed a new and sensitive LC–MS/MS method for simultaneous estimation of these three drugs in human plasma. This method is validated as per FDA regulations (US Food and Drug Administration, Center for Drug Evaluation and Research (CDER) 2001) and can be used for pharmacokinetic study.

Methods

Chemicals and reagents

Lamivudine (purity: 99.8 %), zidovudine (purity: 98.9 %), and nevirapine (purity: 100 %) were purchased from Vivan Life Sciences, Mumbai, India. Lamivudine 13C 15N2 D2 (purity: 99.13 %), zidovudine 13C D3 (purity: 99.33 %), and nevirapine D4 (purity: 98.07 %) used as internal standards were also from Vivan Life Sciences, Mumbai, India.

Methanol (HPLC-grade), ammonium acetate, disodium hydrogen phosphate, and formic acid of the highest purity grade were purchased locally. Milli-Q purified water (Millipore, Milford, MA) was used throughout the study.

Strata-X, 33 μ, polymeric reversed phase 30 mg/1 ml cartridges were procured from Phenomenex.

Plasma lots collected in house were used for the experiments.

Preparation of solutions

Stock solution of individual analyte (200 μg/ml) was prepared in methanol. This concentration was then corrected by taking into account its potency and actual amount weighed.

The stock solutions of lamivudine and zidovudine were then diluted together with 50 % methanol in water to concentration ranges of 472.85 to 73,333.43 ng/ml (lamivudine) and 515.84 to 80,000.00 ng/ml (zidovudine).

Similarly nevirapine stock solution (200 μg/ml) was further diluted with 50 % methanol in water to concentration ranges of 752.27 to 121,333.33 ng/ml.

Preparation of calibration standards

To prepare calibration curve standards, 20 μl of the diluted samples of lamivudine and zidovudine was added to 960 μl of K_2EDTA pooled plasma. Then, 20 μl of each dilution of nevirapine was added. Final concentration ranges were 9.46 to 1466.67 ng/ml for lamivudine, 10.32 to 1600.00 ng/ml for zidovudine, and 15.05 to 2426.67 ng/ml for nevirapine, respectively. All these bulk spiked samples were stored below −20 °C in aliquot of 200 μl.

Preparation of quality control samples

Stock solutions of analytes were diluted as mentioned above with 50 % methanol in water to obtain the concentration ranges of 478.52 to 56666.67 ng/ml for lamivudine, 523.56 to 62000.00 ng/ml for zidovudine, and 752.27 to 121333.33 ng/ml for nevirapine. Nine hundred sixty microliters of K_2EDTA pooled plasma was then spiked with 20 μl of each of quality control dilution of lamivudine and zidovudine mixture and nevirapine solution.

Final concentration ranges were 9.63 to 1146.90 ng/ml for lamivudine, 10.44 to 1243.43 ng/ml for zidovudine, and 15.24 to 1845.48 ng/ml for nevirapine.

Preparation of solution of internal standards

Lamivudine 13C 15N2 D2, zidovudine 13C D3, and nevirapine D4 were used as internal standards. Two hundred micrograms per milliliter stock solutions in methanol were prepared individually for each of them. The respective stock solution was diluted with 50 % methanol in water to obtain a mixture of internal standards containing lamivudine (1 μg/ml), zidovudine (1 μg/ml), and nevirapine (2 μg/ml).

Sample preparation

Fifty microliters of internal standard mixture (lamivudine 13C 15N2 D2 + zidovudine 13C D3 + nevirapine D4) was added to all RIA vials except blank. One hundred microliters of sample was then added to respectively labeled RIA vials. Five hundred microliters of 100-mM anhydrous disodium hydrogen phosphate was added to all samples and mixed by vortex. After centrifugation at 4000 rpm for 5 min in refrigerated centrifuge at 4 °C, the samples were then subjected to solid phase extraction (SPE).

SPE cartridges (Strata-X, 33 μ, polymeric reversed phase 30 mg/1 ml) were conditioned with 1 ml each of methanol and Milli-Q water. The cartridges were then transferred to respectively labeled RIA tubes, and the samples were loaded onto the SPE column by centrifuging at 1000 rpm for 5 min. Washing was performed with 1 ml each of Milli-Q water and then 5 % methanol in water (*v*/*v*), respectively, by centrifuging at 2000 rpm for 5 min. Finally, the cartridges were transferred to fresh labeled RIA tubes and eluted with 1 ml of methanol: water mixture (80:20, *v*/*v*) by centrifugation at 500 rpm for 5 min. Vials were loaded into auto-

sampler for chromatographic operation. Sample processing was done under yellow monochromatic light.

Chromatography

Ten microliters of sample was injected on a reversed phase column (Synergi, 4 μ, Polar-RP 80A, 100 × 4.6 mm) which is connected in series with a cation exchange column (Biobasic SCX, 50 × 4.6 cm, 5 μm). An isocratic mobile phase (10 mM ammonium acetate in 0.2 % formic acid: Methanol:30:70, *v*/*v*) was used at a flow rate of 1.0 ml/min with splitter in Shimadzu HPLC attached to API 4000 Mass spectrometer (Applied Biosystems, USA). The reversed phase column was maintained at 40 °C in the column oven whereas the SCX column was kept at ambient temperature. The run time was 4.8 min.

Mass spectrometry

Electrospray ionization (ESI) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM). By infusing diluted stock solutions of each analyte, the operational conditions were optimized as follows (Table 1).

Source temperature was set at 250 °C. Nebulizer gas (GS1) and auxiliary gas (GS2) flows were 45 and 55 psi, respectively. Quadrupoles Q1 and Q3 were set on unit resolution. Acidic mobile phase (due to addition of formic acid) has improved the protonation of all compounds and has shown a positive impact on signal intensities.

MRM transitions monitored were as follows: m/z 230.1 → 112.1 (LAM), m/z 235.0 → 115.1 (LAM 13C 15 N2 D2), m/z 268.2 → 127.1 (ZDV), m/z 272.2 → 131.1 (ZDV 13C D3), m/z 267.1 → 226.1 (NEV), and m/z 271.2 → 230.2 (NEV D4).

Sample concentrations were calculated by linear regression analysis using the analyst software 1.5.1. Data was processed by peak area ratio. The concentration of unknown was calculated from the equation ($Y = mx + c$) using regression analysis of spiked plasma calibration standards with reciprocal of the square of the drug concentration ($1/X^2$).

Results and discussion

Method development

Specific and effective sample clean-up procedures are required for sensitive and selective LC–MS/MS assays for the determination of very low concentration levels of pharmaceutical targets present in biological samples. Three methods, e.g., protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE), are generally used for preparing biological specimen. Protein precipitation method using organic solvent is the simplest one but the chances of matrix effect prevail. Since the extraction efficiency for highly polar xanalytes is lower, the LLE method is ruled out for lamivudine and zidovudine. We therefore used SPE technique using reverse phase cartridge for sample extraction. Moreover, this technique was further simplified by introducing centrifugation step during washing/elution as we could centrifuge a large number of samples at the same time to save time. Since there were no evaporation and reconstitution steps, time requirement for this method was much less compared to that described by Kumar et al. (Valluru Rajani et al. 2013). This technique was shown to be robust, provided clean samples, and gave good and reproducible recoveries of all analytes and IS. The extraction recovery of analytes was determined by comparing peak areas from plasma samples ($n = 6$) spiked before extraction with those from aqueous samples. The mean overall recoveries across QC levels (with precision) were 76.09 ± 2.953 % (3.88 %) for LAM, 94.38 ± 4.605 % (4.88 %) for ZDV, and 97.30 ± 2.956 % (3.04 %) for NVP (Table 2). The recovery of each IS was more than 74 % (data not shown).

During optimization of chromatographic conditions, we noticed that both LAM and ZDV being highly polar molecules were not retained in the RP column and were eluting either in the void volume or very early. These short retention times were also noticed in the methods described earlier by others (Murali Krishna et al. 2012; Valluru Rajani et al. 2013). This warrants attention about the possibility of improper separation of analytes as well as the matrix effect. Keeping this in mind, we used

Table 1 Optimized mass parameters for analytes and internal standards

Analyte/IS	Dwell time (ms)	Declustering potential (DP) (V)	Entrance potential (EP) (V)	Collision energy (CE) (V)	Collision cell exit potential (CXP) (V)	Collision activated dissociation (CAD) (psi)	Ion source voltage (V)	Curtain gas flow (CUR) (psi)
Lamivudine	400	30	10	20	12	08	5500	30
Lamivudine IS	400	30	10	20	12	08	5500	30
Zidovudine	400	20	10	17	07	08	5500	30
Zidovudine IS	400	35	10	13	07	08	5500	30
Nevirapine	400	40	10	35	12	08	5500	30
Nevirapine IS	400	40	10	35	17	08	5500	30

Table 2 Recovery of analytes in spiked human plasma

Analyte	LQC			MQC			HQC		
	Unextracted peak area[a]	Extracted peak area[a]	Mean percentage recovery	Unextracted peak area[a]	Extracted peak area[a]	Mean percentage recovery	Unextracted peak area[a]	Extracted peak area[a]	Mean percentage recovery
Lamivudine	33,232	24,856	74.80	556,655	411,977	74.01	1,299,965	1,033,108	79.47
Zidovudine	7586	7007	92.37	127,866	116,498	91.11	297,863	296,796	99.64
Nevirapine	52,217	52,567	100.67	889,635	854,959	96.10	2,192,647	2,085,960	95.13

[a] $n = 6$

column coupling technique in which we passed the elute from C_{18} column through SCX column consecutively. Nevirapine being a lyophilic molecule bound tightly with the RP column and eluted late. To make the method simpler, we used the same mobile phase for both the columns instead of different mobile phases. The total run time was only 4.5 min. The short run time is also ideally suited for being considered in high throughput analysis. This method provided good separation of analytes as well as internal standards. The retention times for lamivudine, zidovudine, and nevirapine were 3.28, 2.23, and 2.88 min, respectively. The retention times for internal standards were 3.27 min (LAM), 2.2 min (ZDV), and 2.85 min (NVP), respectively.

Method validation

The current LC–MS/MS assay was validated as per FDA guidelines for specificity, linearity, intra- and interday precision and accuracy, and stability.

Selectivity

Selectivity of the method was evaluated in eight individual human K_2 EDTA plasma lots along with one lipemic and one hemolytic lot. Negligible interferences were observed at the retention times of analytes and internal standards when peak responses in blank lots were compared against the response of spiked LLOQ containing IS mixtures. Representative chromatograms in Fig. 1a–c (blank plasma) and Fig. 2a–c (blank plasma spiked with analytes/IS) demonstrate the selectivity of the method.

Linearity and sensitivity

Eight-point calibration curves were prepared with concentration ranging from 9.505 to 1473.327 ng/ml for LAM, 10.373 to 1607.896 ng/ml for ZDV, and 15.066 to 2418.780 ng/ml for NVP. The peak-area ratio (y) of analytes to internal standards was plotted against the nominal concentration (x) of analytes to determine the linearity of each calibration curve. Excellent linearity was achieved with correlation coefficients greater than 0.999 for all validation batches.

The concentrations of calibration standards were back calculated to obtain the accuracy of each calibration point. The ranges of the calibration points' accuracy for LAM, ZDV, and NVP were 98.4–102.1, 97.7–103.7, and 97.7–101.7 %, respectively.

Precision and accuracy at the LLOQs were respectively 7.12 and 96.83 % for LAM, 8.19 and 98.64 % for ZDV, and 3.84 and 102.40 % for NVP. The LLOQs of the method is 9.634 ng/ml for LAM, 10.445 ng/ml for ZDV, and 15.236 ng/ml for NVP, respectively, which are at par with the reported ones (Murali Krishna et al. 2012; Valluru Rajani et al. 2013). Limits of detection were found to be 2.376 ng/ml (for LAM; signal to noise ratio > 26.780), 2.593 ng/ml (for ZDV; signal to noise ratio > 13.605), and 3.767 ng/ml (for NVP; signal to noise ratio > 154.045). This indicates that this method is sensitive enough for a pharmacokinetic study. Moreover, the good signal-to-noise obtained at this concentration indicates that the LLOQ of the method can be lowered further or the volume of plasma can be decreased. This further widens the scope of this method even to the pediatric patients.

Precision and accuracy

Precision and accuracy for intra- and interday batches for all analytes were determined by six replicate analyses of QC samples ($n = 6$) at four different concentrations—lower limit of quantification (LLOQ), low quality control (LQC), middle quality control (MQC), and high quality control (HQC). The respective concentrations for LAM, ZDV, and NVP were 9.634, 10.445, and 15.236 ng/ml for LLOQ, 27.526, 29.842, and 44.292 ng/ml for LQC, 458.761, 497.370, and 738.192 ng/ml for MQC, and 1146.902, 1243.426, and 1845.480 ng/ml for HQC. Results of precision and accuracy were presented in Table 3. The intraday and interday precision were within 8.4 % for all analytes. The assay accuracy was 89.2–106.4 % of the nominal values. The accuracy of the assay was expressed by [(mean observed concentration)/(spiked concentration)] × 100 %, and precision was evaluated by relative standard deviation (RSD).

Matrix effect

Matrix effect was investigated by extracting blank plasma from eight different sources, including one hemolytic and one lipemic lot. One hundred microliters of blank plasma from each lot was processed as per the procedure mentioned in sample preparation. Aqueous

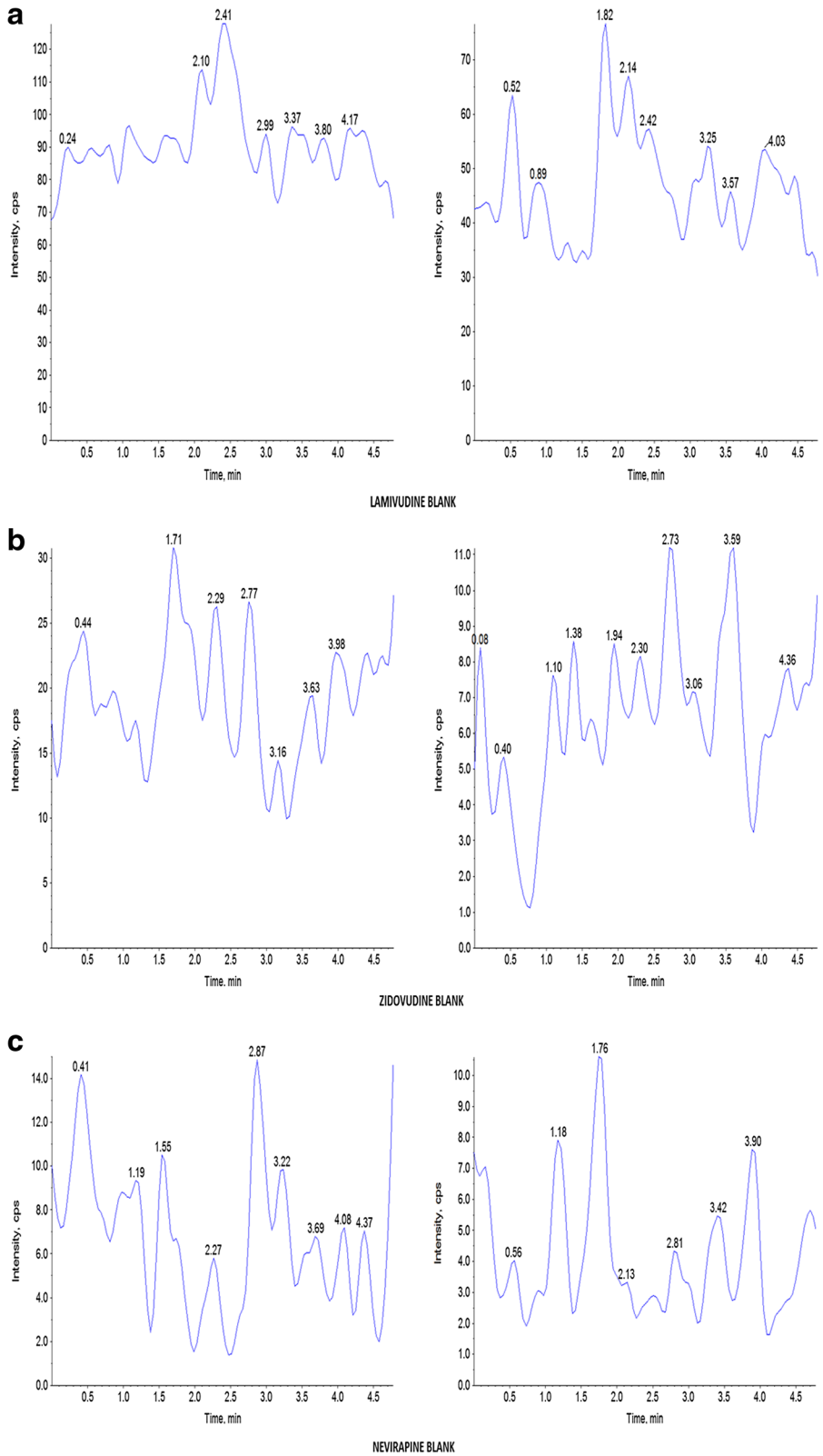

Fig. 1 **a** Blank plasma for lamivudine and its IS. **b** Blank plasma for zidovudine and its IS. **c** Blank plasma for nevirapine and its IS

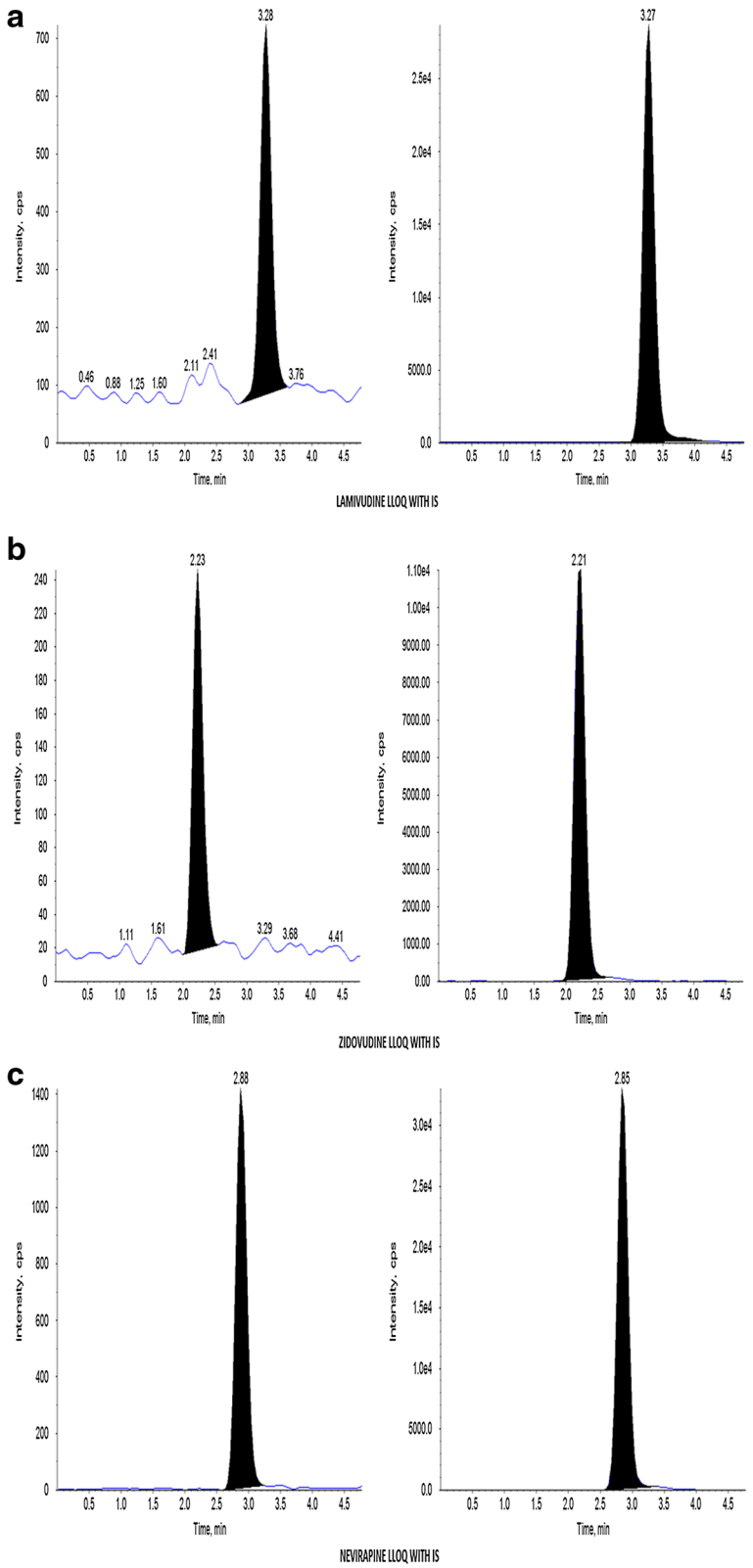

Fig. 2 **a** Chromatogram of lamivudine and its IS. **b** Chromatogram of zidovudine and its IS. **c** Chromatogram of nevirapine and its IS

Table 3 Accuracy and precision of analysis in the quality-control samples

			Intraday			Interday		
Analyte	QC sample	Actual conc. (ng/ml)	Estimated conc. (ng/ml)	Accuracy (%)	% CV	Estimated conc. (ng/ml)	Accuracy (%)	% CV
Lamivudine	LLOQ	9.63	10.05	104.36	3.01	9.33	96.83	7.12
	LQC	27.53	28.06	101.95	2.01	27.92	101.43	3.20
	MQC	458.76	457.46	99.72	2.26	457.49	99.72	1.92
	HQC	1146.90	1152.58	100.49	1.67	1157.65	100.94	2.49
Zidovudine	LLOQ	10.44	9.81	93.95	5.77	10.30	98.64	8.19
	LQC	29.84	27.83	93.25	3.52	29.59	99.15	5.59
	MQC	497.37	483.61	97.23	2.56	494.18	99.36	2.75
	HQC	1243.43	1227.03	98.68	2.42	1244.60	100.09	3.42
Nevirapine	LLOQ	15.24	15.06	98.86	2.96	15.60	102.40	3.84
	LQC	44.29	45.00	101.60	1.89	45.74	103.28	2.83
	MQC	738.19	748.98	101.46	0.86	747.47	101.26	2.24
	HQC	1845.48	1878.40	101.78	1.17	1864.04	101.01	2.59

solution of individual analyte either at LQC or HQC level was added to each of the final eluent. These samples were considered as post-extracted samples (presence of matrix).

Similarly, the aqueous solution of individual analyte either at LQC or HQC level was prepared with the elution solvent and was considered as aqueous samples (absence of matrix). Six replicates each of aqueous samples were injected along with post-extracted samples of LQC and HQC.

Individual analyte area response and IS area response of each post-extracted sample were compared with the mean analyte area response and mean IS area response of the aqueous sample, respectively. The matrix effect was calculated via the formula: Matrix effect (%) = $A_2/A_1 \times 100(\%)$, where A_1 = response of aqueous concentrations and A_2 is the response of post-extracted concentrations.

Average ($n = 6$) matrix factor ranges from 94.66–101.09 % with a CV range of 1.21–4.24 % for all analytes at LQC level and internal standards. At HQC level, the range was 101.99–103.97 with a CV range of 1.66–2.09 % which is within the accepted limit (% CV ≤ 15) (Table 4).

Dilution integrity

Dilution integrity of the method was evaluated after diluting twofold and fourfold with interference free human plasma. Six replicates of these samples were processed and analyzed against a set of freshly spiked calibration standards. The upper concentration limits were shown to be extendable up to 2325.98 ng/mL for LAM, 2515.90 ng/mL for ZDV, and 3690.96 ng/mL for NVP by dilution with blank plasma. The mean back calculated concentrations for twofold and fourfold dilution samples were within 92.18–111.60 % with a % CV of ≤3.43 for all three analytes.

Carryover effect

Carryover effect was evaluated in order to evaluate the cleaning ability of rinsing solution used for the injection needle and port. This avoids any carryover of injected sample in subsequent runs. The experiment was carried out by placing samples in the following order: LLOQ of individual analyte, blank plasma, and upper limit of quantitation (ULOQ) of individual analyte and blank plasma. No carryover was observed during the experiment.

Stability

Stability evaluations were performed in both aqueous- and matrix-based samples. For aqueous solution, both short-term and long-term stabilities were determined as follows:

Table 4 Matrix effects for analytes in eight different lots of human plasma

Analyte	LQC analyte average peak area in absence of matrix	LQC analyte average peak area in presence of matrix	LQC matrix factor for analyte	% CV	HQC analyte average peak area in absence of matrix	HQC analyte average peak area in presence of matrix	HQC matrix factor for analyte	% CV
Lamivudine	33,007	31,718	96.09	1.43	1,281,899	1,332,156	103.92	2.09
Zidovudine	6848	6721	98.15	4.24	273,050	283,906	103.98	1.68
Nevirapine	57,547	54,476	94.66	1.21	2,070,858	2,112,144	101.99	1.66

Table 5 Stability studies of aqueous stock solutions of analytes

Stability check	Analytes	Concentration	Average peak area for stored Solution[a]	Average peak area for fresh Solution[a]	% CV	% Stability
STSS (25 h)	Lamivudine	MQC	536,739	535,814	1.07	99.36
	Zidovudine	MQC	121,203	121,766	1.07	99.49
	Nevirapine	MQC	907,909	912,565	1.16	99.44
LTSS (40 days)	Lamivudine	MQC	551,425	550,174	1.20	100.32
	Zidovudine	MQC	125,575	124,388	1.68	100.95
	Nevirapine	MQC	928,136	915,249	1.36	101.23

[a]$n = 6$

a) *Stability in aqueous solution*
   i. *Short-term stock solution stability (STSS)*
   MQC concentration of each analyte was prepared by dilution of respective stock solution and stored at 25 °C for 24 h. Six replicate injections were given for MQC sample. No significant differences were noticed when these results were compared with those obtained from the freshly prepared MQC samples indicating that all analytes were stable at 25 °C (Table 5). Accepted criteria for the ratio of mean response for stability samples should be between 90 and 110 %.
   ii. *Long-term stock solution stability (LTSS)*
   Aqueous MQC sample of each analyte, prepared by dilution from respective stock solution which was stored at 2–8 °C for 40 days was injected. Mean area response of MQC of stored stock solution was then compared against MQC from

Table 6 Stability studies of analytes in human plasma

Analyte	Stability check	Samples	Average[a] nominal conc. (ng/ml)	Average[a] observed conc. (ng/ml)	% CV	% Stability
Lamivudine	Bench top (7.0 h)	LQC	27.53	27.76	2.34	100.84
		HQC	1146.90	1161.87	1.77	102.31
	Freeze thaw (4 cycles)	LQC	27.53	28.16	2.52	102.26
		HQC	1146.90	1155.10	2.97	101.71
	In-auto-sampler (22 h)	LQC	27.53	27.95	4.44	101.50
		HQC	1146.90	1159.58	1.63	102.11
	Wet extract (7 h)	LQC	27.53	27.72	2.76	100.69
		HQC	1146.90	1150.12	1.65	101.27
Zidovudine	Bench top (7.0 h)	LQC	29.84	28.43	3.38	97.79
		HQC	1243.43	1212.32	2.06	100.06
	Freeze thaw (4 cycles)	LQC	29.84	30.02	1.50	103.24
		HQC	1243.43	1206.04	1.71	99.54
	In-auto-sampler (22 h)	LQC	29.84	28.97	5.08	99.66
		HQC	1243.43	1212.50	1.43	100.07
	Wet extract (7 h)	LQC	29.84	28.32	5.96	97.40
		HQC	1243.43	1210.84	2.51	99.94
Nevirapine	Bench top (7.0 h)	LQC	44.29	45.33	2.18	101.73
		HQC	1845.48	1821.13	1.08	102.24
	Freeze thaw (4 cycles)	LQC	44.29	46.25	2.82	103.80
		HQC	1845.48	1816.07	1.34	101.96
	In-auto-sampler (22 h)	LQC	44.29	45.38	3.30	101.84
		HQC	1845.48	1829.70	1.44	102.73
	Wet extract (7 h)	LQC	44.29	44.48	1.54	99.81
		HQC	1845.48	1795.11	1.76	100.78

[a]$n = 6$

Table 7 Extended accuracy and precision analysis

Analyte	QC sample	Actual conc. (ng/ml)	Estimated conc. (ng/ml)	Accuracy (%)	% CV
Lamivudine	LQC	27.53	26.88	97.65	2.72
	HQC	1146.90	1153.16	100.55	2.52
Zidovudine	LQC	29.84	29.07	97.42	4.71
	HQC	1243.43	1211.79	97.46	2.12
Nevirapine	LQC	44.29	44.85	101.25	2.28
	HQC	1845.48	1815.04	98.35	2.47

freshly prepared stock solution. Mean percent stability (100.83) was well within accepted limit (90–110 %). This indicated the stability of each analyte solution for 40 days at 2–8 °C (Table 5).

b) *Stability in human plasma*

i. *Bench-top stability*
Six aliquots of each analyte in human plasma (at LQC and HQC concentrations) from −20 °C were allowed to thaw unassisted at room temperature (25 °C) for 7 h and processed along with a set of freshly prepared calibration standards as well as LQC and HQC samples. The stability for LQC and HQC samples of each analyte were found in the range of 97.79–101.73 % and 100.06–102.31 %, respectively.

ii. *Freeze thaw stability*
After four freeze thaw cycles, the stability for LQC and HQC samples of each analyte were in the range of 102.26–103.80 % and 99.54–101.96 %, respectively.

iii. *In-injector stability*
The stability for LQC and HQC samples of each analyte kept in auto-sampler at 10 °C for 22 h were in the range of 99.66–101.84 % and 100.07–102.73 %, respectively.

iv. *Wet extract stability*
The stability for LQC and HQC samples of each analyte after 7 h at 25 °C were in the range of 97.40–100.69 % and 99.94–101.27 %, respectively. Accepted range for all the abovementioned stability studies is that the mean concentration for stability samples should be 85–115 % of the mean concentration of freshly prepared samples. Thus, all the analytes were stable during the analysis process. Results of the stability evaluations were presented in Table 6.

Extended precision and accuracy run

Extended precision and accuracy run was performed by processing and analyzing one set of CC and 40 sets of LQC and HQC as a batch (total 90 samples). Results of precision and accuracy were presented in Table 7. The precisions for lamivudine, zidovudine, and nevirapine were 2.72, 4.71, and 2.28 % for LQC and 2.52, 2.12, and 2.47 % for HQC, respectively. The accuracies were 97.65, 97.42, and 101.25 % for LQC and 100.55, 97.46, and 98.35 % for HQC, respectively.

Conclusions

A fast, sensitive, and specific LC–MS/MS method for simultaneous determination of lamivudine, zidovudine, and nevirapine in human plasma was developed and validated. This is the first method using column coupling technique to estimate simultaneously these three drugs in human plasma. It also utilizes a more selective solid phase extraction technique, offering consistent and reproducible recoveries for all three analytes with insignificant interference and matrix effect. Moreover, this is the first method where labeled internal standards were used. As per FDA guidelines, internal standard should preferably be identical to the analyte (US Food and Drug Administration, Center for Drug Evaluation and Research (CDER) 2001). By using 100 μL-plasma samples, the lower limits of quantification were achieved. It demonstrates that the method is reproducible, sensitive, and suitable for high-throughput sample analysis. This method has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SR designed the experiment. LT, SK. and NN were involved in the method development and validation. AM prepared the manuscript after the analysis of the results, and ST reviewed the manuscript. All authors read and approved the final manuscript.

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