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DEVELOPMENT AND VALIDATION OF A SENSITIVE LCMS/MS METHOD FOR THE DETERMINATION OF STAVUDINE, LAMIVUDINE AND NEVIRAPINE IN HUMAN PLASMA

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Abstract: A simple and sensitive liquid chromatography tandem mass spectrometry (LCMS/MS) method for the determination of stavudine, lamivudine and nevirapine in human plasma was developed and validated to support the pharmacokinetic studies. Didanosine (for stavudine) and emtricitabine (for lamivudine and nevirapine) are used as internal standards and solid phase extraction was employed as the extraction technique with the sample volume of 300 μ l. 2 mM ammonium acetate buffer and methanol in the ratio of 5:95 was used as mobile phase using C18 zorbax eclipse (4.6x150mm, 5 μ) column for chromatographic separation. ESI/MSMS in the positive mode with the mass transitions (m/z) of 223.00/125.00 for stavudine, 228.10/133.90 for lamivudine, 264.90/181.90 for nevirapine, 234.90/134.90 for didanosine and 245.90/151.90 for emtricitabine were used for detection. The method linearity range was established as 5 to 1500 ng/ml for stavudine, 5 to 2000 ng/ml for lamivudine and 10 to 4000 ng/ml for nevirapine with the weighting factor of $1/X^2$. The method was validated as per the regulatory guidelines and the results of the method validation were in compliance to the acceptable limits specified by the regulatory guidelines confirming the method readiness for the pharmacokinetic studies.

Keywords: Stavudine, Lamivudine, Nevirapine, LCMS/MS, method validation.



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INTRODUCTION

It is estimated that since the beginning of AIDS epidemic almost 60 million people have been infected with HIV and 25 million people have died of HIV related causes. In response to the AIDS epidemic, many developing countries have introduced antiretroviral programs. Because of the high cost of these medications, many programs rely on less costly generic antiretroviral therapy to achieve treatment goals consistent with the World Health Organization (WHO). (Mina et al., 2007). A regimen consisting of lamivudine, stavudine and nevirapine has been shown to be efficacious and safe in both treatment-experienced and treatment-naive patients, regardless of baseline viral loads. (Vishal et al., 2005).

Stavudine is chemically known as 1-(2R,5S)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione and is analogue of thymidine. It is phosphorylated by cellular kinases into active triphosphate. Stavudine triphosphate inhibits the HIV reverse transcriptase by competing with natural substrate, thymidine triphosphate. It also causes termination of DNA synthesis by incorporating into it. Lamivudine is chemically known as 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one and is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of

hepatitis B. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated. Nevirapine is chemically known as 11-cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one. Nevirapine also inhibits the reverse transcriptase enzyme which transcribes viral RNA into DNA. Unlike other reverse transcriptase inhibitors, nevirapine bind allosterically at a distinct site away from the active site termed the NNRTI pocket.

Numerous bioanalytical methods are reported in literature for the estimation of either of the single molecule viz. stavudine or lamivudine or nevirapine (Wiesner et al., 2002; Sarasa et al., 2010; Burger et al., 1992; Jayaseelan et al., 2010; Kano et al., 2005; Purnima et al., 2010; Chinmoy et al., 2011) by using HPLC and LCMSMS. Methods are also reported simultaneous estimation of stavudine and lamivudine (Triporn et al., 2010) and combination of lamivudine with other anti retrovirals like zidovudine (Joseph et al., 2010; Estrela et al., 2004; Tarinas et al., 2007). Few of the methods are also reported for the simultaneous estimation of three molecules using HPLC

(Rafaella et al., 2008) and LCMSMS. (Vishal et al., 2005, Byakika et al., 2008; Ramakrishna et al., 2012; Mistri et al., 2007; Monif et al., 2006; Mina et al., 2007; Li et al., 2010).

The reported literature was established with the lower limit of quantification of around 10 to 20 ng/ml for stavudine and lamivudine and nevirapine with the plasma sample volume of around 300 to 500 μ l. In the present study an attempt was made to develop and validate the bioanalytical method with the lower limit of quantification of 5-10 ng/ml for stavudine, lamivudine and nevirapine with the lower sample volume and shortest possible runtime. The method was validated in compliance to current international regulatory guidelines (USFDA 2001, EMA 2011, ANVISA 2003)

EXPERIMENTAL

Chemicals and reagents

Drug and internal standards are procured from APL research centre and are of acceptable characterization and purity. HPLC grade methanol and acetonitrile are procured from commercial suppliers. HLB cartridges are procured from Waters and human plasma is obtained from commercial supplier. All other solvents/materials are of analytical grade.

LCMS/MS Instrumentation and conditions

LCMS/MS system configuration includes API-4000 mass spectrometer of AB Sciex

attached to Shimadzu HPLC system with auto sampler. Zorbax Eclipse XDB-C18, 4.6x150mm, 5 μ column was used for chromatographic separation and the column oven temperature was maintained at 35°C. Mobile phase consists of 2mM ammonium acetate buffer (pH 7.0 \pm 0.3): Methanol in the ratio of 5:95 v/v and the set flow rate was 0.8 ml/min. Auto sampler set temperature was 10°C and the injection volume was 10 μ l. Methanol water solution in the ratio of 50:50 v/v was used as rinsing solution. Analytes and respective internal standards are detected in the negative mode using multiple reaction monitoring (MRM) mode. Analyst software version 1.4.2 was used for the handling of LCMSMS system. Peak area ratio of analyte to internal standard was used to perform the calculations using the calibration curve constructed with linear least square regression with appropriate weighting.

Preparation of calibration standards, internal standard, and quality controls

2mM ammonium acetate buffer (pH 7.0 \pm 0.3) was prepared by dissolving around 0.15 mg of ammonium acetate in 1000 ml of milli-Q water and after filtration pH of the solution was adjusted to 7.0 \pm 0.3. Mobile phase was prepared by mixing 95 parts of methanol and 5 parts of 2mM ammonium acetate buffer (pH 7.0 \pm 0.3). Methanol:water mixture (50:50v/v) was prepared by mixing equal amounts of methanol and water. 0.1% formic acid solution was prepared by adding water to

the 500 ml volumetric flask containing 0.5 ml of formic acid. Washing solution was prepared by mixing 50 ml of methanol and 950 ml of milli-Q water. Stock solutions of stavudine, lamivudine, nevirapine, emtricitabine and didanosine were prepared by dissolving the appropriate amount of respective working standard in methanol to obtain 1 mg/ml solution.

Calibration curve samples and quality control samples are prepared by spiking respective analyte dilutions to get the desired concentrations. Separate stock solutions are used for the preparation of calibration curve standards and quality control samples. Calibration curve was prepared in the range of 5.00 to 1500 ng/ml for stavudine, 5.00 to 2000 ng/ml for lamivudine and 10 to 4000 ng/ml for nevirapine. Calibration curve consists of eight standards spanning over the range and the quality control samples (LLOQ, LQC, MQC and HQC) are prepared in the concentration range of near to LOQ for LLOQ, around three times of LOQ for LQC, around 40-60% of ULOQ for MQC and around 70-85% for the HQC.

Sample preparation:

Sample preparation was performed by using solid phase extraction technique. Internal standard dilution (50 μ l) was added to the tubes containing 0.3 ml of plasma and to the vortexed tubes 0.3 ml of 0.1% formic acid was added. On the solid phase extraction unit (SPE), HLB (30mg/1cc) cartridges were conditioned with 1 ml

methanol and 1 ml of milli Q water. To the conditioned cartridges, plasma sample was added and washed with 1 ml of milli Q water followed by 1 ml of washing solution. Finally cartridges are eluted with 1 ml of acetonitrile. Eluted solution was evaporated under dry nitrogen gas at about 50°C till the tubes are dried. Evaporated tubes are reconstituted with 0.4 ml of mobile phase and vortex and the solution is transferred into auto sampler vials.

Method validation:

Method validation was performed as per the current regulatory guidelines. During the method validation, parameters evaluated include accuracy, precision, linearity, selectivity, sensitivity, dilution integrity, matrix effect and various stability evaluations. Stability evaluations include, stock solution stability, stock dilution stability, freeze-thaw stability, bench top stability, auto sampler stability, dry extract stability and long term stability.

RESULTS AND DISCUSSION:

Optimization of Chromatographic and sample processing conditions:

Optimization of chromatographic conditions and sample clean up plays an important role in the successful development of bioanalytical methods. Various columns are tested to have a acceptable chromatographic selectivity and sensitivity and the length of the columns ranged from 50 mm to 150 mm with the particle size variation of 3.5 μ to 5 μ and with different

stationary phase (C8, C18). After optimization, the Zorbax Eclipse XDB-C18, 4.6x150mm, 5 μ was finalized to yield acceptable chromatography. During the selection of mobile phase, the effect of buffer composition, strength, various organic solvents, pH on chromatography were studied. Based on peak characterization and signal intensity, 2 mM ammonium acetate buffer and methanol in the ratio of 5:95 with the flow rate of 0.8 ml/min was selected as mobile phase. Different extraction techniques like protein precipitation, liquid-liquid extraction and solid phase extraction with various composition cartridges was evaluated to extract the three analytes and two internal standards. In consideration to avoid matrix extracts and to get cleaner sample extract and optimum recovery, solid phase extraction with HLB cartridges was considered for further analysis. With the above optimized chromatographic conditions, the retention time of all the three analytes and respective internal standards are in the range of 1.8 to 2.1 minutes. Representative chromatographs of blank sample, Lower QC sample and High QC sample was shown in figures 1 to 3.

Method Validation:

Method validation was carried in compliance to various international regulatory guidelines (US FDA,EMA and ANVISA).

Screening and selectivity:

Suitability of the blank plasma lots was tested by evaluating six different lots along with one haemolytic and lipemic lots. The blank plasma lots are processed and analysed and the least interference plasma lot was selected for preparing the LLOQ samples. All the evaluated plasma lots met the acceptance criteria of having the interference at respective analyte retention time less than 20% of mean analyte response of six LLOQ samples and interference at the respective internal standard retention time is less than 5% of mean internal standard response of LLOQ samples.

Spiking solutions check:

Spiking solutions check was performed to confirm the accurate preparation of serial dilutions of calibration and quality control samples before preparing the calibration standard and quality control samples in plasma.

Linearity:

Linearity of the method was established using eight calibration curve standards and the calibration range of the three analytes include 5.00 to 1500 ng/ml for stavudine, 5.00 to 2000 ng/ml for lamivudine and 10 to 4000 ng/ml for nevirapine. Area ratio of analyte and internal standard was plotted against the concentration. Linearity was evaluated using the linear least square regression method with the weighing of $1/(\text{concentration})^2$ to obtain the best fit

with the regression values above 0.98. The average regression value of the four calibration curves evaluated inter and intraday was found to be 0.9996.

The calculated concentrations of the four calibration curves and regression values are reported in table 1.

Accuracy and Precision:

Accuracy and precision of the method was evaluated by analyzing the quality control samples at four levels (Limit of Quantification, low, medium and high) inter and intraday. Six replicates of each quality control samples are analysed against the calibration curve and four accuracy and precision batches were evaluated and the results are reported in table 2. The results are evaluated for meeting the acceptance criteria of accuracy and precision with the range of 15% for the low, middle and high quality control samples and for the lower limit of quantification it was 20%. The results confirm that the method was accurate and precise for the quantification of stavudine, lamivudine and nevirapine in the established concentration range.

Recovery:

Recovery of the method was evaluated by comparing the extracted samples against the unextracted samples. Six replicates of at three QC levels (low, middle and high) are processed and analysed and the mean response at each level was compared against the mean response of six replicates of unextracted samples. Internal standard

recovery was also evaluated at MQC level. The mean recovery of three levels was calculated. The mean recovery of stavudine, lamivudine and nevirapine were 99.4%, 67.8% and 85.4% respectively. The recovery of the didanosine and emtricitabine were found to be 69.7% and 88.8% respectively.

Dilution integrity:

Dilution integrity was evaluated by preparing the QC samples at the concentration of approximately 2 times of the upper limit of quantification and later the sample was diluted 2 fold and 4 fold using the blank plasma to evaluate the impact of dilution. The results confirm that the dilution of the samples with the blank plasma will not have significant impact on the quantification.

Matrix Effect:

Matrix effect was evaluated in six different plasma lots and one haemolytic and one lipemic plasma lot using post extracted and aqueous samples. Six replicates of post extracted samples at low, middle and high QC levels were compared with the six replicates of aqueous samples. Post extracted samples are prepared by processing the blank plasma samples upto evaporation step and then spiking with respective aqueous dilutions to obtain equivalent concentration of extracted samples. Matrix factor of analyte and internal standard was calculated by using the response of post extracted sample and aqueous samples. Internal standard

normalized matrix factor was calculated by the ratio of analyte matrix factor to internal standard matrix factor. The internal standard normalized matrix factor was close to 1 and in the range of 0.85 to 1.15 confirming the insignificant matrix effect.

Stability:

The stability of stavudine, lamivudine and nevirapine and their respective internal standards was investigated in the stock solutions, stock dilutions and in plasma at various stress conditions during storage, processing and analysis. The stability evaluations in plasma samples were evaluated at low and high QC levels and the freshly prepared comparison samples are used for calculating the % stability and both stability and comparison samples are analysed against the freshly prepared calibration curve. % stability between 85% to 115% was considered acceptable. For the

stock solution and stock dilution stability % stability of 90% to 110% was considered acceptable. Summary of the stability data is presented in table 3.

CONCLUSIONS

A simple, selective, sensitive, accurate and precise method using LC-MS/MS for the determination of stavudine, lamivudine, nevirapine using didanosine and emtricitabine as internal standards was developed and validated. Present method achieved the lower limit of quantification of 5 ng/ml for stavudine and lamivudine and 10 ng/ml for nevirapine as desired during the bioequivalence studies for the appropriate characterization of concentration profile. The method was validated in compliance to international regulatory guidelines and thus can be readily applicable for pharmacokinetic studies.

Figure-1:

Representative chromatogram of blank sample:

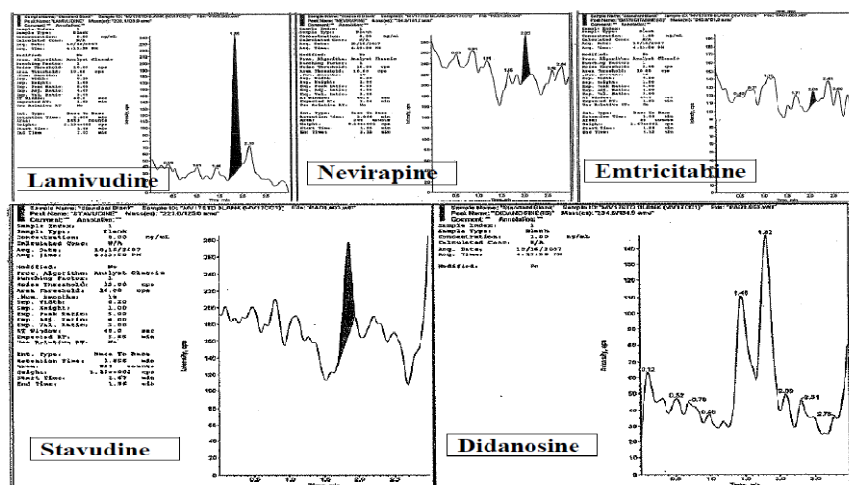


Figure-2:

Representative chromatogram of LLOQ QC sample:

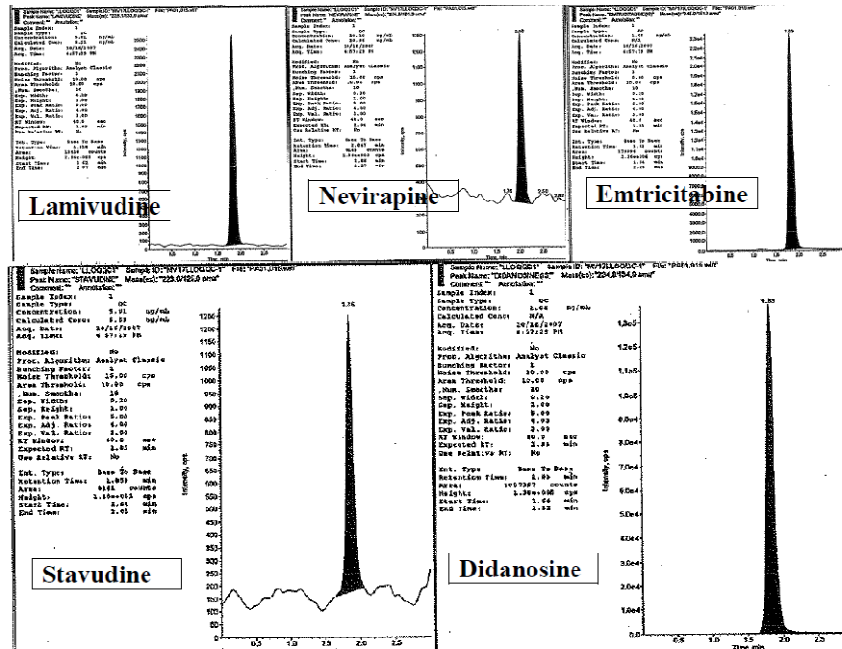


Figure-3:

Representative chromatogram of HQC sample:

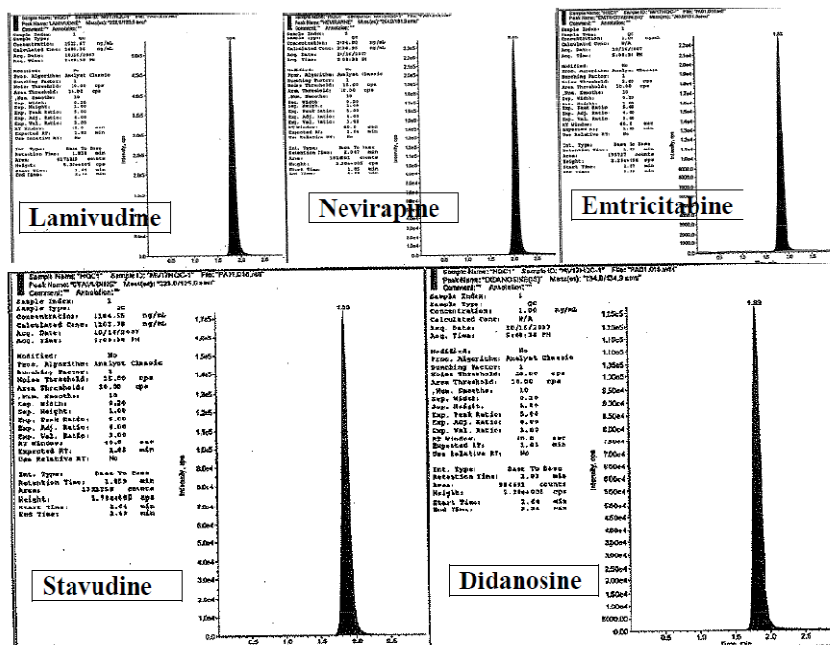


Table 1: Back calculated concentrations of calibration curve (Inter and Intra day)

Back calculated concentrations of calibration curve								
Stavudine								
CC- ID	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8
Nominal Con.	5.00	10.00	50.00	250.00	500.00	999.99	1201.91	1502.39
CC-1	5.07	9.62	52.23	251.44	497.24	962.82	1208.63	1518.12
CC-2	4.94	10.29	49.11	249.46	485.39	1009.27	1216.04	1519.98
CC-3	4.90	10.45	48.85	246.45	490.15	1019.36	1195.37	1529.01
CC-4	4.81	10.73	50.64	249.13	488.76	992.32	1180.76	1507.04
Mean	4.930	10.273	50.208	249.120	490.385	995.943	1200.200	1518.538
SD	0.1080	0.4715	1.5626	2.0516	4.9879	24.7401	15.5265	9.0207
%CV	2.2	4.6	3.1	0.8	1.0	2.5	1.3	0.6
%Nominal	98.6	102.7	100.4	99.6	98.1	99.6	99.9	101.1
Lamivudine								
CC- ID	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8
Nominal Con.	5.00	10.00	50.00	100.01	500.03	1000.07	1603.96	2004.95
CC-1	4.79	10.81	50.51	101.23	508.11	1001.74	1576.20	1881.16
CC-2	4.83	10.53	52.04	106.87	513.05	965.97	1530.33	1855.15
CC-3	4.77	10.78	52.66	105.87	518.59	963.19	1500.00	1848.42
CC-4	4.74	10.93	52.15	105.08	513.99	971.93	1519.25	1844.03
Mean	4.783	10.763	51.840	104.763	513.435	975.708	1531.445	1857.190
SD	0.0377	0.1680	0.9269	2.4663	4.2967	17.7338	32.3613	16.6215
%CV	0.8	1.6	1.8	2.4	0.8	1.8	2.1	0.9

%Nominal	95.7	107.6	103.7	104.8	102.7	97.6	95.5	92.6
Nevirapine								
CC- ID	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8
Nominal Con.	10.0	19.9	49.8	199.1	995.3	1990.6	3201.3	4001.6
CC-1	9.9	20.7	47.1	199.5	994.7	2054.5	3197.0	3971.8
CC-2	9.8	20.6	49.8	221.5	974.6	1892.7	3146.4	3847.7
CC-3	10.0	20.0	46.9	223.3	993.7	1925.0	3139.8	3935.5
CC-4	10.3	18.8	47.8	217.1	1002.5	1970.6	3196.0	3916.3
Mean	10.00	20.03	47.90	215.35	991.38	1960.70	3169.80	3917.83
SD	0.216	0.873	1.324	10.883	11.855	70.226	30.951	52.107
%CV	2.2	4.4	2.8	5.1	1.2	3.6	1.0	1.3
%Nominal	100.0	100.6	96.2	108.2	99.6	98.5	99.0	97.9

Table 2: Back calculated mean concentrations of Quality Control samples (Inter and Intra day)

Back calculated concentrations of Quality Control samples				
QC-ID	LLOQQC	LQC	MQC	HQC
Stavudine				
Nominal Con (ng/ml)	5.01	14.91	677.57	1144.55
Mean	5.522	15.865	653.852	1036.887
SD	0.3661	0.9028	22.6366	40.0510
%CV	6.6	5.7	3.5	3.9
%Nominal	110.2	106.4	96.5	90.6
Lamivudine				
Nominal Con (ng/ml)	5.01	14.92	819.87	1512.67
Mean	4.842	14.226	784.475	1409.257
SD	0.1961	0.4171	15.9897	34.9530
%CV	4.0	2.9	2.0	2.5
%Nominal	96.6	95.3	95.7	93.2
Nevirapine				
Nominal Con (ng/ml)	10.1	29.6	1609.3	3094.8
Mean	9.663	30.438	1685.875	3175.896
SD	0.9663	1.9587	54.4007	72.4229
%CV	10.0	6.4	3.2	2.3
%Nominal	95.7	102.8	104.8	102.6

Table 3: Summary of the stability data:

Short term stock solution stability	9 hours at room temperature
Long term stock solution stability	5 days at 1-10°C
Stock dilution stability	16 hours at room temperature
Bench top stability in plasma	13 hours at room temperature
Freeze-Thaw stability	6 cycles
Auto sampler stability	43 hours in auto sampler (10°C)
Dry extract stability	45 hours at 1-10°C
Long term stability in plasma	75 days at -20°C and 70°C

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