VALIDATED HPLC METHOD FOR DETERMINATION OF LAMIVUDINE AND STAVUDINE IN THEIR FORMULATIONS

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ABSTRACT

A simple, rapid, precise and accurate isocratic reverse phase stability indicating RP-HPLC method was developed and validated for the simultaneous estimation of Lamivudine and Stavudine in commercial tablets. The method has shown adequate separation for Lamivudine (RT-3.087) and Stavudine (RT-6.09) respectively. Separation was achieved on an YMC pack, C8, 150mmX4.6mm, 5µ column using a mobile phase consisting of buffer pH 3.5 and methanol in the ratio of 90:10v/v at a flow rate of 1.0ml/min. the detection was carried out by PDA detector at the wavelength maximum of 265 nm. The drugs were subjected to acid degradation, base degradation, peroxide degradation, thermal degradation, photolytic degradation and humidity degradation. The linearity of proposed method was investigated in the range of 5-50 µg/ml (r= 0.99989) for Stavudine and 20-220µg/ml (r= 0.99997) for Lamivudine, respectively.

KEY WORDS: *Stavudine, Lamivudine, Degradation, RP-HPLC.*

INTRODUCTION

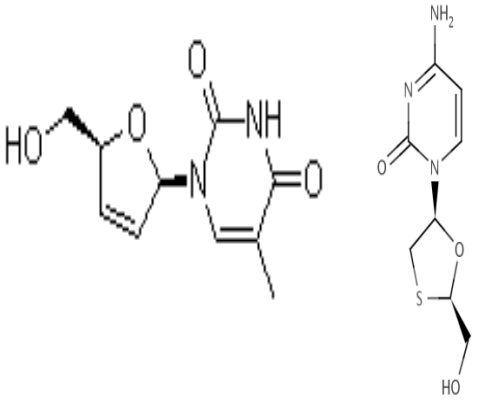


Fig.1.Chemical structures of Stavudine and Lamivudine

Lamivudine is a synthetic nucleoside analogue, showing a potent inhibition of the human immunodeficiency virus (HIV–1) the causative agent of acquired immunodeficiency syndrome (AIDS). Chemically Lamivudine is a (2*R-cis*)-4-Amino-1-[2-(hydroxymethyl)-1, 3-oxathiolan-5-yl-2(1H)-pyrimidinone having molecular formula $C_8H_{11}N_3O_3S$. Lamivudine is a nucleoside reverse transcriptase (NRT) inhibitor of human immunodeficiency virus type 1 (HIV-1). It is a chain terminator, and all three phosphorylation events to give the triphosphate are carried out by cellular enzymes. The chemical structures of Stavudine and Lamivudine was shown in Fig.1. The first phosphorylation is by a cellular *deoxy cytidine kinase* rather than a *viral kinase* .Intracellularly, Lamivudine is phosporylated to its active 5^1 – triphosphate metabolite, Lamivudine triphosphate (L-TP). The principal mode of action of L-TP is the

inhibition of HIV-1 reverse transcriptase (RT) via DNA chain terminator after incorporation of the nucleoside analogue into viral DNA phosphorylation of Lamivudine to the triphosphate form (3TCTP) is enhanced by hydroxy urea, methotrexate or fludarabine. Stavudine, a nucleoside analogue of thymidine, inhibits the replication of HIV in human cells in vitro. Stavudine is phosporylated by cellular kinases to the active metabolite Stavudine triphosphate. Stavudine triphosphate inhibits the activity of HIV reverse transcriptase both by competing with the natural substrate deoxythymidine triphosphate and by its incorporation into viral DNA causing a termination of DNA chain elongation because Stavudine lacks the essential 3`-OH group. Stavudine triphosphate inhibits cellular DNA polymerase beta and gamma, and markedly reduces the synthesis of mitochondrial DNA. There are number of analytical methods have been evoked for the estimation of Stavudine and Lamivudine either individually or combination with other anti viral agents in biological fluids and stability indicating assays. Literature survey reveals [1-19] that no other simultaneous isocratic RP-HPLC method stability indicating assay developed for the estimation of Stavudine and Lamivudine in bulk and pharmaceutical dosage forms. In this present investigation the authors made an attempt to develop a simple, rapid, precise and accurate stability indicating assay for estimation of Stavudine and Lamivudine.

EXPERIMENTAL

Pure standards of Stavudine and Lamivudine obtained from Aurabindo laboratories, Hyderabad. The purities of the standards were found to be 99.87% and 99.96% for Stavudine and Lamivudine, respectively. The HPLC separation made on system consisting of a isocratic, high performance liquid chromatograph with alliance(waters 2690 separation module) equipped with universal rheodyne injector with injection volume 10µl , PDA detector with empower soft ware. An YMC pack, C8, 150mmX4.6mm, 5µ column was used for the separation. Detection was carried out by PDA detector at 265 nm.

CHROMATOGRAPHIC CONDITIONS:

Freshly prepared buffer pH 3.5 and methanol in the ratio of 90:10v/v was used as mobile phase. These were filtered through 0.45 m membrane filter and sonicated before use. The flow rate of mobile phase was 1 ml / min. The column was maintained at ambient temperature. The detection was carried out by at 265 nm. The injection volume was 20 µl .

PREPARATION OF BUFFER:

Dissolve 3.85g of ammonium acetate in 800ml of water and adjust the pH to 3.5 with glacial acetic acid. Make up the volume to 1000ml with water. Filter through 0.45µ membrane filter or fine porosity membrane filter.

PREPARATION OF MOBILE PHASE:

The prepared buffer and methanol was mixed in the ratio of 90:10v/v

PREPARATION OF STANDARD AND STOCK SOLUTIONS:

Stock solutions of Stavudine and Lamivudine approximately equivalent to 1mg/ml were prepared in methanol. The stock solutions were protected from light using Aluminium foil and stored for three weeks at 4^{o}c with no evidence of decomposition. Aliquots of standard stock solutions of Stavudine and Lamivudine were transferred using a A-grade bulb pipettes into 10 ml volumetric flasks and the solutions were made up to volume with mobile phase to get final concentrations of 5-50 µg/ml (r= 0.99989) for Stavudine and 20-200µg/ml (r= 0.99997) for Lamivudine, respectively.

PREPARATION OF TABLETS FOR ASSAY:

Twenty tablets were taken in to a 250 ml volumetric flask add 20 ml of water and disperse the tablets. Add about 150 ml of methanol, sonicate for 30 minutes and make up to the volume with methanol and mix. Centrifuge the solution at 5000RPM for 5 minutes. Dilute 5 ml of the supernatant solution to 100 ml with water. Finally filter the solution through 0.45µ membrane filter. The filtered solution was transferred in to 10 ml volumetric flask and made up to the volume with mobile phase to get the concentrations of each of

the two drugs in the range of linearity previously described.

FORCED DEGRADATION STUDIES OF API AND TABLETS:

In order to establish whether the analytical method and the assay were stability indicating tablets and pure active pharmaceutical ingredients of both Stavudine and Lamivudine were stressed under various conditions to conduct forced degradation studies. As these drugs are freely soluble and stable in methanol and methanol was used as co solvent in all forced degradation studies. All solutions were prepared to use in forced degradation studies were prepared by dissolving API or drug product in small volume methanol and later diluted with aqueous hydrogen peroxide, distilled water, aqueous hydrochloric acid or aqueous sodium hydroxide to achieve concentration of 100 µg/ml each of Stavudine and Lamivudine. After the degradation these solutions were diluted with mobile phase to get starting concentration of 10µg/ml.

OXIDATION STUDIES:

Solutions for use in oxidation studies were prepared in methanol and 30% hydrogen peroxide (20:80v/v) at 85°c about five minutes and the resultant solutions were analyzed after five minutes of the preparation.

ACID DEGRADATION STUDIES:

The solutions for acid degradation studies were prepared in methanol and1 M hydrochloric acid (20:80v/v) at 85°c about five minutes and the resultant solutions were analyzed after five minutes of the preparation.

ALKALI DEGRADATION STUDIES :

The solutions for acid degradation studies were prepared in methanol and1 M sodium hydroxide (20:80,v/v) at 85oc about sixty minutes and the resultant solutions were analyzed after five minutes of the preparation.

THERMAL DEGRADATION STUDIES:

The tablets and API were exposed to 1050c for about 97 hrs and the tablets and powder was removed from oven and the tablets were crushed and mixed and an aliquot of the powder equivalent to the weight of one tablet and API powder were then prepared for analysis as previously described.

PHOTO STABILITY STUDIES:

Tablets and API powder and solutions of each drug were prepared and exposed to light to determine the effects of irradiation on the stability of the two drugs in solution and in the solid state. Approximately 50 mg of each API was spread on a glass dish in a layer that was less than 2 mm in thickness. A solution of each API (1 mg/ml) was prepared in methanol and HPLC grade water (20:80, v/v). Tablets were prepared in the same way. All samples for photo stability testing were placed in a light cabinet (Sun test CPS/CPS$^+$, Atlas Material Testing Technology, Germany) and exposed to light for 97 h resulting in an overall illumination of ≥200 W h/m^2 at 25 °C with UV radiation at 320–400 nm. Control samples which were protected from light with aluminum foil were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

HUMIDITY DEGRADATION:

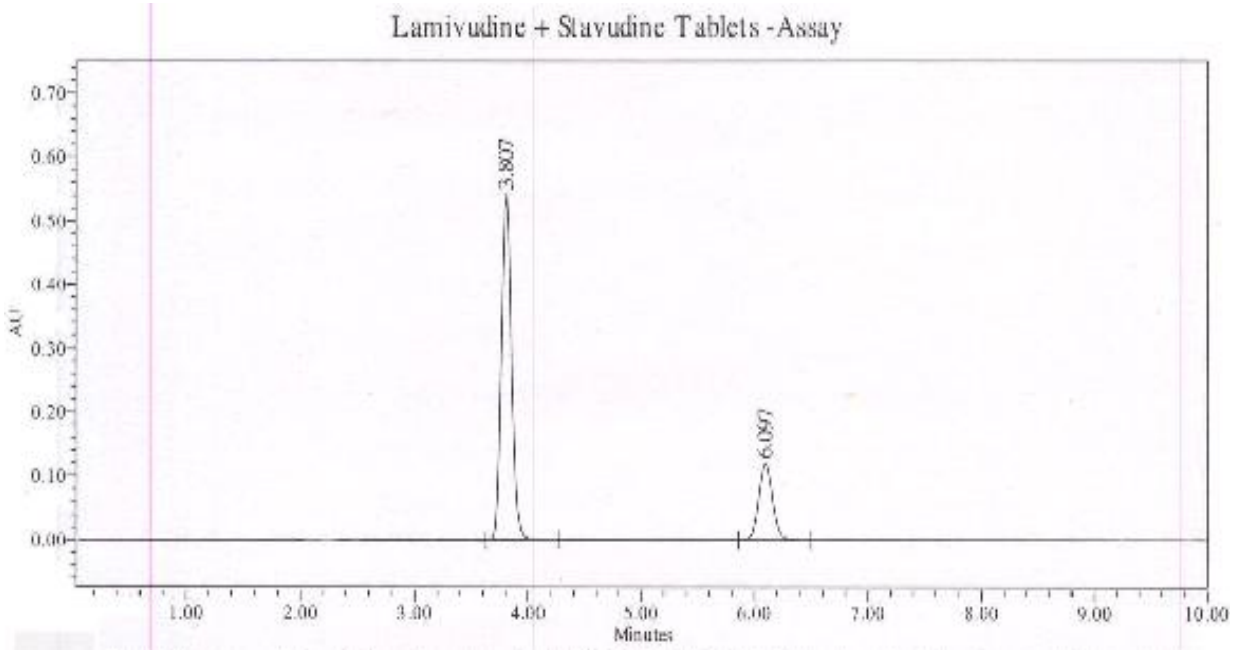
The tablets and API were exposed to 92% RH/ 25°c/97 hrs.

RESULTS AND DISCUSSION

Method development

A simple, rapid, accurate stability indicating assay methanol in the ratio of 90:10v/v was optimized for the effective separation of Stavudine and Lamivudine. The typical chromatogram of Stavudine and Lamivudine was shown in Fig.1. Hence, the method was considered as Selective, Specific and Stability indicating.

Fig.1. Typical chromatogram of Lamivudine and Stavudine



Method validation

The test method was validated for Specificity, Linearity, Precision, Accuracy, Range, Stability of sample solution, Ruggedness and Robustness were found to be meeting the predetermined acceptance criteria. The validated method was found to be Specific, Linear, Precise, Accurate, Robust and Rugged for the assay of Lamivudine and Stavudine in Lamivudine and Stavudine Tablets. Hence, this method can be introduced into the use for the assay of Lamivudine and Stavudine in Lamivudine and Stavudine Tablets

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Specificity

The peak purity data (based on forced degradation data) of Lamivudine and Stavudine peaks at every degradation sample shows that the peaks were homogeneous and there were no co-eluting peaks indicating that the method was stability indicating and specific. The values were shown in Table-1.

Table 1: Specificity of the method for Lamivudine

*Degradation Mechanism	Degradation Condition	Assay (mg/ Tablet)	% Degradation
-	Un degraded	39.2*	-
Acid degradation	1M HCl / 85°C /5 min	35.2	10.2
Base degradation	1M NaOH / 85°C /60 min	38.7	1.3
Peroxide degradation	30% H2O2 / 85°C /5 min	39.1	0.3
Thermal degradation	105°C / 97 Hours	38.9	0.8
Photolytic degradation	10K Lux / 97 Hours	39.0	0.5
Humidity degradation	92% RH / 25°C / 97 Hours	39.4	Nil

Specificity of the method for Stavudine

Degradation Mechanism	Degradation Condition	Assay (mg/ Tablet)	% Degradation
-	Un degraded	150.8*	-
Acid degradation	1M HCl / 85°C /5 min	150.8	Nil
Base degradation	1M NaOH / 85°C /60 min	124.7	17.3
Peroxide degradation	30% H_2O_2 / 85°C /5 min	121.9	19.2
Thermal degradation	105°C / 97 Hours	150.8	Nil
Photolytic degradation	10K Lux / 97 Hours	149.8	0.7
Humidity degradation	92% RH / 25°C / 97 Hours	148.1	1.8

Linearity

The value of correlation coefficient obtained from the linearity studies (CC for Lamivudine was 0.99997 and for Stavudine was 0.99989) indicating that the Response was Linear for Lamivudine and Stavudine peaks over the concentration range from 70% to 130% of test concentration.The values were shown in Table-2.

Concentration (µg/mL)	Average area	Statistical Analysis	
100	2351984	Slope	22500
120	2694554		
140	3032523	Intercept	6371
160	3375692		
180	3697761	Residual Sum of Squares	5754
200	4035640		
220	4371299	Correlation coefficient(CC)	0.99997

Table-2: Linearity of Lamivudine

Concentration (µg/mL)	Average area	Statistical Analysis	
20	687602	Slope	24557
25	783043		
30	889989	Intercept	-10057
35	989432		
40	1088375	Residual Sum of Squares	3507
45	1188816		
50	1281264	Correlation coefficient(CC)	0.99989

Linearity of Stavvudine

Precision

The % RSD evaluated from the areas of Lamivudine, Stavudine peaks from six replicated injections of Standard solution indicating that the measurement system was precise for the estimation of Lamivudine and Stavudine in Lamivudine and Stavudine Tablets. Also, the % RSD evaluated from the % assay of Lamivudine, Stavudine from six replicated preparations of Test sample solutions indicating that the method was precise for the estimation of Lamivudine and Stavudine in Lamivudine and Stavudine Tablets. Test results were showing that the Test Method was precise. The values were shown in Table-3.

Area	
40mg and 150mg / Tablet	
Lamivudine	Stavudine
3413795	1022598
3411295	1032114
3409721	1013654
3412568	1023542
3407658	1022546
3407564	1012361

Statistical Analysis		
Mean	3410434	1021136
SD	2571	7258
%RSD	0.1	0.7
95%Confidence Interval	2699	7618

Table 3: Precision

Ruggedness

The Overall %RSD evaluated from the results of Method Precision and Ruggedness experiments, showing that the method was rugged for system to system, column to column, system. The values were shown in Table- 4.

Sample	Assay (mg / Tablet)			
	Set I		Set II	
	Lamivudine	Stavudine	Lamivudine	Stavudine
1	148.9	39.6	148.1	39.4
2	149.1	39.3	148.1	39.2
3	148.9	39.5	148.1	39.8
4	148.8	39.7	150.4	38.8
5	149.0	39.6	148.7	39.1
6	149.2	39.7	148.9	39.4
Statistical Analysis				
Mean	149.0	39.6	148.7	39.3
SD	0.15	0.15	0.90	0.34
%RSD	0.1	0.4	0.6	0.9
95%Confidence Interval	±0.2	±0.2	±0.9	± 0.4

Table 4: Ruggedness (Intermediate Precision)

Accuracy (Recovery studies)

The recovery results indicating that the test method has an acceptable level of accuracy for the assay of Lamivudine and Stavudine in Lamivudine and Stavudine Tablets from 80 % to 120 % of test concentration. The values were shown in Table-5.

Stability of sample solution

From the data it was concluded that, the sample solution was stable for at least 15 Hours at room temperature (~ 25°C).Hence, the % assay results can be considered reliable, even the sample solution was injected after 15 hours of the preparation. The values were shown in Table-6.

Robustness

The results indicating that the test method was robust for all variable conditions Hence, the method was sufficiently robust for normally expected variations in chromatographic conditions.

Table 5: Accuracy (Recovery) For Lamivudine

Concentration / Sample	Amount added (mg)	Amount found (mg)	% Recovery	Statistical Analysis	
80% Level Sample 1	602.4	598.7	99.4	Mean	99.9
80% Level Sample 2	601.0	601.4	100.1	SD	0.40
80% Level Sample 3	600.7	601.4	100.1	% RSD	0.4
100% Level Sample 1	752.9	743.0	98.7	Mean	99.9
100% Level Sample 2	752.5	750.8	99.8	SD	1.20
100% Level Sample 3	752.7	760.7	101.1	% RSD	1.2
120% Level Sample 1	903.5	903.6	100.0	Mean	100.2
120% Level Sample 2	904.5	911.6	100.8	SD	0.49
120% Level Sample 3	904.3	903.3	99.9	% RSD	0.5
Overall Statistical Analysis					
Mean	100	SD	0.7	% RSD	0.7	95% Confidence Interval	± 0.5

Accuracy (Recovery) For Stavudine

Concentration / Sample	Amount added (mg)	Amount found (mg)	% Recovery	Statistical Analysis	
80% Level Sample 1	159.4	159.3	99.9	Mean	100.0
80% Level Sample 2	159.0	159.3	100.2	SD	0.21
80% Level Sample 3	159.5	159.2	99.8	% RSD	0.2
100% Level Sample 1	199.0	197.3	99.1	Mean	99.9
100% Level Sample 2	198.8	198.7	99.9	SD	0.80
100% Level Sample 3	198.6	200.0	100.7	% RSD	0.8
120% Level Sample 1	238.4	240.2	100.8	Mean	100.9
120% Level Sample 2	238.7	240.7	100.8	SD	0.23
120% Level Sample 3	238.8	241.7	101.2	% RSD	0.2
Overall Statistical Analysis					
Mean	100.3	SD	0.66	% RSD	0.7	95% Confidence Interval	± 0.5

Room temperature ~ (ι 25°C)				
Time in Hours	Lamivudine		Stavudine	
	Area	% Difference	Area	% Difference
Initial	3376489	~	989316	~
1	3361985	0.4	985647	0.4
2	3369546	0.2	988125	0.1
3	3362541	0.4	987014	0.2
4	3363241	0.40	989321	0.0
5	3368542	0.2	987698	0.2
6	3371652	0.1	986258	0.3
7	3370985	0.2	987987	0.1
8	3373658	0.1	987654	0.2
9	3378624	0.1	988123	0.1
10	3379654	0.1	986258	0.3
11	3370963	0.2	986369	0.3
12	3382258	0.2	986589	0.3
13	3378214	0.1	986369	0.3
14	3384125	0.2	987898	0.1
15	3386104	0.3	986987	0.2

Table 6: Stability of Sample Solution

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