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IJPBS |Volume 1| Issue 4 |OCT-DEC |2011|551-559

Analytical method development and method validation for the Simultaneous Estimation of Lamivudine and Stavudine in tablet dosage form by RP-HPLC

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PHARMACEUTICAL SCIENCES ***RESEARCH ARTICLE***

RECEIVED ON 09-11-2011 **ACCEPTED ON 24-11-2011**

ABSTRACT

A high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of two antiretroviral drugs viz. Lamivudine and Stavudine that constitute one of the first line regimens in antiretroviral therapy. The different analytical performance parameters such as linearity, precision, accuracy, specificity, were determined according to International Conference on Harmonization ICH Q2B guidelines. Chromatography was carried out by isocratic technique on a reversed-phase BDS HYPERSIL C18 column with mobile phase comprising of 0.1M disodium hydrogen phosphate anhydrous buffer: methanol, adjusted to pH 5 with glacial acetic acid in the ratio of 50:50, v/v and the chromatographic condition was set at a flow rate of 1ml/min with the UV detector at 238 nm. The retention time Lamivudine and Stavudine was 4.8 and 5.7 minute, respectively. The linearity of the calibration curves for each analyte in the desired concentration range is good (r2 > 0.999) by RP-HPLC method. The method was accurate and precise with recoveries in the range of 98 and 102% for all the two drugs and relative standard deviation (R.S.D.) <1%. The proposed methods were highly sensitive, precise and accurate and hence it can be successfully applied for the simultaneous estimation of Lamivudine and Stavudine in tablet dosage form.

KEYWORDS: *Lamivudine, Stavudine, reverse phase, high performance liquid chromatography, validation, simultaneous estimation*

INTRODUCTION

Multi-drug therapy has become the standard treatment for acquired immunodeficiency syndrome (AIDS)[1]. The situation is imposed by the need to delay the development of resistance by the human immunodeficiency virus (HIV), the causative virus of AIDS, to single anti-HIV drugs and to minimize potential dose dependent side effect[2]. The current typical regimen for treating HIV infection is to use a combination of at least two drugs, a practice known as 'highly active antiretroviral therapy' (HAART)[3].

Lamivudine is chemically known as (2*R*,5*S*)-4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5yl]-2(1*H*)-pyrimidinone[4] and stavudine is chemically 2',3',didehydro-3'-deoxythymidine[5]. Lamivudine is a nucleoside analog having potent in-vitro and in-vivo inhibitory activity against HIV reverse transcriptase[6]. Lamivudine specifically refers to the (-) enantiomer of the *cis*-racemate.

Literature survey reveals several methods that have been used for the quantitative determination of the two drugs individually or in combination with other drugs in pharmaceutical dosage forms or in human plasma by high performance liquid chromatography[7-10], spectrophotometry[11], LC/MS/MS[12] etc. RP-HPLC method with solid phase extraction procedure has been reported for simultaneous determination of six nucleoside analog reverse transcriptase inhibitors[13] and 13 HIV suppressing drugs[14] of which 3TC and d4T are a part. Besides, simultaneous quantification of several antiretroviral agents including these two drugs has been reported by a solid-liquid extraction procedure using RP-HPLC system[15,16].

HPLC methods are useful in the determination of drugs in pharmaceutical formulations especially those containing more than one active components. Therefore, the aim of this work was to develop a relatively simple HPLC method for simultaneous quantification of 3TC and d4T in antiretroviral FDCs without the necessity of sample pre-treatment. This

paper describes the development and validation of reliable, simple, stable and economic reverse phase HPLC assay, using UV detection for the simultaneous determination of 3TC and d4T in FDC tablets. The method appears to be suitable for quality control in pharmaceutical industry due to its sensitivity, simplicity, selectivity and lack of excipients interference.

MATERIALS & METHODS

Chemicals and Reagents: All chemicals and reagents used were of HPLC grade. Methanol, disodium hydrogen phosphate anhydrous, glacial acetic acid and reference standards were obtained from CHANDRA LABS (Hyderabad,A.P,India). Stavex-40 L tablets containing 3TC-15 mg and d4T- 3mg were provided by Aurabindo Pharmaceuticals Ltd., Hyderabad.

Instruments: The instrument used for the study was a Shimadzu High Performance Liquid Chromatography equipped with UV-Visible detector.

Chromatographic Conditions:
Column: BDS HYPERSIL C18, 250×4.6mm, 5µ
Mobile phase: 0.1M disodium hydrogen phosphate anhydrous buffer: methanol, adjusted to pH 5 with glacial acetic acid (50:50, v/v)

Flow rate : 1ml/min

Detection wavelength : 238nm

Pump mode : Isocratic

Run Time : 10 minutes

Retention Time : 4.8 and 5.7 minute respectively

Injection Volume : 20µL

Preparation of Standard Solution: Accurately weighed about 15mg and 3mg of Lamivudine and Stavudine working standards and transferred into a 100ml volumetric flask, added 60ml of diluents, and sonicated to dissolve. Cooled to room temperature and diluted to volume with diluent.

Preparation of Sample Solution: 20 tablets of Lamivudine and Stavudine were weighed and powdered in glass mortar. The powder equivalent to 50mg was transferred into a 100 ml volumetric flask, 70 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for further dilution. 5 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into the HPLC system.

Procedure for Assay: 20 µl of the Standard, Sample and Blank preparations in duplicate were injected separately into the HPLC system and the peak responses for Lamivudine and Stavudine were measured. The quantities from the peak area in mg of Lamivudine and Stavudine were calculated per tablet taken.

RESULTS: The composition of the mobile phase for development of chromatographic method was optimized by using different solvent mixtures of varying polarity. The best results were obtained using 0.1M disodium hydrogen phosphate anhydrous buffer: methanol, adjusted to pH 5 with glacial acetic acid in the ratio 50:50, v/v. This mobile phase showed good resolution of Lamivudine (3TC) and Stavudine (d4T) peak. The wavelength of detection selected was 238 nm, as both the drugs showed optimum absorbance at this wavelength. By our proposed method the retention time of 3TC and d4T was about 4.8 and 5.7 minute, respectively and none of the impurities were interfering in its assay (**Fig. 1 & 2**).

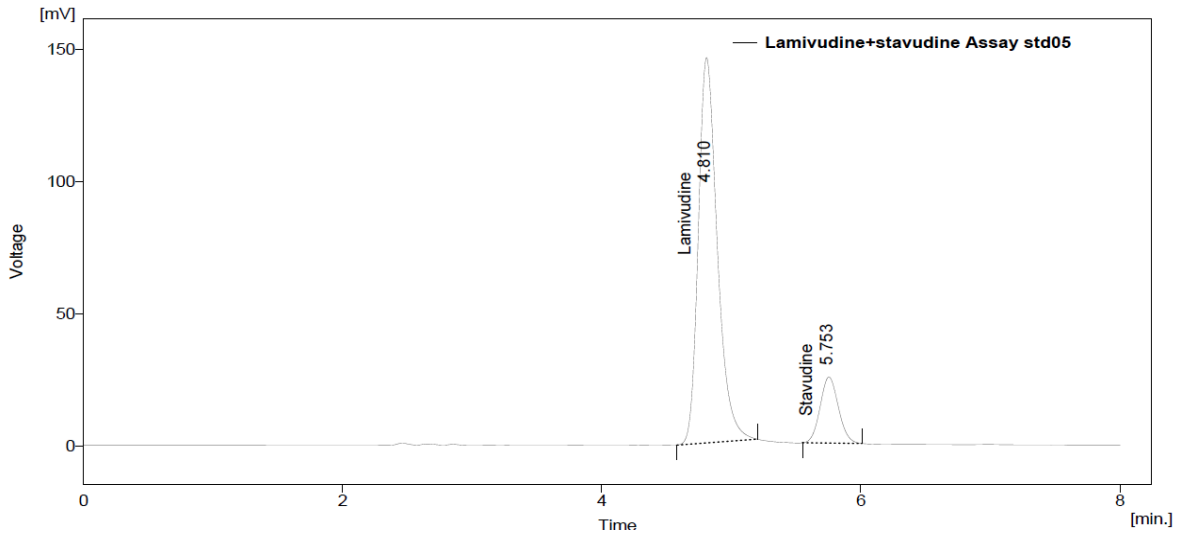

Figure 1: A typical chromatogram of Lamivudine and stavudine standard

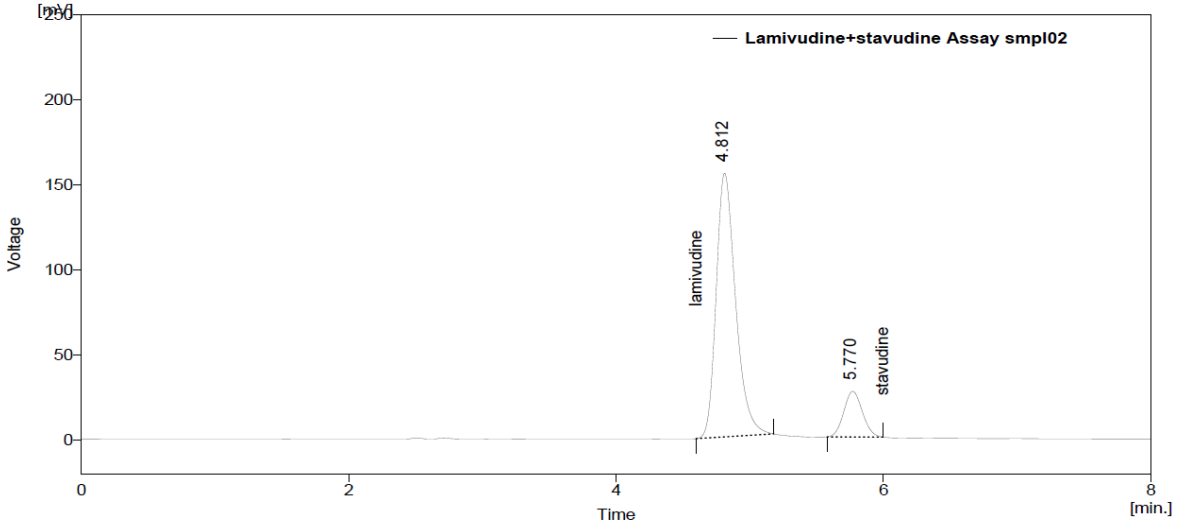

Figure 2: A typical chromatogram of Lamivudine and stavudine sample

Validation of the method: The developed method has been validated for the assay of 3TC and d4T as per ICH guidelines[17,18] by using following parameters.

Specificity and Selectivity: Specificity and selectivity were studied for the examination of the presence of

interfering components. It was checked by subjecting the drug solution in different stress conditions like Acid, Base, Peroxide and the degradation was noted.

Acid Stress (0.1 M HCl)

Table 1: Specificity testing (Acid stress)

Concentration (µg/ml)		Time (hrs)	Retention time(min)		RT of degraded product
3TC	d4T		3TC	d4T	
15	3	0	4.81	5.75	-
		24	4.86	5.80	-

Base Stress (0.1M NaOH)

Table 2: Specificity testing (Base stress)

Concentration (µg/ml)		Time (hrs)	Retention time(min)		RT of degraded product
3TC	d4T		3TC	d4T	
15	3	0	4.81	5.75	-
		24	4.92	5.89	-

Peroxide stress (5% H_2O_2)

Table 3: Specificity testing (Peroxide stress)

Concentration (µg/ml)		Time (hrs)	Retention time(min)		RT of degraded product
3TC	d4T		3TC	d4T	
15	3	0	4.81	5.75	-
		24	4.87	5.82	-

Linearity: Linearity was studied by preparing standard solutions of 3TC and d4T at different concentration levels (**Fig. 3 & 4**). The responses were found linear in the range of 15-90 µg/ml and 3-18 µg/ml for 3TC and d4T, respectively.

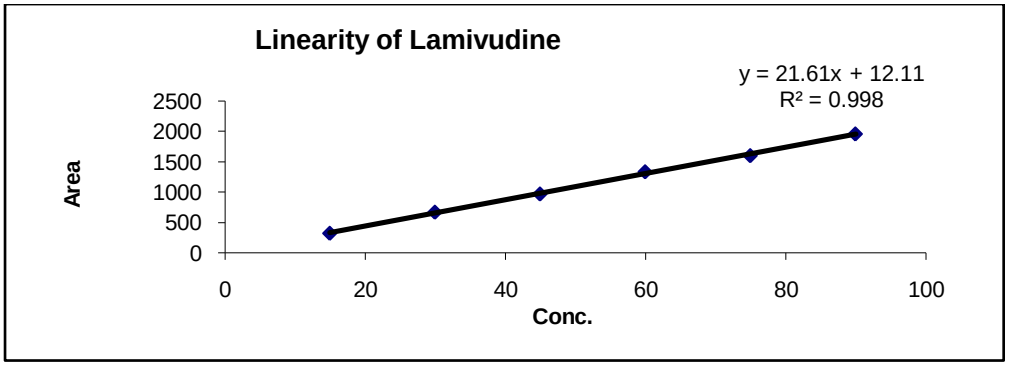

Figure 3: Linearity curve of standard Lamivudine

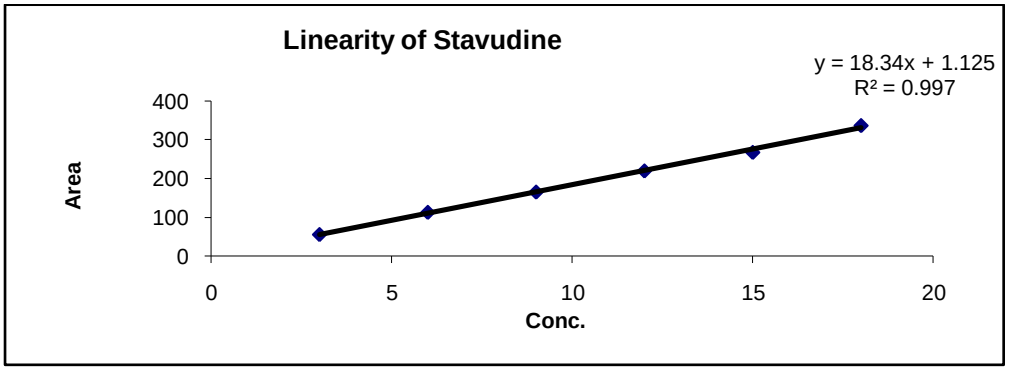

Figure 4: Linearity curve of standard Stavudine

Accuracy:

Accuracy was performed in triplicate for various concentrations of Lamivudine and Stavudine equivalent to 40, 50 and 60 % of the standard amount was injected into the HPLC system per the test procedure. The average % recovery of Lamivudine and Stavudine was calculated. **Table 4:** Results of Analysis of Formulation and Recovery Studies

Table 4: Results of Analysis of Formulation and Recovery Studies

Accuracy 40%

LAMIVUDINE

Sl.no		Area	Amt recovery	%Amt recovery
1	Std	1397.28		
2	Recovery 01	1392.57	39.86	99.66%
3	Recovery02	1408.49	40.32	100.8%
4	Recovery03	1387.31	39.71	99.28%

STAVUDINE

	Area	Amt Recovery	%Amt recovery
Std	233.348		
Recovery 01	234.234	40.15	100.38%
Recovery 02	232.258	39.81	99.53%
Recovery 03	232.496	39.85	99.63%

Accuracy 50%

LAMIVUDINE

Sl.no		Area	Amt recovery	%Amt Recovery
1	Std	1581.69		
2	Recovery 01	1593.16	50.36	100.72%
3	Recovery 02	1585.617	50.12	100.24%
4	Recovery 03	1591.74	50.31	100.63%

STAVUDINE

	Area	Amt Recovery	%Amt recovery
Std	264.50		
Recovery 01	265.83	50.25	100.5%
Recovery 02	264.65	50.02	100.05%
Recovery 03	264.05	50.67	99.83%

Accuracy 60%

LAMIVUDINE

Sl.no		Area	Amt recovery	%Amt Recovery
1	Std	2026.36		
2	Recovery 01	1998.16	59.16	98.6%
3	Recovery 02	2026.75	60.01	100.01%
4	Recovery 03	2009.26	59.49	99.15%

STAVUDINE

	Area	Amt Recovery	%Amt recovery
Std	331.25		
Recovery 01	334.04	60.5	100.84%
Recovery 02	332.19	61.07	100.28%
Recovery 03	333.13	60.7	100.56%

Precision:

A) Method Repeatability: Six sample solutions of the same concentration (50%) were prepared and injected into the HPLC system as per test procedure.

Table 5: Results from determination of precision of analysis of 3TC and d4T

LAMIVUDINE

Sl.no	Rt	Area
1	4.888	1563.914
2	4.84	1543.855
3	4.89	1560.421
4	4.862	1550.717
5	4.81	1528.764
6	4.798	1541.009
Avg	4.848	1548.113
Std dev	0.038905	13.04546
%RSD	0.802496	0.842668

STAVUDINE

Sl.no	Rt	Area
1	5.783	232.314
2	5.787	232.443
3	5.852	232.645
4	5.812	231.611
5	5.753	234.953
6	5.75	233.284
Avg	5.789	232.875
Std dev	0.03835	1.152155
%RSD	0.662401	0.494753

B) Intermediate Precision (Analyst to Analyst variability): Two analysts as per test method conducted the study. For Analyst-1 Method Repeatability and for Analyst-2 six sample solutions of the same concentration (50%) were prepared and injected into the HPLC system as per test procedure.

Table 6: Results from determination of precision of analysis of 3TC and d4T

LAMIVUDINE

Sl.no	Rt	Area
1	4.827	1605.185
2	4.821	1615.218
3	4.831	1609.503
4	4.828	1613.581
5	4.826	1610.821
6	4.795	1612.165
Avg	4.821	1611.079
Std dev	0.01333	3.51739
%RSD	0.27599	0.21832

STAVUDINE

Sl.no	Rt	Area
1	5.792	261.381
2	5.782	263.138
3	5.794	262.162
4	5.79	264.786
5	5.791	260.234
6	5.752	265.754
Avg	5.783	262.909
Std dev	0.01597	1.90254
%RSD	0.27616	0.72365

Robustness and Ruggedness: Robustness was done by small deliberate changes in the chromatographic conditions and retention time of 3TC and d4T was noted. The factors selected were flow rate and % methanol in the mobile phase. The results remained unaffected by small variations in these parameters. Ruggedness of the method was checked by using different analysts and instruments. The relative standard deviation of the results obtained from different analysts and instruments was < 1.0%.

Validation parameter

The method was validated by using the following parameters as shown in **Table 7.**

Table 7: Validation parameter of HPLC method for Lamivudine and Stavudine

Validation Parameter	Lamivudine (3TC)	Stavudine (d4T)
Linearity Range (µg/ml)	15-90	3-18
Regression equation	Y= 21.612x+12.113	Y=18.343x+1.1252
Correlation Coefficient (r^2)	0.9988	0.9979
Accuracy	98.6-100.8	99.53-100.84
Precision		
Method Repeatability (RSD %)	0.842668-0.802496	0.494753-0.662401
Intermediate Precision (RSD %)	0.21832-0.27599	0.72365-0.27616

CONCLUSION: The proposed method is rapid, accurate and sensitive. It makes use of fewer amounts of solvents and change of set of conditions requires a short time. Many samples can be simultaneously and suitably analysed for the routine quality control analysis of 3TC and d4T in bulk and its tablet dosage forms. It does not suffer from any interference due to common excipients present in pharmaceutical preparation and can be conveniently adopted for quality control analysis.

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