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Selective, Sensitive and High-throughput Simultaneous Method Development of Zidovudine, Lamivudine and Nevirapine in Human Plasma Using 96 well Plate Solid Phase Extraction and Liquid Chromatography Coupled with Tandem Mass Spectrometry

Tangudu Nagabhusana Rao[1]*; Guntuku Girija Sankar[1]; Lade Jyothi Rani[2]

1.College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India - 530003

2.Mallareddy Institute of Pharmaceutical Sciences, Dhulapally, Secunderabad-14, India

ABSTRACT

A high throughput, sensitive, selective, and rugged liquid chromatography coupled with mass spectrometry (LC-MS/MS) method for the quantification of Zidovudine, Lamivudine and Nevirapine, in human plasma was developed and validated. The analytes were extracted from plasma by solid phase extraction technique using Waters Oasis HLB 96 well plate 30 µm (30 mg). Isocratic elution of Zidovudine, Lamivudine and Nevirapine were achieved in 3 min using ACE, C18, 4.6 X 150mm, 5µ column having a mobile phase of 0.1% formic acid: Methanol 30: 70 v/v. The flow rate was 0.7 mL/min at a column temperature of 35 ± 5°C. Electron spray ionization technique in positive mode was selected to improve the selectivity and sensitivity required for this application. The retention times of Zidovudine, Lamivudine and Nevirapine were 1.5, 1.2, and 1.7 min, respectively. The method was validated for linearity, precision, accuracy, specificity, sensitivity, matrix effect, dilution integrity, ruggedness, reinjection reproducibility, and stability. The assay produced linear calibration curves over the concentration range for Zidovudine, Lamivudine and Nevirapine in the ranges of 5 to 1500, 5 to 1500 and 10 to 3000 ng/mL respectively with correlation coefficients greater than 0.9963 using a 1/x2 weighted least square regression analysis of standard plots associated with eight-point calibration standards. The precision and mean accuracy were within the acceptable limits.

Keywords: Zidovudine, Lamivudine and Nevirapine, Solid Phase Extraction and Waters Oasis HLB 96 well plate

*Corresponding Author Email: tangudur@gmail.com

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INTRODUCTION

The combination of Zidovudine (ZDV), lamivudine (3TC) and nevirapine (NVP) is a triple nucleoside reverse transcriptase inhibitor regimen, which is used in the treatment of human immunodeficiency virus type 1 (HIV-1). This combination has low hepatotoxicity and minimal interaction with anti-tuberculosis therapy [1]. It is one of the four recommended first line regimens by WHO for the treatment of HIV[2] in adults and children. It is usually supplied in the form of tablets and tablets for oral suspension (TFOS). All these three drugs work by inhibiting the action of viral specific enzyme, reverse transcriptase, which is required for the replication of virus. Triple combination of HIV drugs prevent the integration of HIV into the DNA of infected CD4+ cell which inhibits the replication of virus, resulting in reduced viral load.

Zidovudine (ZDV), a nucleoside reverse transcriptase inhibitor is chemically a deoxythymidine nucleoside analog. It phosphorylates to form active metabolites, which inhibit the HIV reverse transcriptase enzyme competitively and acts as a chain terminator of DNA synthesis [3] Lack of a 3'-OH group in the incorporated nucleoside analog prevents the formation of the 5' to 3' phosphodiester linkage, which is essential for DNA chain elongation and therefore the viral DNA growth will terminate. ZDV and stavudine are not recommended in combination [4] as they contend each other for activation by intracellular phosphorylation, resulting in diminished antiretroviral activity. Absorption of ZDV is rapid and nearly complete on oral administration [5] and because of first pass metabolism the systematic bioavailability of ZDV is approximately 65%. Lamivudine (3TC) is a synthetic nucleoside analog, acts as a nucleoside reverse transcriptase inhibitor (NRTI) against human immunodeficiency virus type 1 (HIV-1) and hepatitis B (HBV)[6]. 3TC phosphorylates intracellularly to its 5'-triphosphate active metabolite, lamivudine triphosphate. It competitively inhibits the HIV reverse transcriptase enzyme and acts as chain terminator of DNA synthesis. On oral administration, absorption of 3TC is rapid and absolute bioavailability is approximately 86% for both tablet and oral solution. Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor, active against HIV-1. It is used in combination with other nucleoside inhibitors [7] and generally, it is prescribed after the immune system has declined. NVP binds directly to reverse transcriptase and thereby blocks the RNA-dependent and DNA-dependent DNA polymerase activities by disrupting the enzyme's catalytic site.

Reported analytical methods [8-20] were available for the determination ZDV, 3TC and NVP individually or in combination in pharmaceutical formulations, however very few reported methods are available for simultaneous determination of ZDV, 3TC and NVP in biological

matrices. *Malm et al.* [11] developed a combination method on blood samples using gradient HPLC with UV detection. The limit of quantification was 0.11, 0.13 and 1.3 µg/mL for 3TC, ZDV and NVP respectively. *Vandana et al.* [19] developed a gradient HPLC-UV method for the three drugs in plasma using 950 µL sample volume with a quantification limit of 51 ng/mL. *Zhou et al.* [16] developed an LC-MS/MS method for determination of ZDV, 3TC and NVP with a LOQ of 20 ng/mL using protein precipitation technique, which is not selective and has high potential for ion-suppression in LC-MS/MS analysis. *Krishna et al.* [17] developed a simultaneous LC-MS/MS method with a run time of 3.5 min. and LOQ of 25 ng/mL for ZDV and 3TC and 81 ng/mL for NVP. The objective of this study is to develop and validate [21-24] a more sensitive and selective high throughput LC-MS/MS method using 96 well plate solid phase extraction that can be efficiently used in pharmacokinetic studies, to evaluate bioavailability and bioequivalence for this potent combination of ZDV, 3TC and NVP. As part of it developed and validated an isocratic LC-MS/MS method with simple and reproducible solid phase extraction in human plasma using Didanosine (DDI) (for ZDV), Emtricitabine (FTC) (for 3TC) and Abacavir (ABC) (for NVP) as an internal standards.

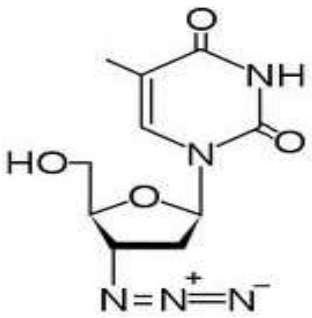

Figure 1: Structure of Zidovudine

Figure 2: Structure of Lamivudine

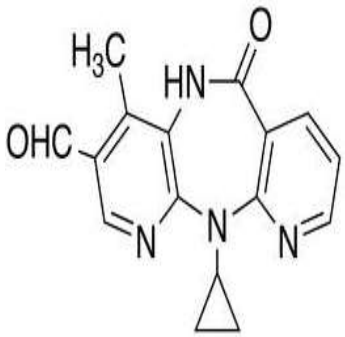

Figure 3: Structure of Nevirapine

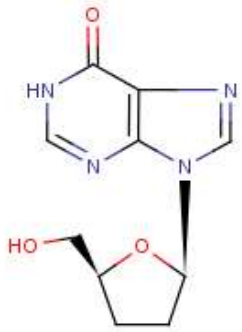

Figure 4: Structure of Didanosine

Figure 5: Structure of Emtricitabine

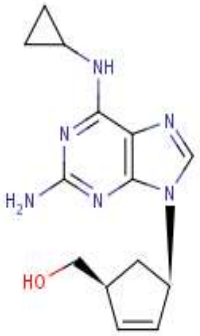

Figure 6: Structure of Abacavir

MATERIALS AND METHOD

Chemicals and reagents

Working standards of Zidovudine, Lamivudine, Nevirapine, Didanosine, Emtricitabine and Abacavir were obtained from sigma Aldrich. Waters Oasis HLB 96 well plates 30 µm (30 mg) were purchased from Waters Corporation (Milford, MA, USA). LC–MS grade methanol was purchased from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). Formic acid was obtained from Sigma Aldrich. HPLC water was obtained from Milli-Q water purification system (Millipore). Human plasma containing K2 EDTA anticoagulant was obtained from Deccan pathological lab (Hyderabad, India).

Instrumentation

Agilent 1200 Series equipped with a binary pump for solvent delivery was used for the analysis. Mass spectrometric detection was performed on API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray inter-face. Quantitation was performed in multiple reaction monitoring (MRM) mode and Analyst software version 1.5.1(SCIEX) was used for controlling the hardware and data handling.

Chromatographic conditions

Chromatographic separation was performed on ACE, C18, 4.6 X 150mm, 5µ analytical columns. Isocratic mobile phase consisting of 0.1% formic acid and methanol in 30: 70 v/v ratio was delivered at a flow rate of 0.7 mL/min. The auto sampler was set at $4^0C \pm 2^0C$ and the injection volume was 5 µL. The column oven temperature was set at 35.0 ± 5.0°C.

The retention times of Zidovudine, Lamivudine and Nevirapine were 1.5, 1.2, and 1.7 min, respectively. The retention time of internal standards Didanosine, Emtricitabine and Abacavir were 1.4, 1.4 and 1.1 min, respectively. The total chromatographic run time was 3.0 min.

Mass spectrometric conditions

- Ionization mode: Positive ionization

- Resolution: Q1 Unit; Q3 Unit
- MRM conditions

Parameters	Q1 (amu)	Q3 (amu)	Dwell Time (msec)	DP (volts)	CE (volts)	CXP (volts)	EP (volts)
Zidovudine	268.2	127.1	200	45	12	12	10
Lamivudine	230.2	112.1	200	40	10	10	10
Nevirapine	267.2	226.1	200	20	9	45	10
Didanosine	237.1	137.1	200	40	10	12	10
Emtricitabine	248.1	130.0	200	40	10	15	10
Abacavir	287.2	191.2	200	40	10	30	10

2.4.4 Source/ Gas parameters

Parameters	CUR (psi)	GS1 (psi)	GS2 (psi)	IS (Volts)	CAD (psi)	TEMP (°C)
Source/Gas	30	45	45	5200	5	400

Preparation of calibration standards and quality control samples

Standard stock solutions of Zidovudine, Lamivudine, Nevirapine, Didanosine, Emtricitabine and Abacavir were prepared by dissolving their accurately weighed amounts in Methanol to give a final concentration of 1mg/mL. Working solutions of analyte (Mixture) were prepared by appropriate dilution of their stock solutions in 50:50 v/v Methanol: water. All the solutions were stored in refrigerator at below 10◦C and were brought to room temperature before use. The combined working solutions of Zidovudine, Lamivudine, and Nevirapine were prepared with appropriate dilution of the stock solutions in 50% methanol. Internal standard working solution (containing 2500 ng/mL DDI, 600 ng/mL FTC and 350 ng/mL of ABC) was also prepared in 50% methanol and is used in the assay. The prepared working solutions were stored at room temperature and fresh dilutions were made on day-to-day basis during the analysis. All the volumetric measurements were made using calibrated micropipettes.

Calibration standards and quality control (QC) samples were prepared by spiking K2 EDTA human blank plasma with the working solutions (5%) prepared from independent stock weightings. Calibration standards and quality controls were prepared in plasma at following concentrations:

CC standard and QC samples	Zidovudine (ng/mL)	Lamivudine (ng/mL)	Nevirapine (ng/mL)
CS1	5	5	10
CS2	10	10	20
CS3	25	25	50
CS4	100	100	200

CS5	400	400	800
CS6	800	800	1600
CS7	1200	1200	2400
CS8	1500	1500	3000
LQC	15	15	30
MQC	800	800	1600
HQC	1200	1200	2400

Sample Preparation:

Calibration standards, QC's were processed using Ezypress Positive Pressure SPE Manifold by using 100 µL of Plasma Volume

- For CC and QC spike 5 µL of each working solutions into 95 µL of human plasma.
- Add 20 µL of internal standard to each tube except for blank plasma samples.
- Add 20 µL of 50:50v/v MeoH: Water to blank plasma samples
- Add 25 µL of 0.1% formic acid solution to each tube and vortex.
- Condition the Waters Oasis HLB 96 well plate 30 µm (30 mg) with 1 mL of methanol followed by 1 mL of Milli Q water.
- Load the samples onto the plate.
- Wash the cartridges with 500 µL of Milli Q Water followed by 500µL of 10% Methanol in water.
- Elute the sample with 800 µL (two aliquots of 400 µL each) of acetonitrile into 96 well collection plate.
- Evaporate the eluent under a gentle stream of nitrogen using a TurboVap 96, at a temperature of approximately 50°C.
- Reconstitute the dried samples with 100 µL of mobile phase and vortex to mix.
- Inject 5 µL of the sample onto the LC-MS/MS system.

Method validation

A complete method validation for simultaneous estimation of Zidovudine, Lamivudine, and Nevirapine was done following the USFDA and EMEA guidelines. Validation runs were performed on different days to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity, ruggedness and stability. Each validation run was organized with a set of spiked standard samples, blank (with ISTD and without ISTD) and QC samples as per the validation parameter. Standard samples were analyzed at the beginning of the run and QC samples were distributed consistently throughout the validation runs.

Selectivity of the method toward endogenous and exogenous components of plasma was evaluated

in 6 different plasma lots. The blank plasma lots were extracted (without addition of ISTD), and injected for LC–MS/MS detection. Later selectivity in each lot was evaluated by comparing the blank peak responses against the mean peak response observed in plasma spiked LLOQ sample (n = 6).

Linearity of the method was assessed using three calibration curves analyzed on three different days. Each plot was associated with a eight point non-zero concentrations spread over the dynamic range. A linear regression analysis with reciprocate of drug concentration as weighing factor (1/X2) was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentration of analytes over the established range.

Intra batch (within day) and inter batch (between day) precision and accuracy was evaluated at four distinct concentrations (LLOQ, LQC, MQC, HQC). Precision and accuracy at each concentration level was evaluated in terms of %CV and relative error respectively. The extraction recovery of analytes was determined at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with that of un-extracted samples (blank extracts spiked after extraction).

The matrix effect was checked at low and high QC level using six different blank plasma lots (including one hemolytic and one lipemic lot). Matrix factor for analytes and internal standard was calculated in each lot by comparing the peak responses of post extraction samples (blank extracts spiked after extraction) against the peak responses of equivalent aqueous samples prepared in mobile phase. Internal standard normalized matrix factor in each lot was later evaluated by comparing the matrix factor of analyte and internal standard.

Stability of analytes in both solutions and in biological matrix was evaluated after subjecting to different conditions and temperatures that could encounter during regular analysis. Stability in plasma was evaluated in terms of freeze–thaw stability, bench top stability, long-term stability, and extracted sample stability. Freeze–thaw stability was evaluated after eight freeze (at -70^0C) thaw (at room temperature) cycles. Bench top stability was assessed at room temperature and the long-term stability was evaluated at both -70^0 C and -20^0C. Stability of extracted samples was determined after reconstitution (in-injector stability at 4^0C). Reinjection reproducibility was proved for 49 Hrs. All the stability assessments were made at LQC and HQC level by comparing the stability samples against freshly prepared samples.

Stability of analytes in stock solutions and in working solutions was assessed at room temperature (short-term stability) and at 2-8^0C (long-term stability). All comparisons were made against freshly

prepared stock solutions or working solutions. Before each analytical run, system suitability was evaluated by injecting six replicates of MQC sample to check the system precision and chromatography. System suitability was considered acceptable when the coefficient of variation for response ratios was less than 4.0%.

RESULTS AND DISCUSSION

Selection of internal standards

For reliable and precise analytical method, it is necessary to use internal standard that behave closely with analyte. Compounds with close pKa and logP values were selected as ISTDs, to nullify the variations in sample preparation step, as it is the most susceptible area for variations.

Method development

For consistent and reliable estimation of analytes it was necessary to give equal importance for optimization of extraction procedure along with chromatographic and mass spectrometric conditions. Analytes and ISTD were tuned in positive polarity mode using electrospray ionization technique. The Q1 and the MSMS scans were made in infusion mode and further compound and gas parameters were optimized in flow injection analysis. The [M+H] peaks were observed at m/z of 268.2 for ZDV, 230.2 for 3TC and 267.2 for NVP and the abundant product ions were found at 127.1 for ZDV, 112.1 for 3TC and 226.1 for NVP (Figure 7), with the application of appropriate collision energy. Increase in source temperature beyond 400^0C augmented the intensity. A 5% change in ionspray voltage and gas parameters did not affect the signal intensity.

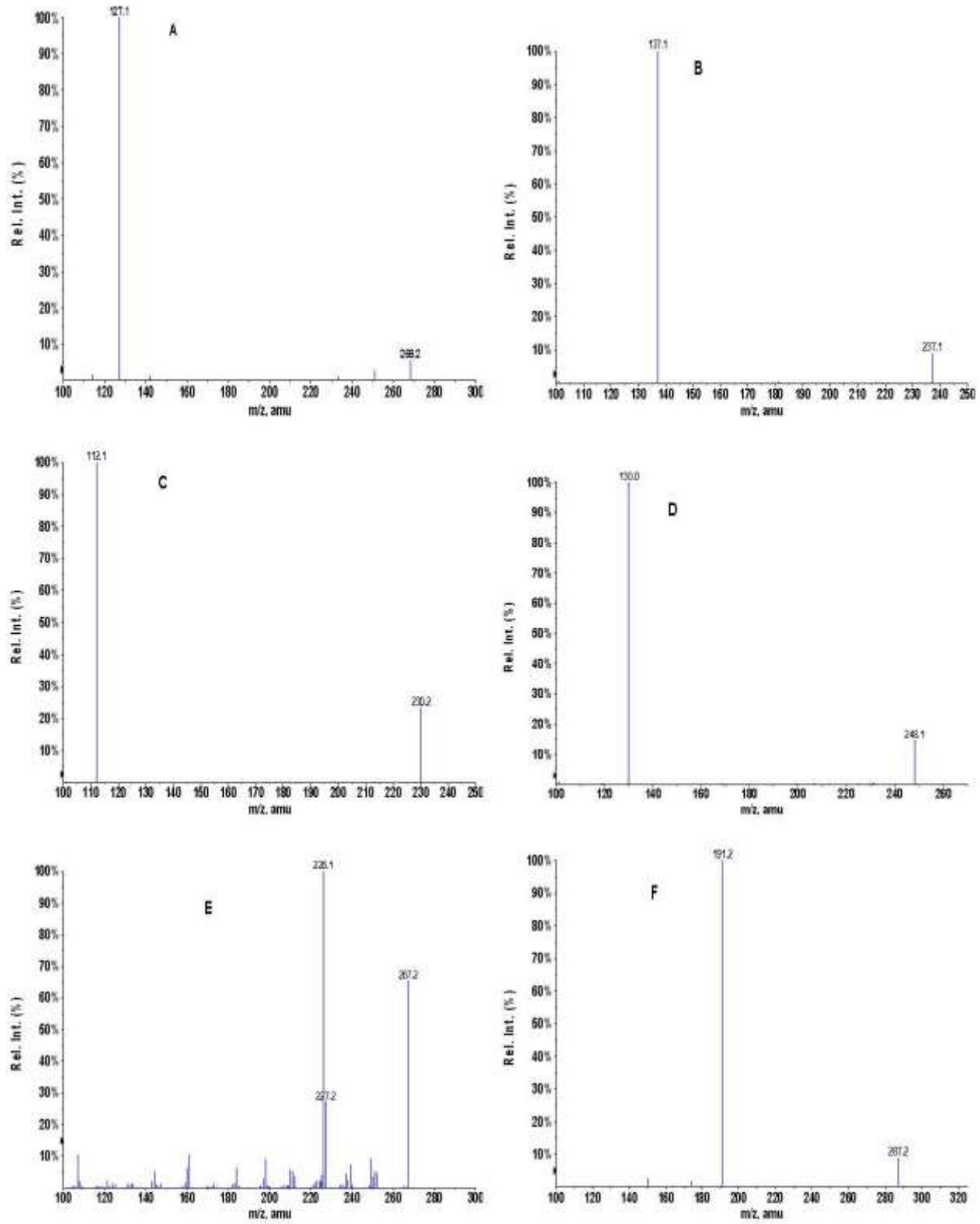

Figure 7: Product ion spectra of Zidovudine (A), Didanosine (B, ISTD for ZDV), Lamivudine (C), Emtricitabine (D, ISTD for 3TC), Nevirapine (E) and Abacavir (F, ISTD for NVP)

In the optimization of chromatographic conditions, isocratic mode was selected as no cross talk was observed between analytes and ISTD. No significant response variation was observed between acetonitrile and methanol in the mobile phase, however consistent chromatography was obtained with methanol. Replacement of milli-Q water with 0.1 % formic acid in mobile phase gave good chromatographic peak shapes and further increase in the buffer concentration was resulted in loss of response. A flow rate of 0.7 mL/min was used to minimize the run time.

Solid Phase extraction was initiated with individual cartridges. Later on the method was shifted to 96 well plate format. Impact of different solutions and their concentration on recovery of analytes was monitored and the final optimized conditions are depicted in Section 2.6. During the optimization of chromatographic conditions and extraction procedure, more emphasis was given to improve the sensitivity and recovery. No significant matrix effects were observed with the proposed chromatographic and extraction conditions.

Selectivity

Selectivity of the method in human K2 EDTA plasma was evaluated in six individual matrix lots along with one lipemic and one hemolytic lot. Peak responses in blank lots were compared against the response of spiked LLOQ and negligible interference was observed at the retention time of analytes and ISTD. Figure. 8 to 10 demonstrate the selectivity of the method with the chromatograms of blank plasma and LLOQ sample respectively.

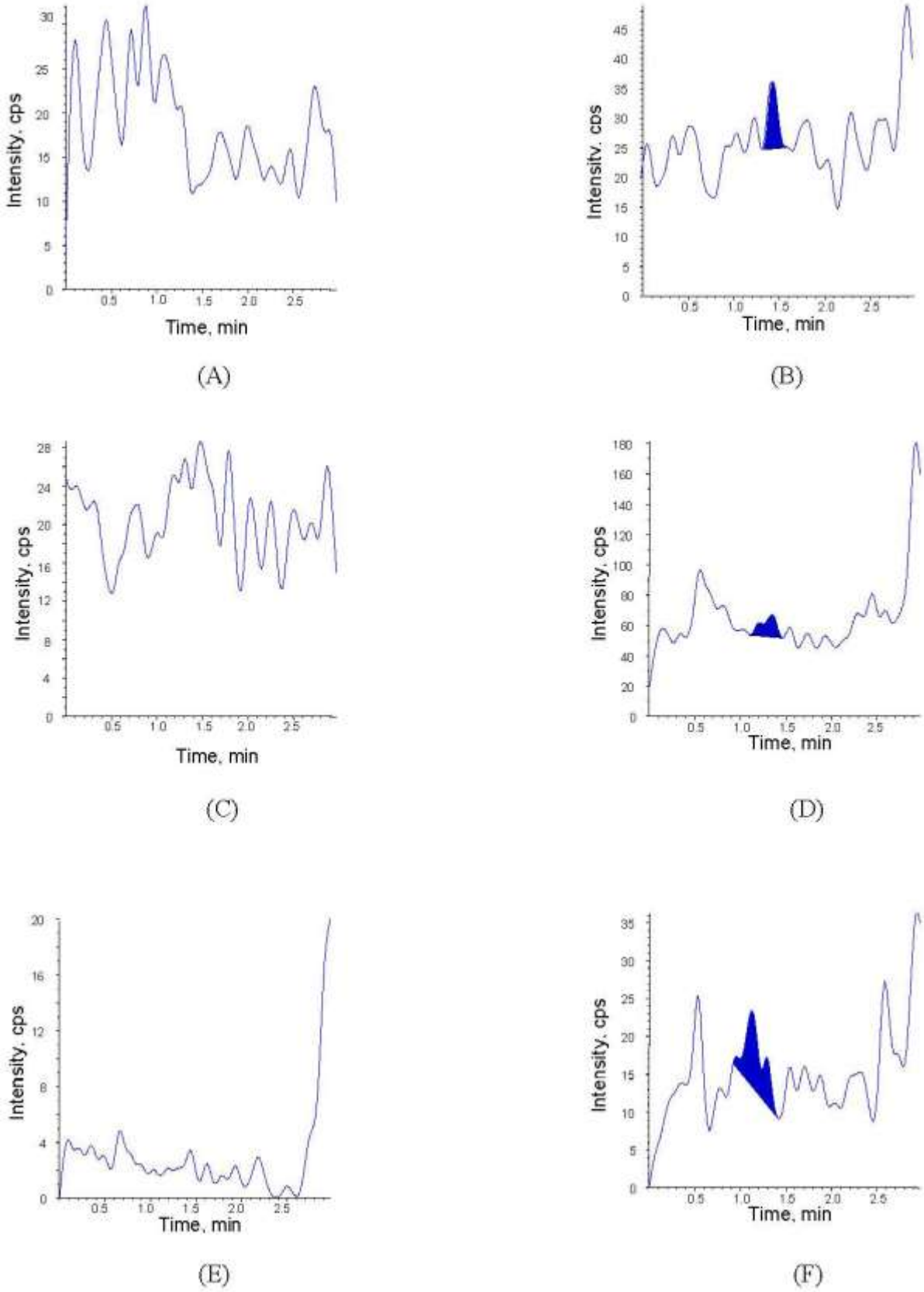

Figure 8: Representative chromatograms of Zidovudine (A), Didanosine (B), Lamivudine (C), Emtricitabine (D), Nevirapine (E) and Abacavir (F) in blank plasma

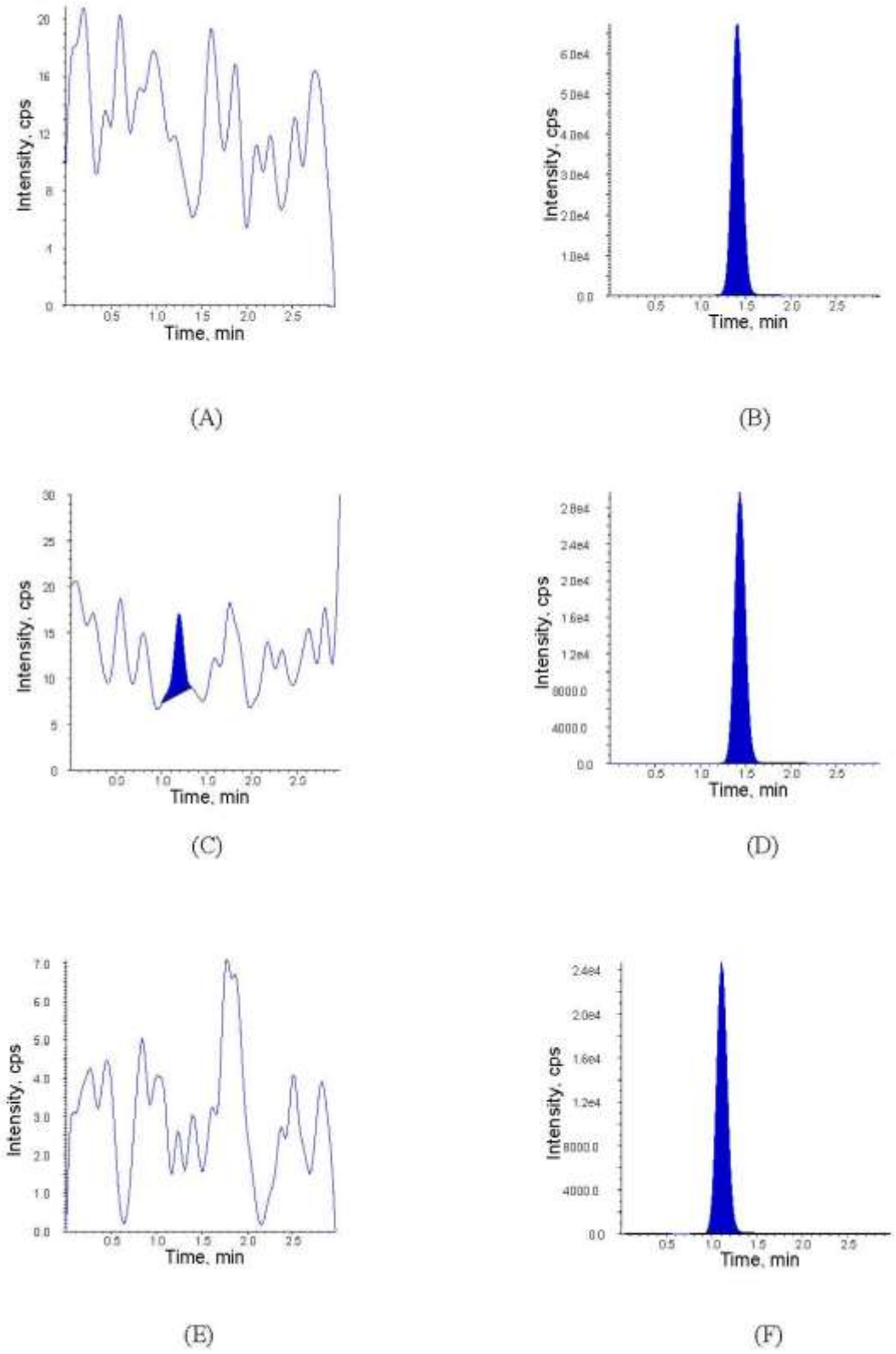

Figure 9: Representative chromatograms of Zidovudine (A), Didanosine (B), Lamivudine (C), Emtricitabine (D), Nevirapine (E) and Abacavir (F) in blank plasma with internal standard

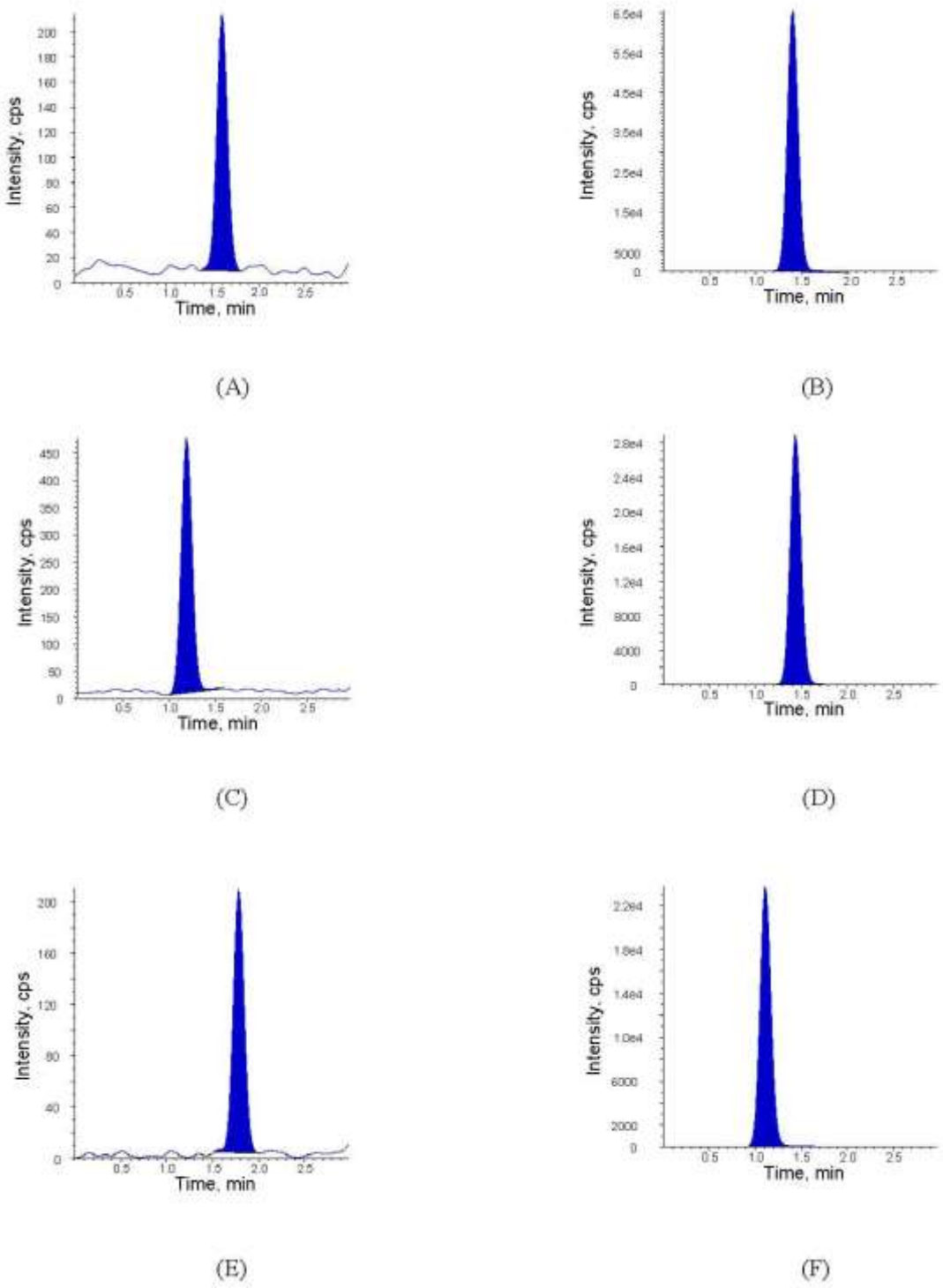

Figure 10: Representative chromatograms of Zidovudine (A), Didanosine (B), Lamivudine (C), Emtricitabine (D), Nevirapine (E) and Abacavir (F) in LLOQ sample

Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to ISTD versus the nominal concentration (x) of analyte. Calibration curves were linear in

the range of 5 to 1500 ng/mL for Zidovudine, Lamivudine and 10 to 3000 ng/mL for Nevirapine with correlation coefficient (r) values more than 0.9973. The r values were calculated from four intra and inter day calibration curves using weighted (1/X2) linear regression analysis. The observed mean back calculated concentrations with accuracy (% Nominal) and precision (%CV) are presented in Table 1. The lower limit of quantitation (LLOQ) for determination of analytes was found to be 5, 5 and 10 µg/mL respectively for Zidovudine, Lamivudine and Nevirapine. At LLOQ (n = 6) accuracy (%RE) was -7.0 to 7.5 with a %CV of <7.8.

Table 1: Summary of Calibration Standards

Analyte	Nominal conc. (ng/mL)	Mean found Conc (ng/mL)	%CV	%RE
Zidovudine	5.01	5.10	1.8	5.2
	10.02	10.74	7.2	2.6
	25.04	22.80	-8.9	4.5
	100.17	102.32	2.1	2.6
	400.67	398.68	-0.5	2.9
	801.34	822.97	2.7	3.5
	1203.21	1202.31	-0.1	0.9
	1504.01	1585.45	5.4	6.2
Lamivudine	5.00	5.06	1.2	2.0
	10.00	9.72	-2.8	3.9
	25.01	25.82	3.2	4.8
	100.03	95.98	-4.0	6.0
	400.11	397.18	-0.7	2.2
	800.21	756.04	-5.5	8.3
	1201.52	1254.30	4.4	2.3
	1501.90	1562.05	4.0	1.9
Nevirapine	10.03	10.10	0.7	0.8
	20.04	19.38	-3.3	-3.4
	50.10	50.68	1.2	1.3
	200.51	211.50	5.5	5.6
	801.84	808.13	0.8	0.9
	1603.75	1533.34	-4.4	-6.2
	2407.97	2371.28	-1.5	-1.9
	3009.98	3028.51	0.6	0.8

[a] - Mean of 4 replicates at each concentration
%CV– Coefficient of variation, %RE – Percent relative error

Precision and accuracy

The precision of a method is defined as the closeness of replicate determinations of an analyte by an assay and accuracy is the closeness of determined value to the true value. Each precision and accuracy run consisting of QC samples (6 replicates each of the LLOQC, LQC, MQC and HQC) were analyzed and back calculated against a set of calibration curve standards. Intra and inter batch precision and accuracy evaluation was done using four different batches analyzed on different days. Results of intra and inter batch accuracy and precision were given in Table 2.

Table 2: Intra batch and inter batch precision and accuracy

Analyte Name	QC level	Nominal conc. (ng/mL)	Intra Batch [a]			Inter Batch[b]		
			Mean Conc Found (ng/mL)	% RE	% Nominal	Mean Conc Found (ng/mL)	% CV	% RE
Zidovudine	LLOQQC	5.01	5.68	11.8	7.2	5.13	2.4	9.6
	LQC	14.79	15.35	3.8	4.3	14.84	-0.3	8.5
	MQC	799.22	838.07	4.9	6.1	810.38	4.9	5.0
	HQC	1200.03	1229.62	2.5	8.7	1242.23	2.5	8.2
Lamivudine	LLOQQC	5.00	5.42	8.4	7.4	5.18	3.6	5.8
	LQC	14.77	13.99	-5.3	1.8	14.78	-0.1	3.9
	MQC	798.58	801.60	0.4	3.7	815.23	2.1	3.0
	HQC	1199.06	1204.46	0.5	3.8	1245.02	3.8	3.2
Nevirapine	LLOQQC	10.03	9.28	-7.4	6.1	9.41	-6.2	7.7
	LQC	29.64	29.73	0.3	1.8	28.16	-5.0	5.1
	MQC	1600.26	1651.16	3.2	3.8	1679.85	5.0	2.0
	HQC	2402.82	2465.55	2.6	3.8	2398.02	-0.2	2.3

%RE, percent Relative error
Conc., Concentration
a 6 replicates at each concentration.
b 24 replicates at each concentration

Matrix effect

Co-eluting matrix components can suppress or enhance the ionization but might not result in a detectable response in matrix blanks due to selectivity of the MS detection, however they can affect the precision and accuracy of the assay. Therefore the potential for variable matrix related ion suppression was evaluated in six independent sources (containing one hemolytic and one lipemic lot) of human plasma, by calculating the IS normalized matrix factor. The mean IS normalized matrix factor between all the analytes was ranged between 0.9727 and 1.1545 with a %CV of 1.5 to 6.6 as shown in Table 3 to 5.

Table 3: Matrix Effect of Zidovudine

Lot #	LQC			HQC		
	MF of Analyte	**MF of ISTD**	**ISTD Normalized Factor**	**MF of Analyte**	**MF of ISTD**	**ISTD Normalized Factor**
1	0.933	1.418	0.658	0.920	0.983	0.936
2	0.976	1.383	0.706	0.919	0.986	0.932
3	0.999	1.465	0.682	0.910	0.951	0.957
4	0.880	1.375	0.640	0.931	0.972	0.958
5	1.019	1.490	0.684	0.914	0.986	0.927
6	0.966	1.364	0.708	0.945	1.015	0.931
Mean	-		0.6796			0.9402
SD			0.02661			0.01374
% CV			3.9			1.5
N			6			6

MF: Matrix Factor

Table 4: Matrix Effect of Lamivudine

Lot #	LQC			HQC		
	MF of Analyte	**MF of ISTD**	**ISTD Normalized Factor**	**MF of Analyte**	**MF of ISTD**	**ISTD Normalized Factor**
1	0.988	1.432	0.690	1.095	0.892	1.229
2	0.954	1.421	0.671	1.079	0.912	1.183
3	1.059	1.562	0.678	1.022	0.815	1.255
4	1.048	1.591	0.659	1.099	0.919	1.196
5	1.197	1.690	0.708	1.054	0.856	1.231
6	1.066	1.644	0.649	1.135	0.944	1.202
Mean	-		0.6759	-		1.2158
SD			0.02155			0.02677
% CV			3.2			2.2
N			6			6

MF: Matrix Factor

Table 5: Matrix Effect of Nevirapine

Lot #	LQC			HQC		
	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	1.022	1.050	0.973	0.989	0.816	1.212
2	0.947	0.994	0.953	1.002	0.817	1.227
3	1.024	1.083	0.946	0.914	0.738	1.240
4	1.006	1.031	0.976	0.966	0.812	1.189
5	1.155	1.254	0.921	0.926	0.779	1.188
6	0.978	0.885	1.105	0.999	0.856	1.168
Mean	-		0.9791	-		1.2039
SD			0.06499			0.02699
% CV			6.6			2.2
N			6			6

MF: Matrix Factor

Extraction recovery and dilution integrity

The extraction recovery of analytes from EDTA plasma was determined by comparing the peak responses of plasma samples (n= 6) spiked before extraction with that of plasma samples spiked after extraction. The mean recovery was found to be 77.6%, 55.9% and 77.1% with %CV of 12.5%, 9.9% and 9.2% for Zidovudine, Lamivudine, and Nevirapine respectively as shown in Table 6. For Internal standards the recovery was found to be 66.2, 71.9 and 78.4% for Didanosine, emtricitabine and Abacavir respectively.

Dilution integrity experiment was carried out at 3 times the ULOQ concentration. After 1/5, 1/10 and 1/50 dilution the mean back calculated concentration for dilution QC samples was within 85–115% of nominal value with a %CV of ≤1.8 as shown in Table 7.

Table 6: Recovery

Analyte	QC Level	A	B	% Recovery	Mean Recovery	% CV
Zidovudine	LQC	4687	5145	91.1	77.6	12.5
	MQC	241699	352159	68.6		
	HQC	362274	495773	73.1		
Lamivudine	LQC	12688	17586	72.1	55.9	9.9
	MQC	585371	1251563	46.8		
	HQC	838202	1718536	48.8		
Nevirapine	LQC	6895	10525	65.5	77.1	9.2
	MQC	424395	489309	86.7		
	HQC	650733	824578	78.9		
Didanosine		297865	449778	66.2	-	
Emtricitabine		248528	345629	71.9		
Abacavir		256288	326854	78.4		

A: Mean Peak response of Extracted Samples, B: Mean Peak response of un Extracted Samples

Table 7: Dilution Integrity

Analyte	Dilution Factor	Nominal conc. (ng/mL)	Mean found conc.[a] (ng/mL)	%RE	%CV
Zidovudine	5	2952.34	2926.41	-0.9	2.5
	10		2953.06	0.0	3.2
	50		2556.23	-13.4	8.9
Lamivudine	5	2926.12	2962.39	1.2	3.9
	10		2862.28	-2.2	4.2
	50		2654.68	-9.3	6.9
Nevirapine	5	6001.85	5688.25	-5.2	2.6
	10		5722.45	-4.7	1.8
	50		5888.25	-1.9	2.5

a: Six replicates at each dilution factor

Ruggedness

Six samples each of LLOQ, LQC, MQC and HQC in human plasma were analyzed along with CC samples. The ruggedness was assessed by changing analyst, solvent lot and different column of same make. Results are summarized in Table 8. The data obtained were within acceptance criteria.

Table 8: Ruggedness

Analyte	QC Level	% RE	% CV
Zidovudine	LLOQ QC	4.4	5.8
	LQC	3.2	2.6
	MQC	1.7	4.8
	HQC	-4.2	3.0
Lamivudine	LLOQ QC	3.6	3.5
	LQC	-0.7	4.6
	MQC	-3.9	3.8
	HQC	-3.0	2.3
	LLOQ QC	-2.9	3.0
Nevirapine	LQC	-6.2	1.5
	MQC	-0.1	4.2
	HQC	-0.7	4.8

N = 6 at each level

Extended Precision and Accuracy Batch

The Extended Precision and Accuracy batch was assessed by processing and analyzing CC samples along with twenty five samples each of LQC,

MQC and HQC in human plasma. Calibration standards were used to determine the accuracy of quality control samples. The batch of 75 samples analysed was found to acceptable for the maximum no of sample that can be processed during routine sample analysis. Results are summarized in Table 9. The data obtained were within acceptance criteria.

Table 9: Extended PA Batch

Analyte	QC Level	% Nominal	% CV
Zidovudine	LQC	-1.4	8.4
	MQC	3.7	4.5
	HQC	-0.1	3.1
Lamivudine	LQC	-2.7	4.9
	MQC	4.5	5.3
	HQC	2.6	5.4
Nevirapine	LQC	-4.9	6.3
	MQC	1.8	5.7
	HQC	-0.4	4.2

N = 25 at each level

Reinjection Reproducibility:

Reinjection reproducibility was performed by injecting the previously passed precision and accuracy batch after a period of 49 hr. The reinjected quality control samples concentrations were back calculated against initially injected CC curve. The % CV of back calculated concentrations for all quality control samples of LQC, MQC and HQC concentration levels ranged from 2.7 to 6.3, 0.9 to 2.0, and 3.0 to 4.9, respectively, for Zidovudine, Lamivudine, and Nevirapine, which are within the acceptance limit of 15.00%. The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels were ranged from 102.0 to 104.2, 93.5 to 96.4, and 95.6 to 99.2, respectively, for Zidovudine, Lamivudine, and Nevirapine, which is within acceptance limit 85.00–115.00%.

Haemolysis Effect

Six samples each of blank matrix, LLOQ, LQC and HQC in 2% and 100% haemolyzed plasma were analyzed along with CC samples (Un-haemolyzed plasma). The overall % nominal were 89.4 to 111.9% with % CV of 1.0 to 7.3%. The data obtained were within acceptance criteria.

Stability

Stability evaluations were performed in both aqueous and matrix based samples. The stock and working solutions were stable for a period of 7 h at room temperature. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The analyte was stable up to 28 h on bench top at room temperature and over 8 freeze-thaw cycles. The processed samples were stable up to 47 h min in autosampler at 4^0C. Reinjection reproducibility is done for 49 h 51 min. The long-term matrix stability was evaluated at both -20^0C and -50^0C over a period of 121 days. The % change shows that there was no significant degradation of analytes was observed over the stability duration and conditions. The stability results presented in Table 6 were within 85-115%.

Table 8: Stability Data

Stability	Analyte	QC Level	A	%CV	B	%CV	%Stability
Bench-top (29 hrs at ~25 °C)	Zidovudine	LQC	14.97	6.9	14.99	6.9	99.9
		HQC	1226.82	6.2	1235.98	7.2	99.3
	Lamivudine	LQC	14.86	8.4	13.79	4.8	107.8
		HQC	1233.59	3.1	1221.06	2.7	101.0
	Nevirapine	LQC	29.87	2.8	29.94	5.1	99.8
		HQC	2425.42	1.5	2497.73	4	97.1
Freeze-thaw (after 8th cycle)	Zidovudine	LQC	14.76	8.4	14.95	6.9	98.7
		HQC	1222.48	2.9	1231.99	7.2	99.2
	Lamivudine	LQC	14.07	4.2	13.89	4.8	101.3
		HQC	1301.77	3.3	1291.06	2.7	100.8
	Nevirapine	LQC	29.02	3.5	28.75	5.1	100.9
		HQC	2343.56	3	2346.75	4	99.9
In-injector (at 10 °C for 42 hrs)	Zidovudine	LQC	15.03	4	15.06	6.7	99.8
		HQC	1166.8	4.4	1136.71	5.4	102.6
	Lamivudine	LQC	13.99	3	13.86	5.9	100.9
		HQC	1205.94	0.5	1214.97	5.4	99.3
	Nevirapine	LQC	30.86	5.7	31.37	5	98.4
		HQC	2363.61	2.5	2500.65	2	94.5
Dry extract (at 1-10 °C for 28 hrs)	Zidovudine	LQC	15.6	2.2	15.06	6.7	103.6
		HQC	1135.32	4.8	1136.71	5.4	99.9
	Lamivudine	LQC	13.83	6.7	13.86	5.9	99.8
		HQC	1188.06	3.2	1214.97	5.4	97.8
	Nevirapine	LQC	30.19	4.9	31.37	5	96.2
		HQC	2431.38	2.3	2500.65	2	97.2
Long-term stability (at -70 °C for 65 days)	Zidovudine	LQC	15.23	4	15.46	6.7	98.5
		HQC	1186.8	4.4	1126.81	5.4	105.3
	Lamivudine	LQC	14.85	3	14.86	5.9	99.9
		HQC	1216.94	0.5	1224.87	5.4	99.4
	Nevirapine	LQC	31.86	5.7	31.27	5	101.9
		HQC	2373.61	2.5	2525.85	2	94.0
Long-term stability (at -20 °C for 65 days)	Zidovudine	LQC	15.6	2.2	15.06	6.7	103.6
		HQC	1135.32	4.8	1136.71	5.4	99.9
	Lamivudine	LQC	13.83	6.7	13.86	5.9	99.8
		HQC	1188.06	3.2	1214.97	5.4	97.8
	Nevirapine	LQC	30.19	4.9	31.37	5	96.2
		HQC	2431.38	2.3	2500.65	2	97.2

A: Mean concentration of stability samples B: Mean concentration of Comparison samples

Whole blood stability

The blood stability was evaluated by comparing the response ratios of stability samples (n=6 at LQC and HQC) against the freshly prepared samples. After spiking in whole blood the samples were placed at room temperature over a period and plasma was separated by centrifugation at 3000 rpm (along with freshly spiked samples, comparison samples) and then processed and analyzed as per

established conditions. The %stability at each low and high QC level is then evaluated by comparing the mean peak response ratio of stability samples against the mean peak response ratio of stability samples. All the three analytes were stable at room temperature up to 2.0 hrs and the % stability was ranged from 92.6 to 96.5.

CONCLUSION

The experiments performed during the validation concluded that the method is validated for the simultaneous quantitation of Zidovudine, Lamivudine, and Nevirapine in human plasma over the concentration range of 5 to 1500 ng/mL for Zidovudine, Lamivudine and 10 to 3000 ng/mL for Nevirapine ng/mL respectively. The precision and mean accuracy are within the acceptable limits. Consistent recoveries were observed for LQC, MQC and HQC. The method is specific enough in the presence of K2EDTA anticoagulant. The method is precise and accurate enough to dilute samples, if necessary. The stability experiments were performed during the validation concluded that the intended analyte and metabolites were stable at different conditions like autosampler (42 hrs), bench top stability (29 hrs), dry extract (28 hrs), long term stability (65 days), and eight freeze and thaw cycles. The analyte, metabolites and ISTD stock solutions were stable at room temperature for 7 hr. Reinjection reproducibility was proved for 49 hr. The method was proved to be rugged by different column. The extended Precision and accuracy batch was proved with 75 QC samples. A rapid, sensitive, high throughput and accurate liquid chromatography with electrospray ionization tandem mass spectrometry method was developed for determination of Zidovudine, Lamivudine, and Nevirapine in human plasma using 96 well plate format solid phase extraction. The extraction method utilizes a low sample volume of 100µL and shown consistent and reproducible recoveries for analyte and ISTD with minimum plasma interference and matrix effect. The validated method can be successfully used to a clinical and tox studies. The method was validated and demonstrated to be robust with high precision and accuracy. The high throughput method can reduce overall processing time and allowing to process and analyze more number of samples in short duration.

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