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Original Research Article

SIMULTANEOUS ESTIMATION OF DIDANOSINE AND STAVUDINE IN COMBINATION IN SYNTHETIC MIXTURE BY RP-HPLC

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Abstract: A validated RP-HPLC analytical method has been developed for the simultaneous estimation of Didanosine and Stavudine in bulk and in a synthetic mixture prepared in the laboratory. The proposed method is fast, simple, accurate, precise, specific, and has ability to separate drug from excipients if found in formulation if developed in future. The method is suitable for routine simultaneous analysis of Didanosine and Stavudine. The method can be successfully applied in simultaneous analyses of the two drugs in case of extensive clinical trials. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC–MS. The proposed method requires no sample pre treatment and is quite economical for routine analyses.

Key Words: Stavudine, Didanosine, Simultaneous estimation, RP-HPLC, Synthetic mixture.

Introduction: The pharmacokinetic studies conducted by Seifert RD et.al.[1] reveal that co-administration of Didanosine 100 mg and Stavudine 40 mg is well tolerated and the drugs do not interact pharmacokinetically. Sophisticated sensitive, high-pressure liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method for the simultaneous determination of Didanosine (ddI) and Stavudine (d4T) in various biological fluids and tissues has been developed by Huang Y et.al.[2] Looking at the multi drug therapy which is preferred in the management of HIV AIDS, it is foreseen that a combination of Stavudine and Didanosine may soon be developed and launched in the market because of the reason that antiretroviral combination therapy defends against resistance by suppressing HIV replication as much as possible.

Stavudine is a dideoxynucleoside analogue that inhibits reverse transcriptase and has in vitro activity against HIV.

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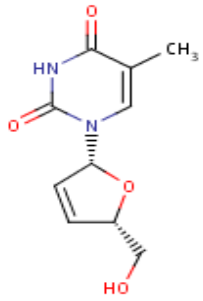

Fig 1 Stavudine

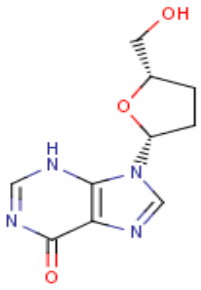

Fig 2 Didanosine

Stavudine is a NRTI with activity against HIV-1. Stavudine 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. Stavudine inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA. Didanosine is a potent inhibitor of HIV replication, acting as a chain-terminator of viral DNA by binding to reverse transcriptase; ddI is then metabolized to dideoxyadenosine triphosphate, its putative active metabolite. It is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Didanosine 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. Didanosine is effective against HIV, and usually used in combination with other antiviral therapy. Switching from long term AZT treatment to Didanosine has been shown to be beneficial[10].

Experimental

Apparatus

Chromatographic analysis was performed with a Shimadzu HPLC (LC-10AT VP) equipped with UV-Visible and PDA detector. Software – Class VP was used for LC peak integration along with data acquisition and data processing. The column used was Phenomenex C18 column (250 x 4.6mm id, 5 µm particle size). Injector was manual injector loop with injection volume of 20 µl. Ultrasonic bath (Frontline FS 4 ultrasonic cleaner, Mumbai) was used for sonication for degassing of mobile phase.

UV-1700, Shimadzu, Japan with UV Probe 2.0 software was used to obtain the overlay spectra of the drugs to determine the analytical wavelength.

The standard samples were obtained as gift samples from Emcure Pharma Ltd., Pune, along with their certificate of analyses. The standard substances were weighed on Sartorius CP224S analytical balance (Gottingen, Germany).

Reagents and Materials

HPLC grade acetonitrile used were purchased from S.D. Fine chemicals, Mumbai.

Water HPLC grade from Finar reagents, Ahmedabad, was used for the preparation of mobile phase.

Whatman filter paper no. 41 was used as a filter medium procured from S.D. Fine chemicals, Mumbai.

Preparation of mobile phase

HPLC grade solvents were used in separate bottle of gradient pumps as mobile phase. A mixture of acetonitrile and water in a ratio of 80:20 v/v with pH of the mixture adjusted to 4.5 with acetic acid were adjusted by gradient pump operated by Class VP LC solution software.

Mixed solvents were filtered through nylon 0.45 µm membrane and degassed by the instrument and used as mobile phase.

Preparation of standard solutions

Preparation of standard stock solution (100 µg/ml)

Accurately weighed 10 mg Didanosine and Stavudine each were transferred to separate 100 ml volumetric flasks, dissolved in 50 ml methanol and diluted up to mark with methanol to prepare standard stock solutions of both the drugs each having a concentration of 100µg/ml.

Preparation of working standard solution

From the standard stock solution of concentration 100µg/ml of Didanosine 0.05, 0.2, 0.4, 0.8, 1.2 and 1.5 ml were taken in separate 10ml volumetric flasks and diluted up to the mark with methanol.

From the standard stock solution of concentration 100µg/ml of Stavudine 0.01, 0.05, 0.1, 0.4, 0.8, and 1.2 ml were taken in separate 10ml volumetric flasks and diluted up to the mark with methanol.

Preparation of synthetic mixture

A synthetic mixture was prepared in laboratory such that the composition of Didanosine and Stavudine was in the ratio of 50:50 w/w. Additives like magnesium stearate and talc were added and mixed properly so that they could be encapsulated in a hard gelatin shell.

Preparation of sample solution

Accurate quantity of the synthetic mixture equivalent to the powder of 10 mg of Didanosine and 10mg of Stavudine was weighed and transferred to a 100 ml volumetric flask, dissolved in methanol (60 ml) and sonicated for 30 min. The solution was filtered through Whatmann filter paper No. 41 and residue was washed with methanol. The solution was diluted up to the mark with methanol.

Determination of wavelength of maximum absorbance

The standard solutions of both Didanosine and Stavudine were injected in the system and spectrum in the range of 200 to 400 nm was recorded

Chromatographic conditions

The final chromatographic conditions are tabulated below as:

Table 1: Final chromatographic conditions

S.no.	Parameter	Description / Value
1.	Stationary Phase	Phenomenex® C_{18} column with 250 mm x 4.6 mm i.d and 5 µm particle size
2.	Mobile Phase	A mixture of acetonitrile and water with pH adjusted to 4.5 with acetic acid in a ratio of 80:20 v/v
3.	Flow Rate	1.0 ml/min
4.	Detection wavelength	260nm
5.	Detector	Photodiode array (PDA) detector
6.	Injector	Manual injector loop
7.	Injection volume	20µl
8.	Column Temperature	Ambient
9.	Run Time	8 min
10.	Diluent	Methanol

Method Validation

The method was validated with respect to the following parameters given below as per ICH guidelines[11].

Linearity was established by triplicate injections of standard solutions containing Didanosine and Stavudine in concentration ranges of 0.5-15 mg/ml and 0.1-12 mg/ml respectively.

The limit of detection (LOD) and the limit of quantification (LOQ) values were calculated from the calibration curves as k.SD / b where k = 3 for LOD and k = 10 for LOQ, SD is the standard deviation of the responses, b is the slope of the calibration curve.

Precision was evaluated in terms of intraday and interday precision. The intraday precision was investigated using three different concentrations of standard solutions. The intraday precision was investigated by analyzing three different solutions of Didanosine (4.0, 8.0 and 12.0 µg/ml) and Stavudine (4.0, 8.0 and 12.0 µg/ml). The interday precision was investigated by analyzing three different standard solutions of Didanosine (4.0, 8.0 and 12.0 µg/ml) and Stavudine (4.0, 8.0 and 12.0 µg/ml) on different days. The results were reported in terms of % coefficient of variance (% CV).

The accuracy of the method was determined by calculating recoveries of Didanosine and Stavudine by the standard addition method. For this known amounts of standard solutions of Didanosine and Stavudine (50, 100, and 150 % level) were added to preanalyzed sample solutions. The amount of Didanosine and Stavudine was analyzed by using the regression equations of the calibration curve.

The specificity of the method was established through resolution factor of the drug peak from the nearest resolving peak and also among all other peaks. Selectivity was confirmed through peak purity data using a PDA detector. To assess the method specificity, synthetic mixture without Didanosine and Stavudine (placebo) was prepared with the excipients as required for commercial preparation and compared with respective drug standard to evaluate specificity of the method. Representative chromatograms of placebo and standard were compared for retention time, resolution factor and purity.

Method robustness was performed by applying small changes in the composition of mobile phase, pH, analytical wavelength and flow rate. Robustness of the method was done at three different concentration levels of 4.0, 8.0 and 12.0 µg/ml for Didanosine and 4.0, 8.0 and 12.0 µg/ml for Stavudine. The results were expressed in terms of % CV.

The system suitability parameters like theoretical plates (T_p), and asymmetry factor (As), capacity factor (K'), resolution (R_s), retention time (RT) and tailing factor (T_f) reported in European Pharmacopoeia[2-3] were calculated by Class VP LC solution software. The HPLC system was equilibrated with the initial mobile phase composition, followed by six injections of the standard solutions having same concentration. These six consecutive injections were used to evaluate the system suitability on each day of method validation. In order to establish system suitability for the instrument, six consecutive injections of Didanosine and Stavudine was prepared from working standard solution and analyzed.

Assay procedure

Appropriate three different aliquots from sample solution were suitably diluted with mobile phase in such a way to get concentrations in a range of 0.5 to 15 µg/ml for Didanosine and 0.1 – 15 µg/ml for Stavudine. The finally prepared solutions were analyzed under chromatographic conditions as described in table 1. The amount of Didanosine and Stavudine present in sample solution was determined by fitting the area response into the regression equation of both the drugs in the method.

Results and Discussion

To determine the analytical wavelength, solutions of Didanosine and Stavudine were scanned between 200 and 400 nm and since UV overlay spectra of both the drugs show maximum absorbance at 260 nm it was selected as the analytical wavelength.

Validation of the Proposed Method

The developed method as described above was validated for various parameters like system suitability, specificity, linearity, precision, accuracy, LOQ and LOD.

Linearity and range

Linearity of the method was evaluated at seven concentration levels by diluting the working standard solution in the concentration range of 0.5 to 15 µg/ml for Didanosine and 0.1 – 15 µg/ml for Stavudine. The results show that an excellent correlation existed between the peak area and concentration of analyte. The calibration curve was prepared by plotting the peak area versus the concentration and regression equation was calculated. The calibration curve was repeated for five times and the average results are mentioned in table 2. The optical and regression characteristics are mentioned in Table 3.

Table 2: Data for calibration curve for Didanosine and Stavudine

Conc. of Didanosine µg/ml	Peak area	Conc. of Stavudine µg/ml	Peak area
0.5	6432	0.1	964
2	24826	0.5	5136
4	50736	1.0	8894
8	96068	4.0	41736
12	134745	8.0	88476
15	174949	12.0	126754

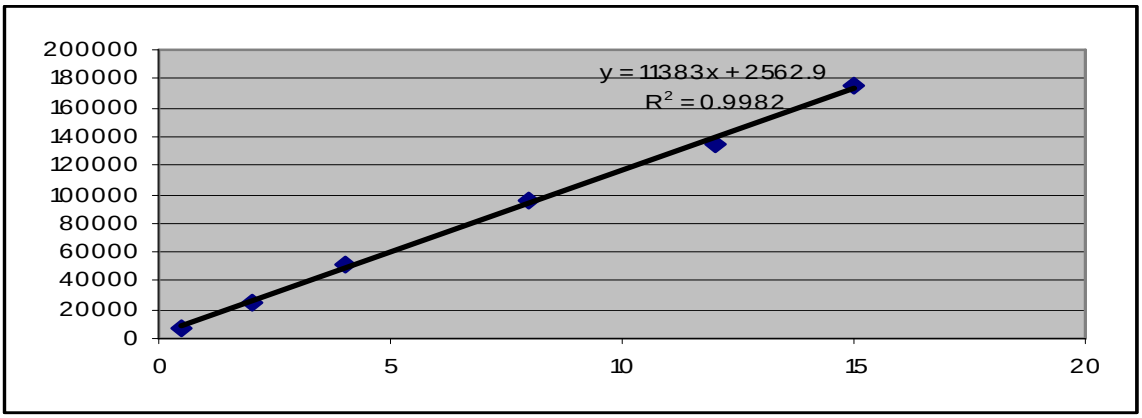

Fig 3: Calibration curve for Didanosine for simultaneous estimation with Stavudine

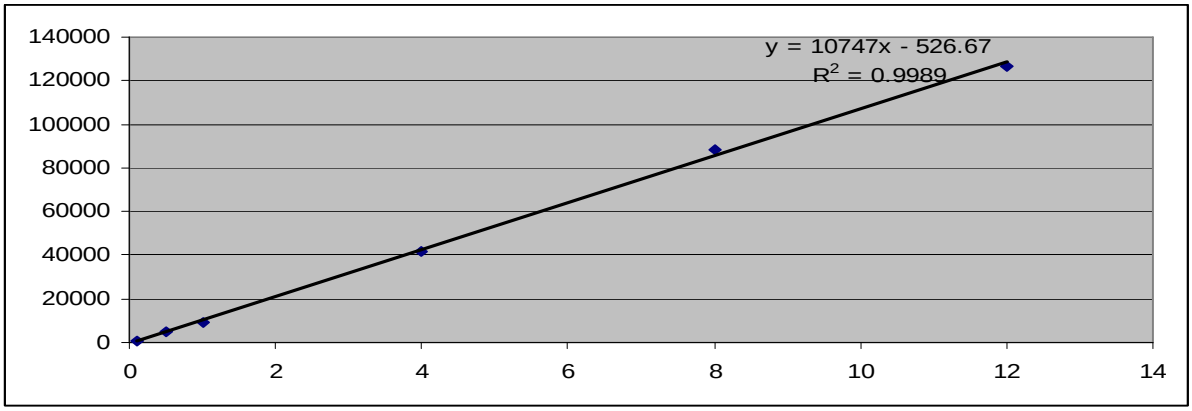

Fig 4: Calibration curve for Stavudine for simultaneous estimation with Didanosine

Table 3: Optical and regression characteristics for analysis of Didanosine and Stavudine by RP-HPLC method

Parameters	Didanosine	Stavudine
Concentration range (µg/ml)	0.5 to 15	0.1 – 15
Limit of Detection (LOD) (µg/ml)	0.06389	0.02865
Limit of Quantification (LOQ) (µg/ml)	0.1936	0.0868
Regression equation (y*= a + bc)		
Slope (b)	11383	10747
Intercept (a)	+ 2562.9	–526.67
Correlation coefficient (r)	0.9982	0.9989
y*= a + bc, where c is the concentration		

Precision

Method precision

The results of repeatability (method precision) experiment are shown in Table 4. Method precision was determined by repeatedly injecting 8.0 µg/ml concentration of Didanosine and 8.0 µg/ml of Stavudine (n = 6). The developed method was found to be precise and the results are expressed in terms of % CV.

Table 4. Method precision data of Didanosine and Stavudine by RP-HPLC method

Drug	Concentration	RT	%CV	Area	%CV
Didanosine	8.0 mcg/ml	3.15	0.2483	96547	0.3780
Stavudine	8.0 mcg/ml	6.35	0.3912	88493	0.2366

Intermediate precision

The results of intermediate precision experiment for both intraday and interday are shown in Table 5. Replicate analyses of three concentrations of the standard solution show good reproducibility. The developed method was found to be precise as the % CV values were within acceptance limit.

Table 5: Intermediate precision data of Didanosine and Stavudine RP-HPLC method

Didanosine µg/ml	Intra-day measured mean area, % CV (n=6)	Didanosine µg/ml	Inter-day measured mean area, % CV (n=6)
4	50529.7; 0.7840	4	48972.8; 1.1754
8	96165.2; 0.9143	8	95159; 1.0680
12	134608.5; 0.2460	12	135571.5; 1.3232
Stavudine µg/ml	**Intra-day measured mean area, % CV (n=6)**	**Stavudine µg/ml**	**Inter-day measured mean area, % CV (n=6)**
4	41502.3; 0.4207	4	41082.3; 1.1479
8	88426.2; 0.7705	8	88632.5; 1.1122
12	126280.8; 0.7054	12	126414; 1.4170

Accuracy (% Recovery)

Good recovery of the spiked drug was obtained at each added concentration, indicating that the method was accurate. A known amount of drug (50, 100, and 150 %) was added to the pre analyzed sample solution. This solution was

analyzed under the chromatographic conditions mentioned in table 1. The assay was repeated over 3 consecutive days to obtain intermediate precision data. The results of accuracy study are shown in Table 6.

Table 6 Recovery study of Didanosine and Stavudine by RP-HPLC method

Drug	Known conc. µg/ml	Added conc. µg/ml	% Recovery
Didanosine	4	2 (50%)	101.0101
	4	4 (100%)	98.7655
	4	6 (150%)	100.3376
Stavudine	4	2 (50%)	100.6176
	4	4 (100%)	99.6713
	4	6 (150%)	98.7010

Robustness

To evaluate the robustness of the proposed method, experimental conditions were deliberately altered and the response of the drugs was recorded. The results of change in ratio of mobile phase, pH of ammonium format buffer and wavelength are shown in Table 7.

Table 7 Intra-day robustness data of Didanosine and Stavudine by RP-HPLC method

Parameter	Modification	% Recovery ± S.D, % CV (n=6) Didanosine	% Recovery ± S.D, % CV (n=6) Stavudine
Flow rate (1 ml/min)	+0.1	99.1262 ± 0.9617, 1.1271	99.5123 ± 0.6921, 0.9832
	-0.1	99.8561 ± 0.5369, 0.6514	99.7436 ± 0.7631, 0.7846
Mobile phase composition Acetonitrile : water (80:20 v/v), pH 4.5	81:19	98.6437 ± 0.3715, 0.4983	98.1672 ± 0.8393, 0.8625
	79:21	98.7947 ± 1.3285, 1.3688	99.1334 ± 1.2543, 1.2695
pH 4.5	+0.1	98.4358 ± 1.0217, 1.0732	98.1521 ± 0.9612, 0.9734
	-0.1	97.8561 ± 1.5149, 1.6132	97.4732 ± 1.4825, 1.4879
Wave length (260 nm)	259	99.1574 ± 0.9154, 1.0527	99.2146 ± 1.0256, 1.1215
	261	98.9513 ± 0.7369, 0.7536	99.1439 ± 0.7642, 0.7851

Limit of detection and quantitation: These data show that the method is sensitive for the determination of Didanosine and Stavudine. The results are shown in table 8 .

Table 8: LOD and LOQ for Didanosine and Stavudine

	Standard Deviation (σ)	Slope of cal. curve	LOD µg/ml	LOQ µg/ml
Didanosine (8µg/ml)	220.3960	11383	0.06389	0.1936
Stavudine (8µg/ml)	93.2981	10747	0.02865	0.0868

Specificity and selectivity

The resolution factor for Didanosine and Stavudine from the nearest resolving solvent peak was > 3 in all samples. The placebo shows no detector response near retention times of 3.132 min and 6.385 min [Figure 5], while the

Didanosine and Stavudine standards display good resoluted peaks [Figure 6and 7] and no interference from excipients present in the formulation [Figure 8] indicate specific nature of the method. The purity curve and data [Figure 9 & Table 9] of Didanosine and Stavudine show that no other excipients are co-eluted with the drug and the peak is pure in nature.

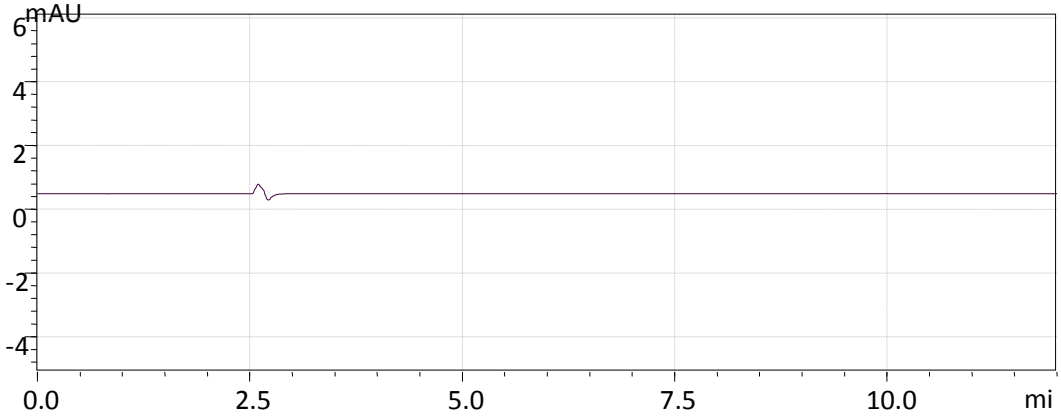

Figure 5 Placebo chromatogram in synthetic mixture at 260 nm

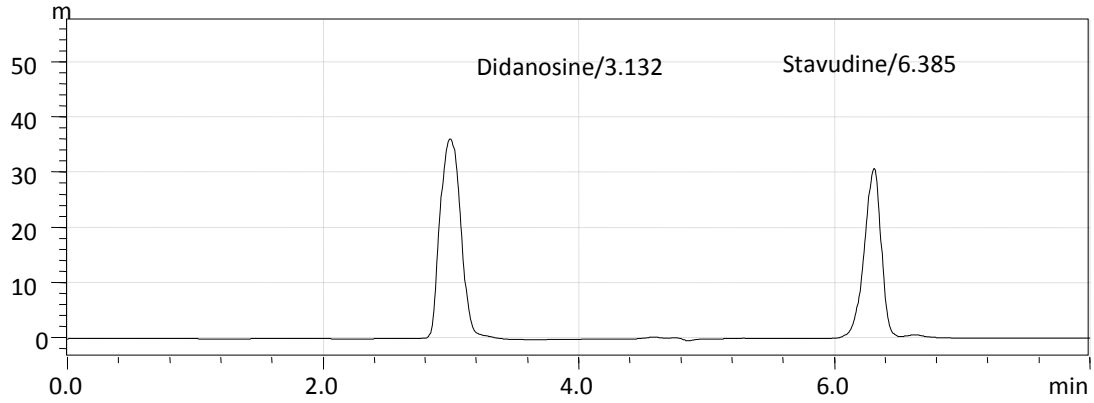

Figure 6 Didanosine and Stavudine chromatogram in synthetic mixture at 260 nm

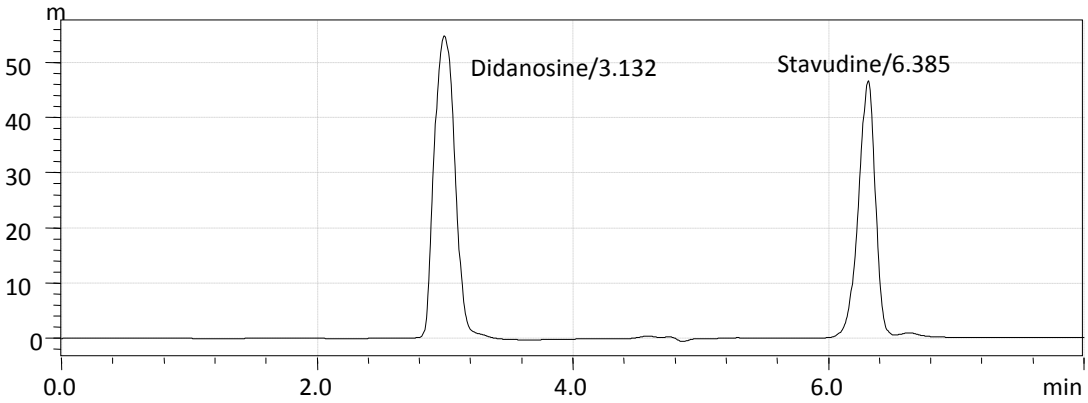

Figure 7 Didanosine and Stavudine chromatogram at 260 nm

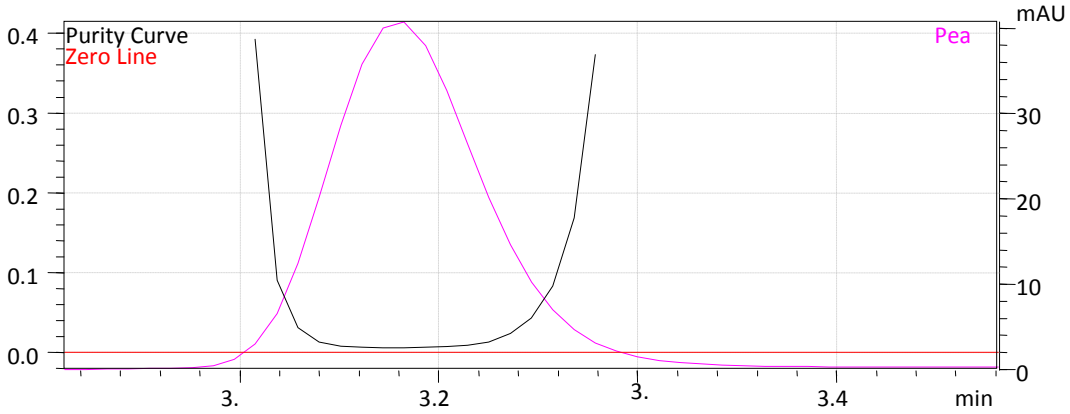

Figure 8 Peak Purity plot of Didanosine with purity 0.9986

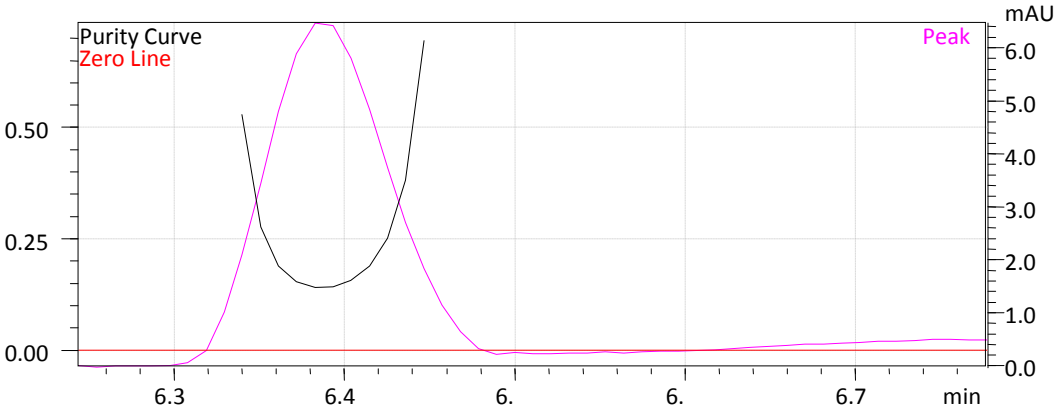

Figure 9 Peak Purity plot of Stavudine with purity 1.0000

Table 9 Table showing Peak purity data

Drug	Peak purity Index	Single point Threshold	Minimum peak purity threshold
Didanosine	0.9986	0.9867	4736
Stavudine	1.00	0.9997	6745

System suitability

As system suitability test was an integral part of chromatographic method development and were used to verify that the system is adequate for the analysis to be performed, the system suitability parameters for Didanosine and Stavudine were evaluated. The suitability of the chromatographic system was demonstrated by comparing the obtained parameter values. The obtained parameters are given in Table 10 and they are found within the acceptance criteria.

Table 10 System suitability parameters for Didanosine and Stavudine by RP-HPLC method

Parameter	Value for Didanosine	Value for Stavudine
Retention time (Minutes)	3.15	6.39
Resolution (Rs)	3.74	4.01
Theoretical plates (T_P)	2857	4879
Tailing factor (T_f)	1.05	0.46
Asymmetric factor (A_f)	1.10	0.63
Capacity factor (K')	3.6	5.6

Solution stability

The % CV of the assay of Didanosine and Stavudine during solution stability experiments were within 2 %. No significant changes were observed in the content of standard drug solution during solution stability and mobile phase stability experiments when performed using the method. The solution stability and mobile phase stability experiment data confirms that the sample and standard in solvent and mobile phases used during assay determination were stable for at least 24 hours. Results of the solution stability are shown in Table 11.

Table 11 Table showing solution stability data for Didanosine and Stavudine

Drug and concentration →	Didanosine 8µg/ml		Stavudine 8µg/ml	
	Time (Hr)	Peak area	Time	Peak area
	00	94068	00	85476
	06	93976	06	85345
	12	93841	12	85291
	18	93649	18	85164
	24	93521	24	85024

Conclusion

A validated RP-HPLC analytical method has been developed for the simultaneous estimation of Didanosine and Stavudine in bulk and in a synthetic mixture prepared in the laboratory. The proposed method is fast, simple, accurate, precise, specific, and has ability to separate drug from excipients if found in formulation if developed in future. The method is suitable for routine simultaneous analysis of Didanosine and Stavudine. The method can be successfully applied in simultaneous analyses of the two drugs in case of extensive clinical trials. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC–MS. Prime importance was given to develop a fast, economic and simple RP-HPLC method. The proposed method requires no sample pre treatment and is quite economical for routine analyses.

The proposed method meets the system suitability criteria, peak integrity and resolution for the drugs. Detection and quantification limits achieved describe that the method is very sensitive. High recoveries and acceptable % CV values confirm that the proposed method is accurate and precise. The analytical results demonstrate the ability of the developed method to assay both the drugs in the presence of common excipients. The results of precision study show that the method is precise. Assay results found from the study show that the method can be successfully applied for the simultaneous estimation of Didanosine and Stavudine in capsule dosage form if developed in future. Hence, the method is recommended for routine quality control analysis of both the drugs in combination, in analyses during clinical trials and in simple capsule dosage form if developed in future.

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