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A Novel and Rapid Stability Indicating Separation Technique for Simultaneous Determination of Potential Impurities Associated with Stavudine, Lamivudine and Nevirapine in Fixed Dose Combination by UPLC

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ABSTRACT

A simple, precise, accurate stability-indicating Ultra-Performance Liquid Chromatography (UPLC) method was developed and validated for the simultaneous determination of known potential impurities present in Stavudine (ST), Lamivudine (LA) and Nevirapine (NE) in fixed dose combination drug. The desired chromatographic separation was performed on Acquity UPLC HSS-T3 (2.1 × 100 mm), 1.8 µ column using gradient elution of 0.1% perchloric acid in water adjusted the pH 2.6 with diluted sodium hydroxide solution as mobile phase A and acetonitrile as mobile phase B at flow rate of 0.2 ml/min. UV detection was performed at 265 nm. Total run time was 18 min in which main actives and eleven known and major unknown impurities were separated. The method was validated according to the International Conference on Harmonization (ICH) guidelines with respect to specificity, precision, accuracy, linearity, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ). The developed and validated Reverse Phase-Ultra Performance Liquid Chromatography (RP-UPLC) method is specific for quantification of all potential impurities present in the stavudine/lamivudine/nevirapine tablets.

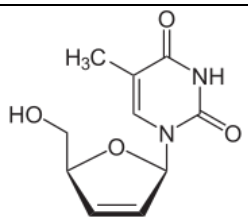
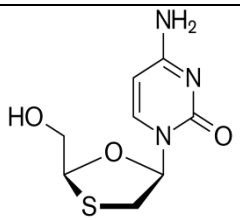
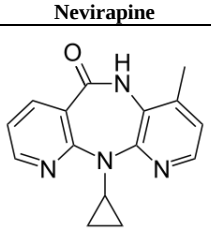
Keywords: Forced Degradation, Lamivudine, Nevirapine, RP-UPLC, Stavudine, Stability Indicating

INTRODUCTION

Stavudine/lamivudine/nevirapine tablets are a fixed dose combination drug product used to treatment for Human Immunodeficiency Virus (HIV). The medication effects by slowing the reproduction of virus cells in the body making it easier for the immune system to control the infection. This treatment is only meant to alleviate health condition and will not completely cure the infection. Stavudine (ST) is a Nucleoside Analogue Reverse-transcriptase Inhibitor (NARTI) active against HIV. Lamivudine (LA) is a potent Nucleoside Reverse Transcriptase Inhibitor (NRTI). Nevirapine (NE) is a non-nucleoside reverse transcriptase inhibitor.

ST is chemically known as 1-[(2R, 5S)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methylpyrimidine-2,4-dione with molecular formula $C_{10}H_{12}N_2O_4$ and molecular weight 224.21 g/mol. It is soluble in water, sparingly soluble in ethanol. LA chemically known as 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1,3-oxathioLn-5-yl] pyrimidin-2-one. LA molecular formula is $C_8H_{11}N_3O_3S$ and its molecular weight is 229.26. LA is highly soluble in water and it's solubility in water is about 70 mg/ml, soluble in Dimethyl Sulfoxide (DMSO), slightly soluble in ethanol. Chemical formula of Nevirapine (NE) is 11-cyclopropyl-4-methyl-5H-dipyrido[2,3-e:2',3'-f][1,4]diazepin-6-one with molecular weight 266.29 and molecular formula $C_{15}H_{14}N_4O$. Nevirapine is practically insoluble in water, sparingly to slightly soluble in dichloromethane Figure 1.

Figure 1: Structural formula of stavudine, lamivudine and nevirapine

Stavudine	Lamivudine	Nevirapine
		

In the course of literature study there are different analytical techniques available for determination of the present actives individually and in combination like high throughput Liquid Chromatography Tandem-mass Spectrometry (LC-MS/MS) method is available for quantification of ST/LA/NE in human plasma [1]. Dissolution method using Ultra Performance Liquid Chromatography (UPLC) [2], normal phase High Performance Thin Layer Chromatography (HPTLC) [3], simultaneous quantification techniques using UV spectrophotometric, High Performance Liquid Chromatography (HPLC) and HPTLC [4] and HPLC method for quantification ST/LA/NE in fixed dose combination product [5], were reported. There is no supported study or complete paper available for determination and quantification of potential impurities present in ST/LA/NE tablets either by using HPLC or UPLC. The present study demonstrates a simple, rapid and accurate method by UPLC having multiple advantages in terms of sensitivity, selectivity, reproducibility and fast analytical technique. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceuticals and biomedical analysis. In the present work, this technology has been applied to the method development and validation study for quantification of potential impurities present in ST, LA and NE in ST/LA/NE tablets.

The combination of ST/LA/NE is not official in any pharmacopoeia. So far, no RP-UPLC stability indicating method has been reported for the rapid simultaneous determination of eleven potential impurities in ST, LA and NE tablets. The proposed method is able to separate eleven potential impurities in ST, LA and NE tablets in 18 min of run time. Thereafter, this method was validated according to International Conference on Harmonization (ICH) recommendation [6-10]. Forced degradation or stress testing studies are part of the analytical development strategy and are also an integral component of validating analytical method that indicate stability of the method. These studies were conducted on finished dosage form to identify degrading species formed during different conditions and storage.

EXPERIMENTAL CONDITIONS AND METHODS

Equipments

Acquity UPLC system consisting of photodiode array detector, column compartment, binary gradient pump, Auto sampler was used for the analysis. The software utilized for the analysis was Empower-3.

Standards, chemicals and reagents

Impurity standards and tablets of ST, LA and NE were sponsored by Aurobindo Pharma limited. Perchloric acid, acetonitrile of gradient grade and sodium hydroxide AR grade chemicals procured from Merck chemicals. The reagents and chemicals in this study were used as such without further purification. The possible potential impurities that may arise from ST, LA and NE tablets are mentioned below. Structures are illustrated in Figure 2.

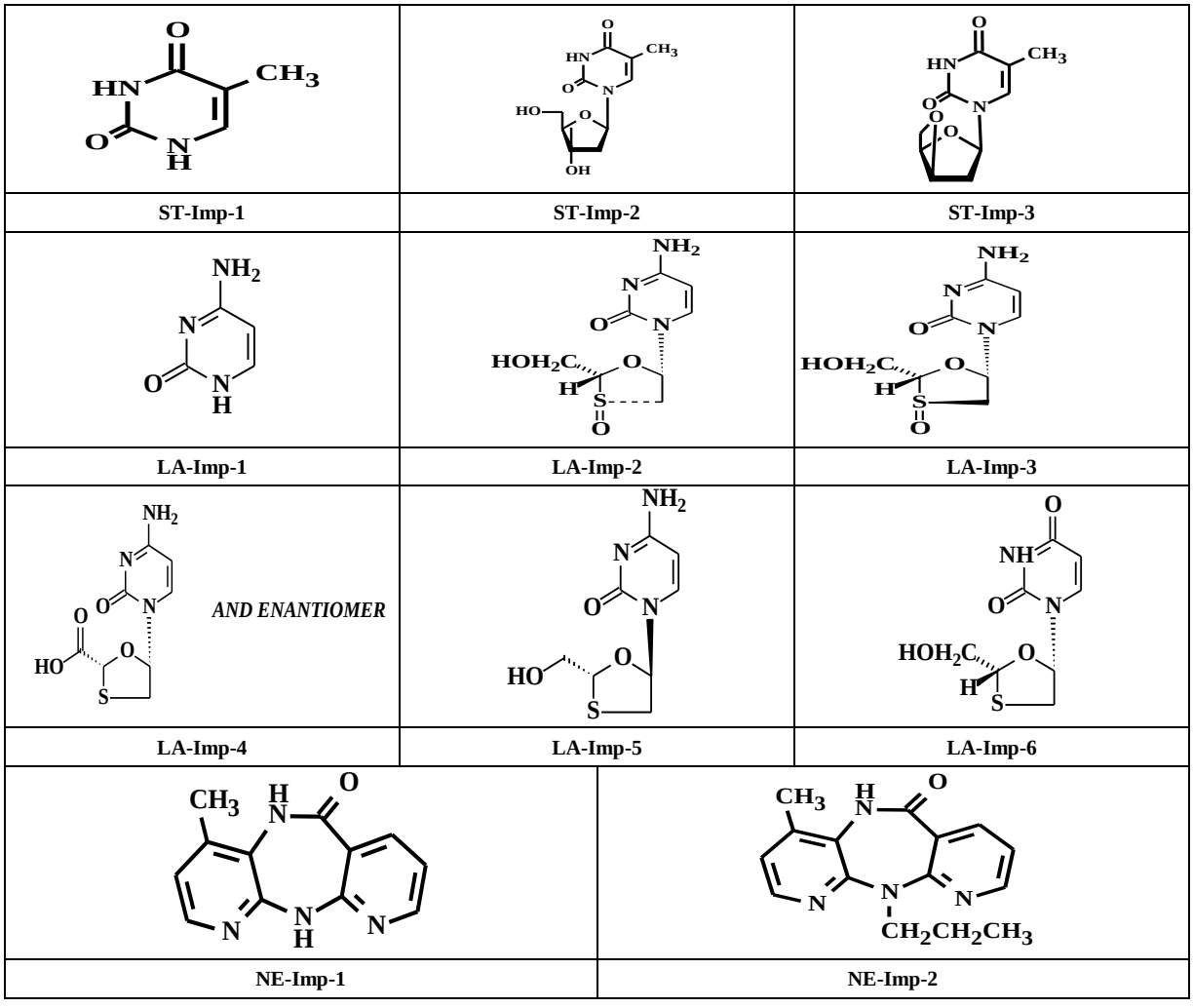


Figure 2: Potential Impurities of stavudine, lamivudine and nevirapine

5-Methylpyrimidine-2,4(1H, 3H)-Dione[Thymine]-ST-Imp-1; 1-(2-Deoxy-β-D-Ribofuranosyl)-5-Methyluracil[β-THYMIDINE]-ST-Imp-2; 3',5'-Anhydro thymidine-ST-Imp-3; 4-Aminopyrimidin-2(1H)-One [Cytosine]-LA-Imp-1; 4-Amino-1-[(2*R*,3*S*,5*S*)-2-(hydroxymethyl)-3-oxo-[1,3]-oxathiolan-5-yl]-pyrimidin-2(1*H*)-one [Lamivudine-(*S*-Sulfoxide)]-LA-Imp-2; 4-Amino-1-[(2*R*,3*R*,5*S*)-2-(Hydroxymethyl)-3-Oxo-[1,3]-Oxathiolan-5-yl]-Pyrimidin-2(1*H*)-one [Lamivudine-(*R*-Sulfoxide)]-LA-Imp-3; *Cis*-5-(4-Amino-2-Oxopyrimidin-1(2*H*)-yl)-1,3-Oxathiolane-2-Carboxylicacid [Lamivudine Acid]-LA-Imp-4; 4-Amino-1-[trans-2-(Hydroxymethyl)-1,3-Oxathiolan-5-YL]-Pyrimidin-2(*1H*)-One[Lamivudine diastereoisomer]-LA-Imp-5; 1-[(2R,5S)-2-(Hydroxymethyl)-[1,3]-Oxathiolan-5-yl]pyrimidin-2,4(1H,3H)-dione[Lamivudine-uracil derivative] [Ph.Eur. Impurity-J]-LA-Imp-6; 5,11-Dihydro-4-Methyl-6H-Dipyrido[3,2-B:2′,3′-E][1,4]Diazepin-6-one[Descyclopropyl Nevirapine (USP Related Compound B)]-NE-Imp-1; 5,11-Dihydro-6H-4-Methyl-11-(N-Propyl)-Dipyrido[3,2-b:2′,3′-e][1,4]Diazepin-6-one[N-Propylnevirapine (USP Related Compound C)]-NE-Imp-2

UPLC method development and optimization of stability indicating method

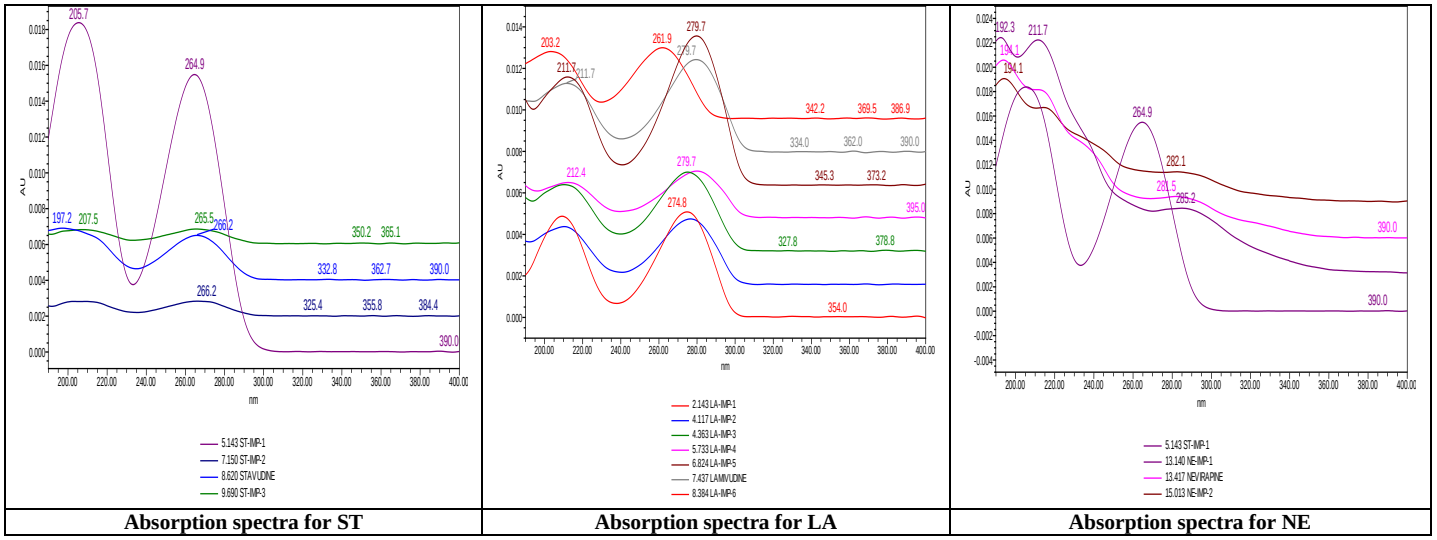
The UPLC method was aimed to develop a method, which is able to resolve the process and degradation impurities of ST, LA and NE. pKa of LA is found to be about 14.29, ST is about 9.95 and for NE is about 10.37 and 5.06. Since LA is highly polar in nature and NE is highly non-polar in nature, in traditional C8 and C18 columns were not able to get the optimum separations between the polar and non-polar impurities. Hence it was proposed use Acquity UPLC HSS-T3 (2.1 × 100 mm) 1.8 µ column which is silica-based bonded phase compatible with 100% aqueous mobile phase and should be used for separations of polar and non-polar compounds. Due to highly polar nature of S it is preferred to choose ion pair reagent for separation purpose.

Trials were initiated using 0.1% perchloric acid in water with pH 2.6 (adjusted with diluted sodium hydroxide solution) as mobile phase-A. Acetonitrile is used as mobile phase-B. Due to the diverse range of polarities for ST, LA, NE and its related impurities, gradient elution mode was preferred over isocratic elution technique.

0.1% perchloric acid in water with pH 2.6 as mobile phase-A provided the adequate and acceptable selectivity pattern between LA, ST, NE and its corresponding impurities. Since ST, LA and NE contains basic amine functional groups, Acquity UPLC HSS T3 (100 × 2.1 mm), 1.8 µ particle size column was selected for the method development which contains high strength silica particles giving the widest usable pH range (pH 1-12), that produces excellent peak shape for bases and the same column was used for method development and validation. The column oven temperature at 25°C was found to be suitable for optimum separaion between impurities.

Impurity mixture solutions were prepared at specification level to know the elution pattern. For ST all impurities prepared at 0.2% level except for ST-Imp-1, prepared at 2.5% level, all LA impurities were prepared at 0.2% level, and NE impurities were prepared at 0.30% and 0.20% for NE-Imp-1, NE-Imp-2 respectively against sample test concentration of 160, 600 and 800 µg/ml for ST, LA and NE respectively. A degassed mixture of water and Acetonitrile in the ratio of 80:20 v/v used as diluent. All impurities were dissolved along with all drug components was found to be suitable for sample extraction with finalized diluent.

The spectral data for majority of impurities of ST, LA and NE has shown wavelength maxima at about 265 nm (Figure 3), the same maxima of 265 nm has been chosen for quantification of impurities. The injection volume 1 µl has been chosen and found satisfactory area counts for impurities as well as main drug. Hence the same injection volume was fixed.



Absorption spectra for ST	Absorption spectra for LA	Absorption spectra for NE

Figure 3: Absorption spectral characteristics of ST, LA and NE impurities

Analytical finalized chromatographic parameters for method validation

The column used was Acquity UPLC HSS T3 (100 × 2.1 mm), 1.8 µ particle size. The mobile phase buffer consists of 1 ml of perchloric acid in 1000 ml of Milli-Q water filtered through 0.22 µ membrane filter as a mobile phase-A and acetonitrile as mobile phase-B with the gradient programme given in Table 1. The column oven temperature was 25°C and the injection volume was 1 µl. The detection was at a wavelength 265 nm. Water and acetonitrile in the ratio of 80:20 v/v was used as diluent.

The typical relative retention times of LA-Imp-1, LA-Imp-2, LA-Imp-3, ST-Imp-1, LA-Imp-4, LA-Imp-5, ST-imp-2, LA, LA-Imp-6, S, ST-Imp-3, NE-Imp-1, NE, NE-Imp-2 are about 0.16, 0.30, 0.32, 0.38, 0.43, 0.51, 0.53, 0.55, 0.62, 0.64, 0.72, 0.98, 1.00 and 1.12 respectively.

Table 1: Gradient program

Time (min)	Flow rate ($ml.min^{-1}$)	Mobile phase-A (%)	Mobile phase-B (%)
0.0	0.20	100.0	0.0
4.0	0.20	95.0	5.0
10.0	0.20	85.0	15.0
12.0	0.20	60.0	40.0
15.0	0.20	60.0	40.0
15.2	0.20	100.0	0.0
18.0	0.20	100.0	0.0

Preparation of solutions

Standard solution

Standard stock solutions of LA, ST, NE (0.04 mg/ml, 0.027 mg/ml and 0.054 mg/ml respectively) were prepared in diluent. Further standard stock solution was diluted to get the concentrations about 0.8, 1.2 and 1.6 µg/ml of ST, LA and NE respectively.

Impurity solutions

All impurities were initially dissolved in an appropriate amount of water and acetonitrile, followed by using diluent to acquire desired concentration levels for validation purpose.

Preparation of sample solution

Ten tablets were crushed to a fine powder by using mortar and pestle. Accurately weighed crushed powder equivalent to about one tablet was transferred into a 250 ml clean, dry volumetric flask. Added about 50 ml of acetonitrile, shanked gently for 10 min by means of hand shaking, without formation of lumps. Added about 150 ml of water, shanked for 5 min with hand shaking and sonicate for 25 min with intermittent shaking in controlled temperature. Diluted to volume with water and mix. Filtered through 0.22 µm membrane filter (Millipore PVDF/mdi Nylon).

Chromatographic system suitability parameters

RSD for peak areas of six replicate injections of the standard solution is NMT 5.0%. The column effectiveness as determined from standard solution for ST, LA and NE is NLT 25000 USP plate count. The Symmetry factor for the same peaks is NMT 2.0 (Table 2).

Table 2: Chromatographic system suitability data

Name of the component	USP theoretical plates	USP tailing factor	% RSD
Stavudine	91371	1.27	1.05
Lamivudine	55058	1.26	1.00
Nevirapine	326606	1.37	0.67

Analytical method validation

ST/LA/NE tablets available in two different strengths 40/150/200 mg and 30/150/200 mg. For validation objective 40/150/200 mg strength has been selected. The method was validated for Specificity, forced degradation studies, precision, sensitivity (LOD and LOQ), linearity, range, accuracy, solution stability and robustness as per ICH general recommendation.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities the specificity of the developed LC method was checked with respect to diluent, placebo used in sample matrix and with eleven potential impurities in presence of ST/LA/NE tablets.

Generation of forced degradation samples

Stress degradation studies were performed for ST/LA/NE in tablet dosage form to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted by exposing it with an acid hydrolysis (5 N HCl/5 ml/RT/60 min), base hydrolysis (5 N NaOH/5 ml/ RT/60 min), oxidation (30% H_2O_2/5 ml/RTC/30 min), thermal stress (80°C/24 h), humidity stress (90% RH/24 h) and photolytic stress (white fluorescent 1.2 million lux hours UV 200 watt hr/m^2 for 7 days).

Precision

Six sample preparations were prepared and calculated the percent Relative standard deviation of each individual impurity as per the ICH limits.

Intermediate precision

The intermediate precision (Ruggedness) of the method was evaluated by different analyst using different column and different UPLC nstrument on different day.

Sensitivity

The Limit of Detection (LOD) of a compound is defined as the lowest concentration that can be detected. The Limit of Quantification (LOQ) is the lowest concentration of a compound that can be quantified with acceptable precision and accuracy. The LOD and LOQ for impurities were calculated from the linearity data using formula $3\sigma/S$ and $10\sigma/S$ respectively where σ is the standard deviation of the response and ST is the slope of the linearity curve. Precision was performed at below predicted LOD and LOQ values due to considerable area counts observed at very low concentration level and calculate as %w/w using sample test concentration.

Linearity and range

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of the analyte in the sample. Standard solutions at 6 different concentration levels were prepared and analyzed in order to demonstrate the linearity for all the impurities. The regression curve was obtained by plotting peak area versus concentration, using the least squares method and regression equation was obtained for all the impurities.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. The standard addition and recovery experiments were conducted to demonstrate accuracy of the method. The study was carried out in triplicate for the determination of recovery at 50, 100 and 150% concentration of specification level for all the impurities. The peak area for each impurity was determined and recovery was calculated from the peak area of impurity standard solution at the same concentration level.

Solution stability

In order to demonstrate the stability of both reference and sample solutions, these solutions were injected immediately after preparation and at periodical intervals by maintaining at cooler temperature.

Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage and the impact of the variation on each impurity was evaluated. The flow rate of the mobile phase was 0.18 and 0.22 ml/min. The effect of column temperature (actual 25°C) was studied at 20 and 30°C. The effect of pH of mobile phase studied pH ± 0.1 for mobile phase A. For gradient programme variation, the composition of mobile phase-B was changed by ± 2 absolute. For wavelength variation, ± 5 nm was changed from the working wavelength i.e., 265 nm.

RESULTS AND DISCUSSION

Specificity and forced degradation

Specificity experiment was performed on ST/LA/NE tablets to evaluate the stability indicating nature of the method. Placebo with individual drug components and sample solutions were prepared on different stress conditions in the diluent viz., Base hydrolysis (5 N NaOH/5 ml/RT/60 min), acid hydrolysis (5N HCl/5 ml/RT/60 min), oxidation (30% H_2O_2/5 ml/RT/30 min), thermal stress (80°C/24 h), photolytic stress (white fluorescent 1.2 million lux hours UV 200 watt h/m^2 for 7 days) and humidity stress (90%RH/24 h).

Significant degradation for LA was observed in alkali, oxidative, humidity, thermal and in photolytic degradation conditions. Slight degradation is observed for ST in acid, alkali, humidity, thermal and in photolytic. There was no significant degradation observed for NE. Unspecified impurities at RRT about 0.52, 0.93, 0.94, 0.96 were generated at low level in acid, base, peroxide, thermal and in humidity degradation conditions of ST (Figures 4 and 5; Table 3).

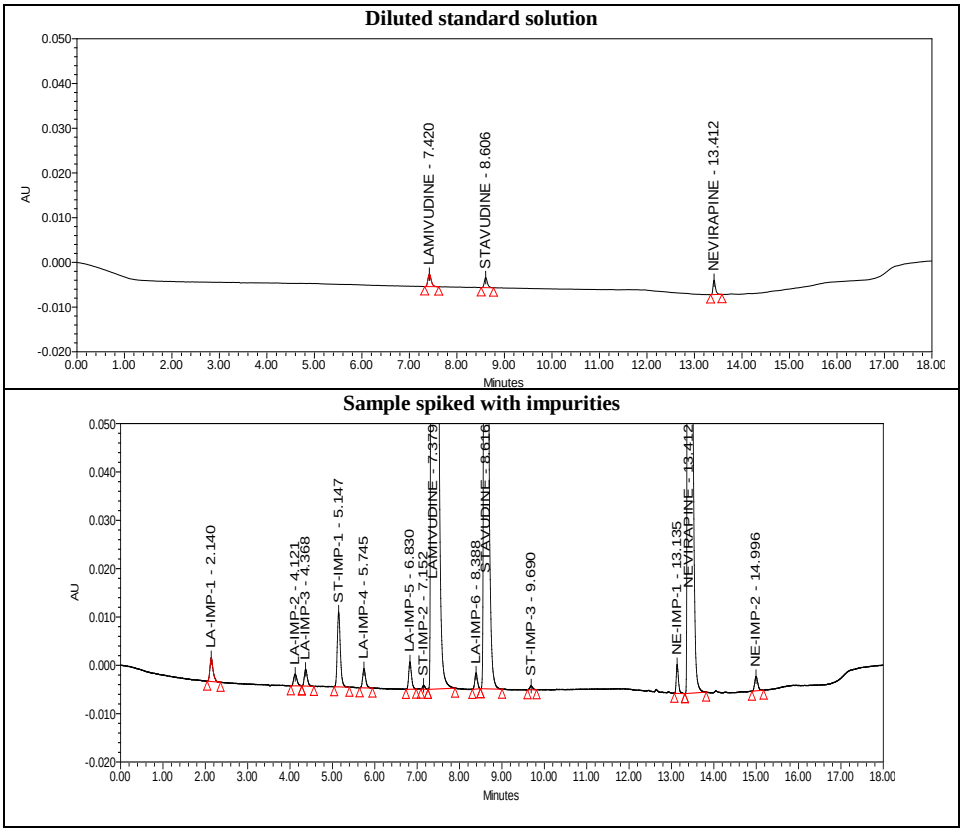
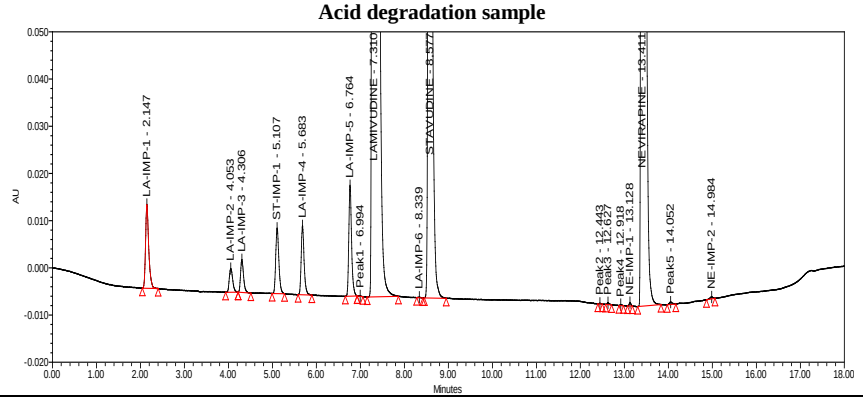
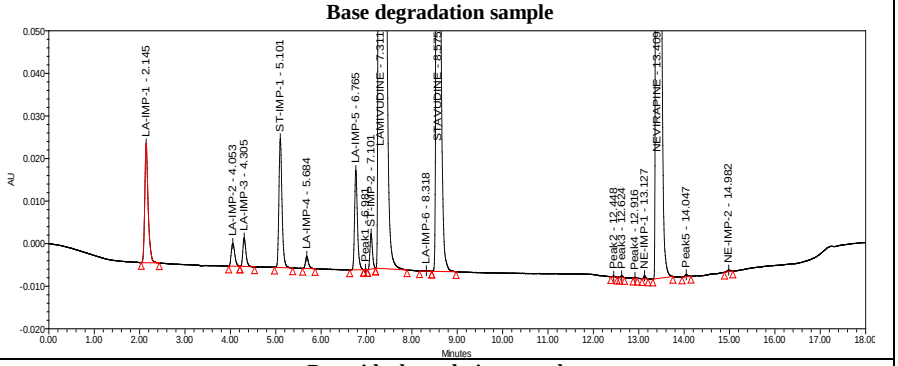


Figure 4: Typical chromatogram of diluted standard solution, ST/LA/NE tablets spiked with impurities

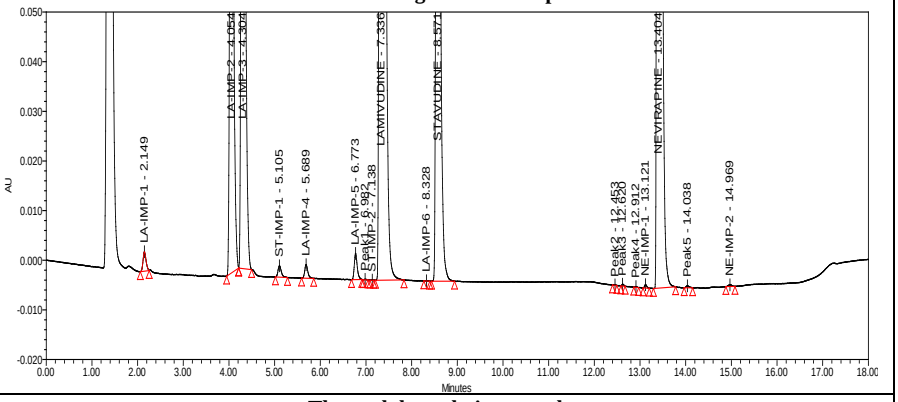
Acid degradation sample



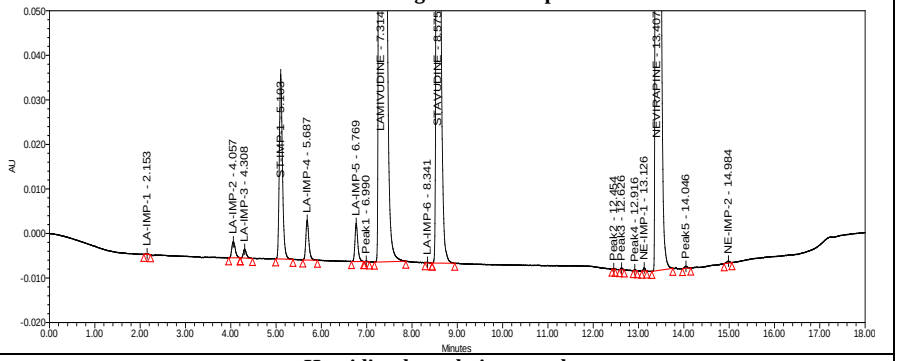
Base degradation sample



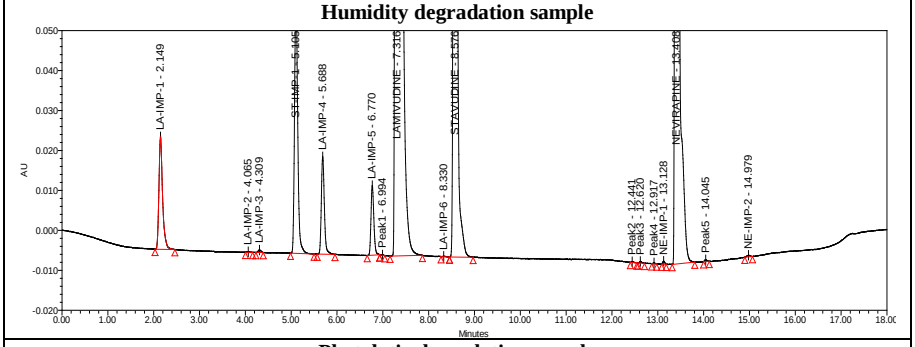
Peroxide degradation sample



Thermal degradation sample



Humidity degradation sample



Photolytic degradation sample

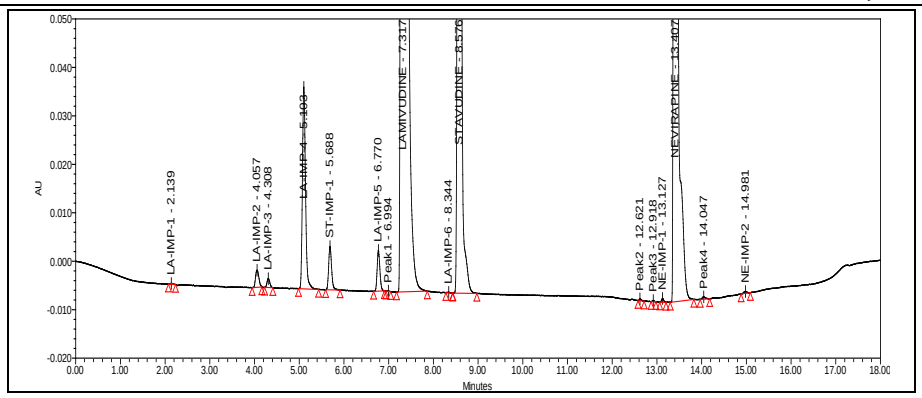


Figure 5: Stress sample chromatograms

Table 3: Summary of forced degradation study for ST, LA and NE

Stress condition and time	% Assay of ST	% Assay of LA	% Assay of NE	% imps+%Deg. Products of ST	mass balance (%Assay+Imps+% Deg products of ST)	% imps+%Deg. products LA	mass balance (%Assay+Imps+% Deg products of LA	% imps+% Deg. products NE	mass balance (%Assay+Imps+% Deg products of NE	Major Appeared impurities
Acid hydrolysis-15 min	99.2	95.9	99.9	0.28	99.5	4.50	100.4	0.03	99.9	ST-ST-Imp-1 LA-LA-Imp-1, LA-Imp-2, LA-Imp-3, LA_Imp-4, LA-Imp-5 NE-No significant degradation
Base hydrolysis - 15 min	97.4	96.5	99.9	2.23	99.6	3.35	99.8	0.04	99.9	St-ST-Imp-1, ST-Imp-2 LA-LA-Imp-1, LA-Imp-2, LA-Imp-3, LA_Imp-4, LA-Imp-5 NE-No significant degradation
Oxidation-15 min	99.8	67.7	99.9	0.08	99.9	32.46	100.2	0.09	100.0	ST-No significant degradation LA-LA-Imp-1, LA-Imp-2, L-Imp-3, LA_Imp-4, LA-Imp-5 NE-No significant degradation
Thermal-24 h	99.2	97.6	99.9	0.71	99.9	2.01	99.6	0.03	99.9	ST-ST-Imp-1 LA-LA-Imp-2, LA-Imp-3, LA_Imp-4, LA-Imp-5 NE-No significant degradation
Humidity-24 h	98.6	94.5	99.3	1.08	99.7	5.16	99.7	0.03	99.3	ST-ST-Imp-1 LA-LA-Imp-1, LA-Imp-4, LA-Imp-5, NE-No significant degradation
Photolytic-7 days	99.4	98.1	99.9	0.76	100.2	1.94	100.0	0.03	99.9	ST-ST-Imp-1 LA-LA-Imp-2, LA-Imp-3, LA_Imp-4, L-Imp-5 NE-No significant degradation

Precision

The precision of the method was checked by injecting six individual preparations of ST/LA/NE tablets spiked with 0.2% level for LA-Imp-1 to LA-Imp-6 and 2.5% for ST-Imp-1 and 0.2% for ST-Imp-2, ST-Imp-3 and 0.3% for NE-Imp-1 and 0.2% for NE-Imp-2. The percentage RSD for impurities found to be 1.4, 1.9, 1.3, 1.9, 1.2, 1.6, 1.1, 3.4, 3.6, 3.4 and 3.3% w/w respectively of each impurity.

Intermediate precision

The intermediate precision (Ruggedness) of the method was evaluated by different analyst using different column and different UPLC instrument on different day. The percentage RSD was 3.7, 0.9, 1.7, 2.3, 1.6, 2.1, 3.6, 1.7, 2.0, 4.0 and 3.7% w/w respectively.

Sensitivity (LOQ and LOD)

The LOD of %w/w for LA-Imp-1 to, LA-Imp-6, ST-Imp-1 to ST-Imp-3, NE-Imp-1, to NE-Imp-2 was 0.010, 0.010, 0.010, 0.011, 0.013, 0.009, 0.024, 0.020, 0.019, 0.015, 0.010 and 0.011 respectively. The LOQ of %w/w for LA-Imp-1 to, LA-Imp-6, to ST-Imp-3, NE-Imp-1, to NE-Imp-2 is 0.020, 0.019, 0.020, 0.021, 0.026, 0.018, 0.049, 0.040, 0.039, 0.030, 0.021 and 0.022 respectively. This indicates observed LOD and LOQ values are found to be at low concentration level. Hence developed method found to be sensitive enough to detect impurities at lowest concentration levels.

Linearity and range

The linear calibration plot is obtained over the calibration range LOQ to 150%. The results show that correlation obtained between peak area and concentration of ST, LA, NE and their corresponding impurities (Table 4).

Table 4: Linearity table

Name of the component	Trend line equation	Range	Correlation coefficient	Intercept	Residual sum of squares
LA-Imp-1	y=20106x+210	0.012-1.826	0.99944	210	460
LA-Imp-2	y=10132x+385	0.012-1.770	0.99943	385	228
LA-Imp-3	y=11946x+325	0.012-1.732	0.99929	325	294
ST-Imp-1	y=175089x+1186	0.004-0.621	0.99944	1186	1359
LA-Imp-4	y=5401x+148	0.012-1.813	0.99781	148	243
LA-Imp-5	y=17603x+428	0.012-1.791	0.99950	428	374
ST-Imp-2	y=10686x+83	0.003-0.478	0.99604	83	172
LA	y=11279x+222	0.012-1.809	0.99921	222	303
LA-Imp-6	y=12574x+462	0.011-1.720	0.99969	462	201
ST	y=31657x+477	0.003-0.485	0.99925	477	223
ST-Imp-3	y=11456x+95	0.003-0.466	0.99442	95	213
NE-Imp-1	y=11775x –216	0.016-2.383	0.99913	216	439
NE	y=7024x+344	0.016-2.422	0.99879	344	315
NE-Imp-2	y=7024x+344	0.016-2.422	0.99879	344	315

Accuracy

Accuracy was evaluated for the levels including LOQ, 50, 100 and 150% of the specification level of the impurities. The results observed from the recovery samples were found in the range between 90-110% with the RSD lower than 5.0%. The method was found to be accurate within the desired range (Tables 5a-5d).

Table 5a: Accuracy study for LA-Imp-1, LA-Imp-2 and LA-Imp-3

Sample spiked at level	LA-Imp-1		% Recovery	LA-Imp-2		% Recovery	LA-Imp-3		% Recovery
	Amount added (%w/w)	Amount recovered (%w/w)		Amount added (% w/w)	Amount recovered (%w/w)		Amount added (% w/w)	Amount recovered (%w/w)	
LOQ sample-1	0.0210	0.0199	94.8	0.0225	0.0229	101.8	0.0262	0.0262	100.0
LOQ sample-2	0.0209	0.0210	100.5	0.0224	0.0222	99.1	0.0273	0.0292	107.0
LOQ sample-3	0.0208	0.0203	97.6	0.0223	0.0219	98.2	0.0261	0.0272	104.2
50% sample-1	0.101	0.094	93.1	0.098	0.091	92.9	0.099	0.097	98.0
50% sample-2	0.101	0.094	93.1	0.099	0.093	93.9	0.099	0.099	100.0
50% sample-3	0.101	0.093	92.1	0.098	0.090	91.8	0.098	0.097	99.0
100% sample-1	0.200	0.192	96.0	0.195	0.192	98.5	0.195	0.192	98.5
100% sample-2	0.201	0.197	98.0	0.196	0.193	98.5	0.196	0.197	100.5
100% sample-3	0.202	0.195	96.5	0.197	0.190	96.4	0.197	0.193	98.0
150% sample-1	0.305	0.292	95.7	0.297	0.294	99.0	0.297	0.296	99.7
150% sample-2	0.305	0.299	98.0	0.298	0.290	97.3	0.298	0.294	98.7
150% sample-3	0.299	0.285	95.3	0.291	0.283	97.3	0.291	0.286	98.3

Table 5b: Accuracy study for ST-Imp-1, LA-Imp-4 and LA-Imp-5

Sample spiked at level	LA-Imp-4		% Recovery	LA-Imp-5		% Recovery	LA-Imp-6		% Recovery
	Amount added (% w/w)	Amount recovered (% w/w)		Amount added (% w/w)	Amount recovered (% w/w)		Amount added (% w/w)	Amount recovered (% w/w)	
LOQ sample-1	0.0192	0.0180	93.8	0.0201	0.0217	108.0	0.0171	0.0163	95.3
LOQ sample-2	0.0192	0.0191	99.5	0.0201	0.0214	106.5	0.0180	0.0175	97.2
LOQ sample-3	0.0192	0.0187	97.4	0.0200	0.0210	105.0	0.0180	0.0177	98.3
50% sample-1	0.072	0.067	93.1	0.097	0.101	104.1	0.092	0.093	101.1
50% sample-2	0.072	0.072	100.0	0.097	0.099	102.1	0.092	0.093	101.1
50% sample-3	0.071	0.066	93.0	0.097	0.100	103.1	0.091	0.092	101.1
100% sample-1	0.190	0.182	95.8	0.193	0.199	103.1	0.182	0.184	101.1
100% sample-2	0.191	0.181	94.0	0.193	0.197	102.1	0.183	0.184	100.5
100% sample-3	0.181	0.171	107.5	0.194	0.198	102.1	0.183	0.185	101.1
150% sample-1	0.308	0.331	106.6	0.0293	0.306	104.4	0.277	0.278	100.4
150% sample-2	0.289	0.308	107.1	0.293	0.303	103.4	0.277	0.281	101.4
150% sample-3	0.283	0.303	99.1	0.287	0.294	102.4	0.271	0.271	100.0

Table 5c: Accuracy study for ST-Imp-1, ST-Imp-2 and ST-Imp-3

Sample spiked at level	ST-Imp-1		% Recovery	ST-Imp-2		% Recovery	ST-Imp-3		% Recovery
	Amount added (% w/w)	Amount recovered (% w/w)		Amount added (% w/w)	Amount recovered (% w/w)		Amount added (% w/w)	Amount recovered (% w/w)	
LOQ sample-1	0.0114	0.0115	100.9	0.0263	0.0254	96.6	0.0348	0.0336	96.6
LOQ sample-2	0.0113	0.0105	92.9	0.0249	0.0243	97.6	0.0347	0.0347	100.0
LOQ sample-3	0.0113	0.0106	93.8	0.0275	0.0276	100.4	0.0346	0.0340	98.3
50% sample-1	0.130	0.129	99.2	0.089	0.085	95.5	0.093	0.091	97.8
50% sample-2	0.130	0.128	98.5	0.085	0.081	95.3	0.094	0.093	98.9
50% sample-3	0.129	0.123	95.3	0.084	0.081	96.4	0.093	0.092	98.9
100% sample-1	0.258	0.256	99.2	0.176	0.175	99.4	0.185	0.186	100.5
100% sample-2	0.259	0.258	99.6	0.177	0.174	98.3	0.186	0.190	102.2
100% sample-3	0.260	0.255	98.1	0.177	0.176	99.4	0.186	0.179	96.2
150% sample-1	0.392	0.387	98.7	0.268	0.261	97.4	0.281	0.283	100.7
150% sample-2	0.393	0.385	98.0	0.268	0.260	97.0	0.282	0.285	101.1
150% sample-3	0.384	0.375	97.7	0.262	0.257	98.1	0.276	0.273	98.9

Table 5d: Accuracy study for NE-Imp-1 and NE-Imp-2

Sample spiked at level	NE-Imp-1		% Recovery	NE-Imp-2		% Recovery
	Amount added (% w/w)	Amount recovered (% w/w)		Amount added (% w/w)	Amount recovered (% w/w)	
LOQ sample-1	0.0185	0.0192	103.8	0.0195	0.0198	101.5
LOQ sample-2	0.0168	0.0164	97.6	0.0194	0.0190	97.9
LOQ sample-3	0.0184	0.0186	101.1	0.0194	0.0196	101.0
50% sample-1	0.095	0.089	93.7	0.106	0.103	97.2
50% sample-2	0.090	0.086	95.6	0.107	0.103	96.3
50% sample-3	0.089	0.087	97.8	0.106	0.103	97.2
100% sample-1	0.187	0.181	96.8	0.211	0.198	93.8
100% sample-2	0.188	0.190	101.1	0.212	0.199	93.9
100% sample-3	0.189	0.191	101.1	0.213	0.202	94.8
150% sample-1	0.285	0.289	101.4	0.321	0.304	94.7
150% sample-2	0.286	0.287	100.3	0.317	0.299	94.3
150% sample-3	0.279	0.280	100.4	0.314	0.292	93.0

Solution stability

Solution stability was performed at cooler temperature of 6°C, it was found that standard solutions were stable up to 34 h and sample solution was stable up to 32 h.

Robustness

The analytical results from the deliberately change chromatographic conditions like flow rate, column temperature, change in pH, wave length and change of organic component in gradient programme revealed that there was no significant change observed in the relative retention times of the main analyte and their corresponding impurities illustrating the method was robust.

CONCLUSION

The present developed and validated study provided a rapid, precise and sensitive method for simultaneous determination and separation of impurities of ST, LA, and NE in ST/LA/NE tablets. The forced degradation studies indicated that method was selective and stability indicating. Recover and Linearity parameters confirmed that the method was accurate and linear. The calibration curves obtained were found to be linear with values of correlation coefficients greater than 0.995.

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