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Stability Indicating Method Development and Validation of Lamivudine, Zidovudine and Nevirapine by Using HPLC

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ABSTRACT

A simple, efficient, and robust stability-indicating RP-HPLC method has been developed and validated to measure Lamivudine, Zidovudine and Nevirapine at a single wavelength (268 nm) in order to assay. The samples were eluted in an isocratic method using an inertsil ODS column (4.6 mm × 250 mm with a particle size of 5 µ) with a mobile phase consisting of 10 mM Ammonium acetate buffer (with a pH adjusted to 3.8 using Acetic acid): acetonitrile (60:40, v/v), acetonitrile and water (1:1, v/v) using as diluent, through ambient temperature delivered at a flow rate 1.2 mL/mins. A good linear response was obtained in the range from 15-75 µg/mL, 30-150 µg/mL and 20-100 µg/mL of Lamivudine, Zidovudine and Nevirapine respectively. The LODs for Lamivudine, Zidovudine and Nevirapine were found to be 0.315, 0.405 and 0.600 µg/mL, respectively and the LOQs for Lamivudine, Zidovudine and Nevirapine were 0.945, 1.080 and 2.100 µg/mL respectively. The method was quantitatively evaluated in terms of accuracy (recovery), linearity, precision, selectivity and robustness in accordance with standard guidelines. The method is simple, suitable and conducive for analyzing Lamivudine, Nevirapine and Zidovudine in bulk and in pharmaceutical formulations.

Keywords: Degradation, HPLC, Lamivudine, Zidovudine, Method development, Validation, Nevirapine

Abbreviations: NRTIs: Nucleotide Reverse Transcriptase Inhibitors; NNRTI: Non-Nucleotide Reverse Transcriptase Inhibitors; UV Detector: Ultraviolet Detector; RSD: Relative Standard Deviation; HPLC: High-Performance Liquid Chromatography; ICH: International Conference on Harmonization; SD: Standard Deviation; PDA: Photodiode array; LOD: Limit of Detection; LOQ: Limit of Qualification; DNA: Deoxyribonucleic acid; RNA: Ribonucleic Acid; HIV: Human Immunodeficiency Virus; LC: Liquid Chromatography; LC-MS: Liquid Chromatography-Mass Spectroscopy; USP: United States Pharmacopeia; AIDS: Acquired Immunodeficiency Syndrome; Rt: Retention time; RT: Room Temperature

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INTRODUCTION

A disease caused by human immunodeficiency virus (HIV) [1-3] is called Acquired Immune Deficiency Syndrome (AIDS) which affects the human immune System. It is the critical clinical effect of infection with HIV, which is a retrovirus that directly and indirectly destroys CD4+ T cells [4]. Present treatment for HIV infection contains highly active antiretroviral therapy that reduces mortality and morbidity of HIV infection Patients [5,6]. The remedy for the treatment of HIV infection is triple drug therapy with a two Nucleoside Analogue Reverse Transcriptase Inhibitors (NRTIs) backbone in combination with a protease inhibitor or a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRT) [7,8]. Both NRTI and NNRTI here inhibit an activity of reverse transcriptase, which is an essential viral enzyme that transcribes viral RNA into DNA. In developing countries, patients are using fixed-dose tablets containing the combination of lamivudine and nevirapine with either Stavudine or Zidovudine [9]. Both Lamivudine and Zidovudine are NRTIs. Zidovudine significantly slows HIV spread, but doesn't stop entirely [10]. This allows HIV to become Zidovudine-resistant over time, for this reason, Zidovudine is usually used in conjunction with other NRTIs and anti-viral drugs to prolong the lifespan of AIDS patients [11-13]. It is highly synergistic to use Lamivudine in combination with Zidovudine. Nevirapine belongs to the class of NNRTI and it is recommended for antiretroviral therapy. It is more effective to use in a combination of three or more antiretroviral drugs as HIV quickly develops resistance to the single antiretroviral drug if it used alone.

In the literature, numerous methods are described to determine separately or in combination of lamivudine, zidovudine and nevirapine with other drugs in pharmaceutical formulation [14-22] still, very few methods are reported to determine nevirapine, zidovudine and lamivudine simultaneously in biological matrices by using High-Performance Liquid Chromatography (HPLC) or LC-MS/MS [23-32].

Our aim was to develop a simple, accurate, sensitive method for simultaneous determination of nevirapine, zidovudine and lamivudine in combined pharmaceutical dosage form by HPLC with UV detection, where simple mobile phase composition was used for chromatographic separation without any ion-pairing agent. Total retention time for analysis was short with a good resolution between these components. All these reasons make this new method was really lucrative. This method was also validated for linearity, accuracy, precision, selectivity, sensitivity and degradation studies according to the ICH guidelines.

EXPERIMENTAL

Chemicals and reagents

Lamivudine, Zidovudine and Nevirapine were obtained from Pharmatrain (Kukatpally, Hyderabad), chemical structures were shown in Figures 1-3. Ammonium acetate, Acetic acid and acetonitrile were used HPLC Grade obtained from Merck (Mumbai, India). Milli-Q-Water resistivity 18.2 MΩ × cm was generated from a Milli-Q-Water purification system manufactured by Millipore (USA).

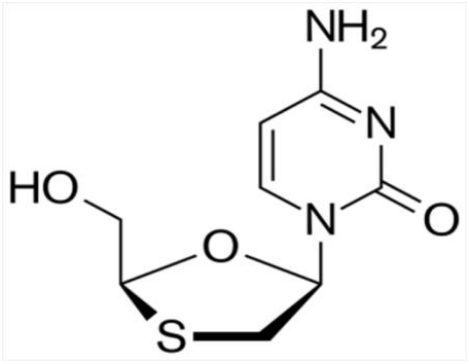

Figure 1: Chemical structure of Lamivudine

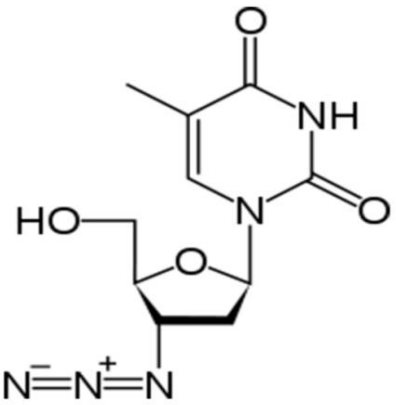

Figure 2: Chemical structure of Zidovudine

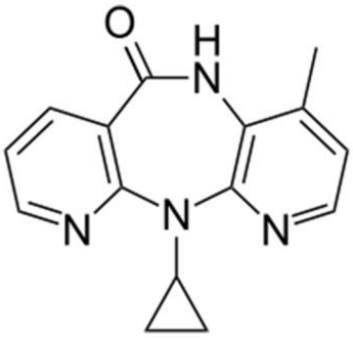

Figure 3: Chemical structure of Nevirapine

Equipment

The HPLC instrument (waters alliance 2695 model) consisted of Quaternary pump (model 2790), low pressure mixing pump and inline vacuum degassing, Flow rates of this system from 50 µl/mins to 5 ml/mins, the maximum capacity of Autosampler has 120 vials with temperature control from 4ºC to 40ºC, column compartment provides ambient to 65ºC and Photodiode array detector (PDA) (model 2996) with a wavelength range of 190-800 nm. The output signal was recorded on the monitor and then quantified by using Empower 2 Software.

Chromatographic conditions

The Chromatographic analysis was performed with an isocratic elution mode for 10 mins run time, with ambient column temperature. The mobile phase consists of 10 mM Ammonium acetate and adjusted pH: 3.8 with Acetic acid- acetonitrile (60:40), the flow rate of pump set 1.2 mL, Inertsil ODS column (length 250 mm × 4.6 inner diameter, 5 µ particle Size), the chromatogram was monitored with UV detection at 268 nm and injection volume 4 µl. Water and acetonitrile in the ratio of 50:50 used as diluents.

METHODOLOGY

Preparation of standard solution

Stock Standard Solution was prepared by taking accurately weighted 15 mg, 30 mg and 20 mg of lamivudine, Zidovudine and Nevirapine working standards into 10 mL cleaned and dried volumetric flask, add diluent let it be dissolved completely and using the same diluent make volume up to the mark. Taken 1.0 mL of the above solution into 10 mL volumetric flask and add diluent to make it up to the mark. For preparing the standard Solution, taken 3.0 mL of solution from above stock solution into 10 mL volumetric flask and add diluent to make it up to the mark.

Assay of pharmaceutical dosage form (sample solution)

For the preparation of the sample solution, the samples of Lamivudine, Zidovudine and Nevirapine are taken in the concentration of 0.045 mg/mL, 0.09 mg/mL and 0.06 mg/mL respectively. The process of preparation is the same as standard solution.

Assay of pharmaceutical formulation

The proposed method was effectively applied to find Lamivudine, Zidovudine and Nevirapine in their tablet dosage form. The results obtained for Lamivudine, Zidovudine and Nevirapine were comparable with the consequently labeled amounts, results were shown in Table 1.

Table 1: Assay results for Lamivudine, Zidovudine and Nevirapine

Drug name	Label Claim (mg)	% Assay
Lamivudine	150	100.89
Zidovudine	300	101
Nevirapine	200	100.25

Method validation

According to the International Conference on Harmonization (ICH) guidelines, this method was validated.

Linearity

Calibration plots for the analytes were prepared with standard stock solutions to yield the concentration of 15-75 µg/mL for Lamivudine, 30-150 µg/mL for Zidovudine, 20-100 µg/mL for Nevirapine into the HPLC system. Taken five concentrations in between the ranges given above, and performed triplicate injections of each concentration. Calibration curves were plotted between concentrations of analytes versus area of that analyte. Linearity regression analysis of the data gave slope, intercept and correlation coefficient value.

Accuracy/Recovery

Standard addition technique was used to perform accuracy by recovery studies. The pre-analyzed samples were spiked with extra 50%, 100%, 150% of each standard Lamivudine, Zidovudine, Nevirapine and the mixtures were analyzed by the method proposed. The recovery studies were conducted in triplicate.

Precision

For the precision, repeatability was carried out for a short time interval under the same chromatographic parameters. For the intermediate precision, repeatability was carried out in a different day under same chromatographic parameters. The sample with the same concentration was injected in six replicates for intraday (precision) and six replicates interday (intermediate precision). The peak area for injection recorded, and calculated RSD for intraday (precision) and interday (intermediate precision).

Robustness

Robustness of the method was carried out by making slight deliberate changes in the analytical methodology like flow rate and wavelength. It was observed that there were no changes in system suitability parameters like USP tailing factor, theoretical plates and resolution, which demonstrated that the developed HPLC method is robust.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The Limit of Detection (LOD) is the lowest amount of analyte in the drug, which can be detected, but not necessarily quantified. The Limit of Quantification (LOQ) is the lowest amount of analyte in the drug, which can be quantitatively determined with suitable precision and accuracy. The LOQ and LOD were determined based on the slope and the standard deviation of the response using the signal-to-noise ratio (S/N) as per ICH Guidelines Q2(R1) 2005.

Degradation studies

According to the ICH guidelines 'Stability testing of new drug substances and products' needs that stress testing be carried out to clarify the inherent stability characteristics of the active substance. The main aim of this work was to carry out the stress degradation studies on the Lamivudine, Zidovudine and Nevirapine using the proposed method.

Hydrolytic degradation under acidic conditions

Pipette 3 mL from a standard stock solution containing 0.15 mg/mL, 0.3 mg/mL and 0.2 mg/mL of Lamivudine, Zidovudine and Nevirapine into a 10 mL flask and added 1.0 mL of 0.1 N HCl. Then, the volumetric flask was kept at room temperature for 6 hrs and then neutralized with 0.1 N NaOH and filled with diluents up to the mark. By using 0.45 microns syringe filters, filter the solution and place in vials.

Hydrolytic degradation under alkaline conditions

Pipette 3 mL from standard stock solution containing 0.15 mg/mL, 0.3 mg/mL and 0.2 mg/mL of Lamivudine, Zidovudine and Nevirapine into a 10 mL flask and added 1 mL of 0.1 N NaOH. Then, the volumetric flask was kept at room temperature for 6 hs and then neutralized with 0.1 N HCl and filled with diluents up to the mark. By using 0.45 microns syringe filters, filter the solution and place in vials.

Thermal induced degradation

The sample was taken in a petri dish and kept in a hot air oven at 105ºC for 48 hrs. Then the sample was taken and diluted with diluents to prepare 45 µg/mL, 90 µg/mL and 60 µg/mL of Lamivudine, Zidovudine and Nevirapine were injected into HPLC and it was analyzed.

Oxidative degradation

Pipette 3 mL from a standard stock solution containing 0.15 mg/mL, 0.3 mg/mL and 0.2 mg/mL of Lamivudine, Zidovudine and Nevirapine into a 10 mL flask and added 1 mL of 3% w/v of hydrogen peroxide as oxidation agent filled with diluents up to the mark. That volumetric flask was then kept at room temperature for 30 mins. By using 0.45 microns syringe filters, filter the solution and place in vials.

RESULTS AND DISCUSSION

To optimize the RP-HPLC parameters, to reach a good resolution and a good peak shape for Lamivudine, Zidovudine and Nevirapine, several chromatographic parameters were tested. Several mobile phases of different ratios were analysed to get sufficient selectivity for the drugs.

Ammonium acetate buffers contributed a higher selectivity and sensitivity than other buffers. Using methanol and acetonitrile as organic components shown results in higher sensitivity, but varying the mobile phase ratio of acetonitrile and methanol were affected in resolution, tailing factor theoretical plates and run time. Varying the pH of the mobile phase resulted in poor peak shapes, not showing good resolution. So, we introduced Ammonium acetate into the mobile phase to adjust the pH of the buffer to 3.8.

The optimized mobile phase consisted of 10 mM ammonium acetate buffer (pH 3.8): acetonitrile (60:40 v/v). The column effluent was monitored at 268 nm. The optimal injection volume was 4 µL. The column's temperature was maintained at 25ºC (ambient). The Inertsil column (4.6 mm × 250 mm with a particle size of 5 µm) was used.

This method is in an isocratic mode with a constant flow rate of 1 mL/mins. Retention times (Rt) of approximately 2.327 ± 0.06 mins, 2.864 ± 0.07 mins and 5.608 ± 0.06 mins were consistently observed for lamivudine, zidovudine and nevirapine respectively, in all the analytical runs. The standard and sample chromatograms were shown in Figures 4 and 5.

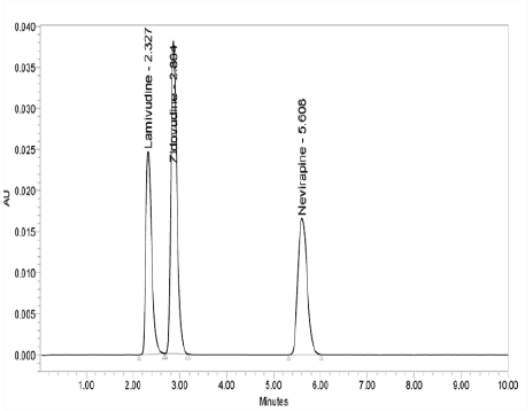

Figure 4: A typical chromatogram of standard solution of Lamivudine, Zidovudine and Nevirapine

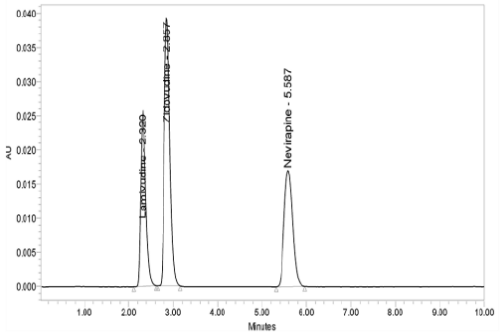

Figure 5: A typical chromatogram of sample solution of Lamivudine, Zidovudine and Nevirapine

Validation of the proposed test

System suitability test

In chromatographic analysis must pass system suitability parameters before the sample analysis can start. The tailing factor (T), theoretical plate number (N) and retention time (Rt) for the principal peak and its degradation product were evaluated for lamivudine, zidovudine and nevirapine. The tailing factors were 1.38 for lamivudine, 1.32 for zidovudine and 1.18 for nevirapine. The theoretical plate numbers (N) were 2715.09 for lamivudine, 3350.07 for zidovudine and 4979.51 for nevirapine. The retention times (Rt) of the drug lamivudine, zidovudine and nevirapine were 2.327 mins, 2.864 mins and 5.608 mins respectively. The results tailing and plate count obtained from the system suitability tests (Table 2) satisfied the USP guidelines and ICH guidelines.

Table 2: System suitability parameters

Parameters	Lamivudine	Zidovudine	Nevirapine
Retention time	2.327	2.864	5.608
Area	212473	338930	228426
USP Plate count	2715.09	3350.07	4979.51
USP Tailing	1.38	1.32	1.18

Linearity

A linear correlation coefficient factor was obtained between the peak area versus concentrations of lamivudine, zidovudine and nevirapine. The calibration curves were linear for concentrations between 15-150 µg/mL. The linearity of the calibration curves was validated by the values of the correlation coefficients (r2).

The correlation coefficients were 0.999 for lamivudine, 0.999 for zidovudine and 0.999 for nevirapine. The results of the linearity experiment are listed in Table 3. Linearity graphs were shown in Figures 6-8.

Table 3: Linearity results for Lamivudine, Zidovudine and Nevirapine

Parameters	Lamivudine	Zidovudine	Nevirapine
Concentration range (µg/mL)	15-75	30-150	20-100
Correlation coefficient	0.999	0.999	0.999
Intercept	518.9	610.7	234.2
Slope	4681	3761	3754

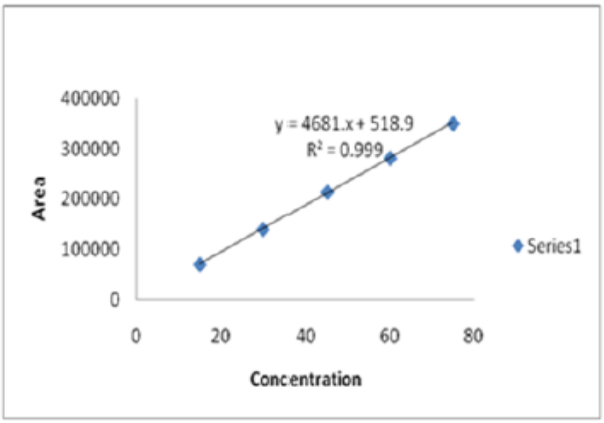

Figure 6: Linearity graph of Lamivudine

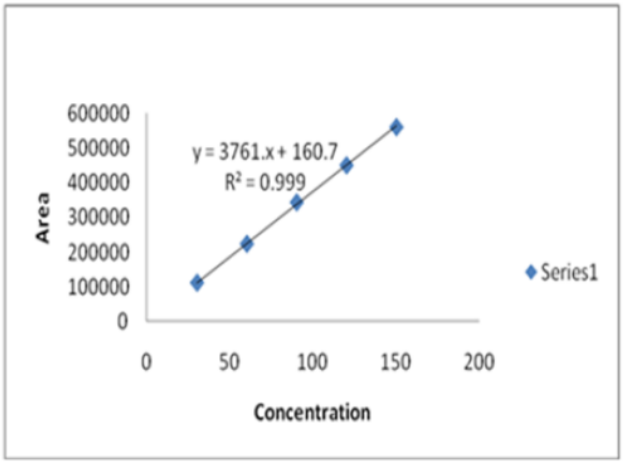

Figure 7: Linearity graph of Zidovudine

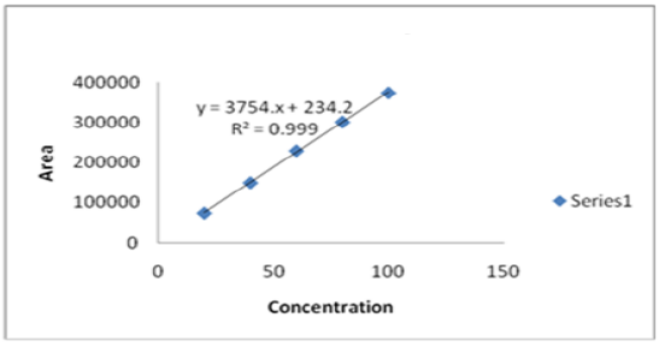

Figure 8: Linearity graph of Nevirapine

Accuracy (% recovery)

The accuracy parameter conducted by using the standard addition method. The proposed method afforded a recovery of 99.49-101.54% after the additional standard drug solution was spiked with the presciently analyzed test solutions. The recovery percentages for lamivudine, zidovudine and nevirapine were in the ranges from 99.80-101.06%, from 100.28 to 101.54% and from 99.49 to 100.63% respectively. The values of the recovery (%) were shown in Table 4, which indicates the accuracy of the proposed method.

Table 4: Accuracy results for Lamivudine, Zidovudine and Nevirapine

Drug Name	% Concentration	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
Lamivudine	50%	169535	7.5	7.49	100.88	100.58
	100%	343335	15	15.16	101.06	
	150%	508669	22.5	22.45	99.8	
Zidovudine	50%	169535	15	15.04	100.28	100.7
	100%	343335	30	30.46	101.54	
	150%	508669	45	45.13	100.29	
Nevirapine	50%	112964	10	9.96	99.55	99.89
	100%	228376	20	20.13	100.63	
	150%	338676	30	29.85	99.49	

Precision

The intra-day precision of the method ranged from 0.5 to 0.7%RSD for lamivudine, zidovudine and nevirapine. The inter-day precision of the method was found to be between 1.4 and 1.6%RSD for lamivudine, zidovudine and nevirapine, which signify that the developed method is precise (Table 5). The lowest values of the RSD (%) indicate that the preferred method is repeatable.

Table 5: Precision and inter-day precision results for Lamivudine, Zidovudine and Nevirapine

Injection	Precision			Inter-day Precision		
	Lamivudine	Zidovudine	Nevirapine	Lamivudine	Zidovudine	Nevirapine
Injection 1	210975	339480	226840	210922	338500	225911
Injection 2	213566	343663	228902	211976	339880	225790
Injection 3	214047	343686	229939	210581	337668	224726
Injection 4	214393	343710	228344	217253	347991	230596
Injection 5	213869	343021	228951	217795	350093	232001
Injection 6	215322	345443	230317	215853	346856	230473
Average	213695	343167	228882	214063	343498	228249
Standard deviation	1463	1980	1236	3276	5422.5	3113
% RSD	0.7	0.6	0.5	1.5	1.6	1.4

LOD and LOQ

The LODs for lamivudine, zidovudine and nevirapine were found to be 0.315, 0.405 and 0.600 µg/mL respectively, and the LOQs for lamivudine, zidovudine and nevirapine were 0.945, 1.080 and 2.100 µg/mL respectively (Table 6).

Table 6: LOD & LOQ results for Lamivudine, Zidovudine and Nevirapine

Drug	LOD Concentration (in µg/mL)	S/N Ratio Value	LOQ Concentration (in µg/mL)	S/N Ratio Value
Lamivudine	0.315	2.98	0.945	9.98
Zidovudine	0.405	2.97	1.08	9.97
Nevirapine	0.6	2.98	2.1	9.97

Robustness

Deliberate changes in the method, i.e., changes in the flow rate and the wavelength, did not significantly affect the peak tailing, the theoretical plates (Table 7).

Table 7: Robustness results for Lamivudine, Zidovudine and Nevirapine

Drug name	Flow Rate (mL/mins)	System Suitability Results		Wavelength (nm)	System Suitability Results	
		USP Plate Count	USP Tailing		USPPlate Count	USP Tailing
Lamivudine	1.08	2828.94	1.42	265	2796.7	1.42
	1.2	2715.09	1.38	268	2715.09	1.38
	1.32	2773.51	1.43	271	2811.61	1.43
	1.08	3528.32	1.35	265	3466.2	1.34
Zidovudine	1.2	3350.07	1.32	268	3350.07	1.32
	1.32	3442.59	1.35	271	3483.38	1.35
	1.08	5312.75	1.21	265	5143.88	1.2
Nevirapine	1.2	4979.51	1.18	268	4979.51	1.18
	1.32	5101.72	1.21	271	5196.02	1.22

Stability indication

Stability testing drug Substances and Product require that stress testing is conducted to clarify the inherent stability characteristics of an active substance and to rapidly identify differences that might result from changes in the manufacturing process or the sample's source. Formulation drug products were exposed to thermal stress, hydrolytic stress under basic and acidic medium and oxidative stress. An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products. So described, different types of stress used were thermal, oxidation, base hydrolysis and acid hydrolysis. Although unknown degradant peaks were observed in the acid, base, peroxide and thermal study, no degradant peaks were reported at the retention time (Rt) of lamivudine, zidovudine and nevirapine. Therefore, lamivudine, zidovudine and nevirapine are stable up to the specified period (12 h) when the proposed method is used, or they are susceptible to acids, alkali, heat and hydrogen peroxide.

Degradation of lamivudine, zidovudine and nevirapine in 0.1 N HCl (acidic conditions) at room temperature (RT) for 6 hrs under reflux conditions

The results showed multiple peaks for the degradation products. The percentages of drug degradations observed were 5.00%, 8.03% and 20.69% for lamivudine, zidovudine and nevirapine (Table 8), here in acidic medium, nevirapine is more degrade than lamivudine and zidovudine. Here no degradant peaks were observed in the retention time (Rt) of lamivudine, zidovudine and nevirapine (Figure 9).

Table 8: Degradation results for Lamivudine, Zidovudine and Nevirapine

Type of degradetion	Lamivudine			Zidovudine			Nevirapine		
	Sample Area	% Recovered	% of Degradation	Sample Area	% Recovered	% of Degradation	Sample Area	% Recovered	%of Degradation
Acid	195408	91.97	5	314828	92.89	8.03	182265	79.79	20.69
Alkali	202579	95.34	14.39	319278	94.2	13.73	214457	93.88	16.66
Thermal	192483	90.59	9.41	297214	87.69	12.31	199367	87.28	12.72
Oxidative	205404	96.67	3.33	304109	89.73	10.27	195093	85.41	14.59

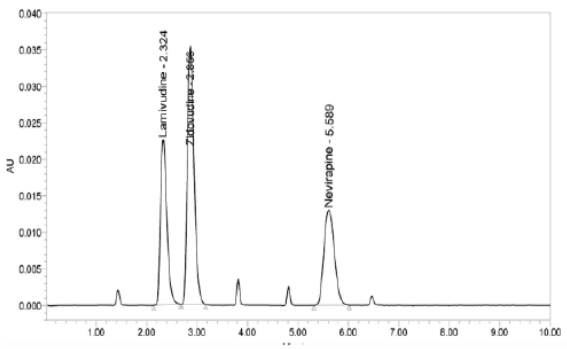

Figure 9: A typical HPLC chromatogram of acid stressed sample of Lamivudine, Zidovudine and Nevirapine

Degradation of lamivudine, zidovudine and nevirapine in 0.1 N NaOH (alkali conditions) at room temperature (RT) for 6 hrs under reflux conditions

The results showed multiple peaks for the degradation products. The percentages drug degradations observed were 14.39%, 13.73% and 16.66% for lamivudine, zidovudine and nevirapine (Table 8), here in alkali medium, nevirapine is more degrade than lamivudine and zidovudine. No degradant peaks were observed here, at the retention time (Rt) of lamivudine, zidovudine and nevirapine (Figure 10).

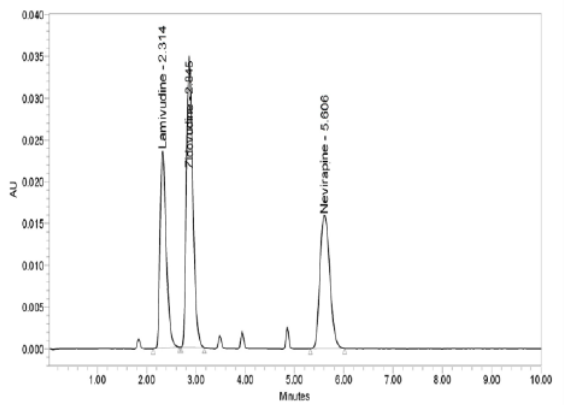

Figure 10: A typical HPLC chromatogram of alkali stressed sample of lamivudine, zidovudine and nevirapine

Oxidation degradation of lamivudine, zidovudine and nevirapine in 3% H2O2 at room temperature (RT) for approximately 30 mins under reflux conditions:

The sample and drug substances were treated with a 3% solution of hydrogen peroxide and kept at room temperature (RT) under reflux conditions for approximately 30 mins. The observed% degradations of lamivudine, zidovudine and nevirapine were 3.33%, 10.27% and 14.59% respectively (Table 8). Therefore, note that nevirapine exhibited the maximum amount of degradation under peroxide degradation conditions. Here no degradant peaks were observed, at the retention time (Rt) of lamivudine, zidovudine and nevirapine (Figure 11).

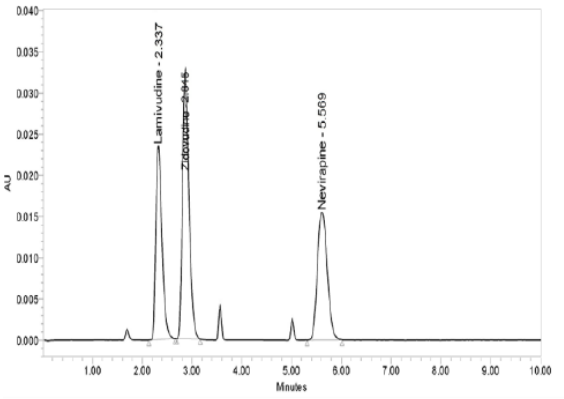

Figure 11: A typical HPLC chromatogram of oxidative stressed sample of Lamivudine, Zidovudine and Nevirapine

Thermal degradation of lamivudine, zidovudine and nevirapine at 105°C for approximately 48 hrs

The thermal degradation of lamivudine, zidovudine and nevirapine at 105ºC for approximately 48 hrs in a hot air oven was studied. Degradation peaks were found in drug products. No degradant peaks were observed here, at the retention time (Rt) lamivudine, zidovudine and nevirapine (Figure 12). The percent degradations of lamivudine, zidovudine and nevirapine were found to be 9.41, 12.31 and 12.72% respectively (Table 8).

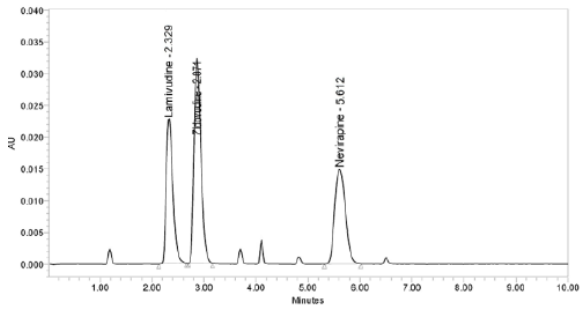

Figure 12: A typical HPLC chromatogram of thermal stressed sample of Lamivudine, Zidovudine and Nevirapine

CONCLUSION

The proposed RP-HPLC method is accurate, precise, rapid, robust, sensitive and selective for lamivudine, zidovudine and nevirapine. The prescribed method adapted the use of an economical and easily available mobile phase, a UV detector, convenient and easy extraction procedures. And also this method is most suitable for analysis of LC-MS. Washing the column with water and acetonitrile (1:1) ratio and conditioning the column with the mobile phase made it an excellent method for the quantification of lamivudine, zidovudine and nevirapine in bulk drugs and in their pharmaceutical dosage forms. A stability-indicating RP-HPLC method for the estimation of lamivudine, zidovudine and nevirapine in their solid dosage forms was established and validated in accordance with the ICH guidelines. The peak purity data and forced degradation experiment data confirmed that there was no interference of the peaks of the active ingredients with those of any other degradation products or other additives. The developed method can be useful for regular analyses of drugs in bulk and in different formulations without any interference of excipients.

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