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DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE DETERMINATION OF ZIDOVUDINE AND RELATIVE SUBSTANCES IN BULK

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Abstract:

A reversed- phase High Performance Liquid Chromatography (RP-HPLC) method for the rapid and accurate quantification of zidovudine (AZT) and related substances. The detection was carried out by Shimadzu RP-C_{18} ODS (250×4.6mm, 5µm) column using mixture of water and Methanol (70:30) with a flow rate of 1ml/min. The eluents were detected at 270nm and the Retention time for zidovudine, zidovudine B and C impurity was found to be 9.5, 11.3 and 3.5 min. A linear responses for zidovudine and impurities B and C were 5-25 µg/ml, 1-5 µg/ml with correlation factor of 0.998, 0.998 and 0.997 respectively. The method was validated as per ICH guidelines for specificity, linearity, accuracy and robustness. The validated method was successfully employed for the simultaneous determination zidovudine (AZT) and related substances.

Keywords: *zidovudine (AZT), impurities B and C, RP-HPLC anti-viral.*

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INTRODUCTION:

zidovudine 1-[(2R,4S,5S)-4-azido-5-(hydroxymethyl) oxolan-2-yl]-5-methylpyrimidine-2,4-dione. [figure1] is a combination with other antiretroviral agents for the treatment of human immuno virus (HIV) infections. Zidovudine impurity B 1-(3-Chloro-2,3-dideoxy-b-D-erythro-pentofuranosyl)-5-methylpyrimidine-2,4(1H,3H)-dione.[figure 2] and Zidovudine impurity C Methylpyrimidine-2,4(1H,3H)-dione. [figure1] [1-10] Literature review reveals very few methods are reported for the assay of zidovudine (AZT) and related substances in Tablet dosage forms using RP-HPLC method. The reported HPLC methods was having disadvantages like high flow rate, more organic phase and use of costly solvents. The RP-HPLC method utilizes economical solvent system and having advantages like better retention time, less flow rate, very sharp and symmetrical peak shapes. The aim of the study was to develop a simple, precise, economic and accurate RP-HPLC method for the estimation of Lamivudine Rilpivirine hydrochlorides in Tablet dosage forms.

MATERIALS AND METHODS:

Instrumentation:

Liquid chromatographic Agilent (LC-1220) system was manufactured by Agilent and equipped with UV detector. Rheodyne injector with20 µl loop volume and HPLC column- Agilent RP-C_{18} ODS (250 X 4.6mm), 5µm. Weighing was done on an Electronics Balance of Single pan balance of model AX-200-shimadzu. p^H of buffer was maintained by p^H analyzer, digital electronics model-7007.

Materials:

Zidovudine (AZT) and related substances were obtained from Indian Pharmacopeia Reference Standards (IP). HPLC grade acetonitrile Merck, HPLC grade water was obtained from fischer scientific, methanol HPLC grade was obtained from Merck, all the above chemicals and solvents are from ranchem.

Chromatographic conditions:

The mobile phase consisted of HPLC grade water and Methanol (70:30) (v/v) at a flow rate of 1.0 ml/min. Shimadzu RP-C_{18} ODS (250×4.6mm, 5µm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of the method for each of three drugs 270nm was selected as the detection wave length for PDA detector. The HPLC system was operated at a room temperature of 30°C

Preparation of zidovudine standard stock solution:

About 10mg of zidovudine standard stock solution were weigh and transferred into a 10ml volumetric flask, add HPLC water, mix for 5min and make up the volume with HPLC water and sonicated for 10 min to dissolve the drug.

Preparation of zidovudine impurity B standard stock solution:

Accurately weigh and transferred 10mg of zidovudine impurity B standard into a 10ml volumetric flask. Add 5ml of methanol and HPLC water to the volumetric flask mix for 5min.

Preparation of zidovudine impurity C standard stock solution:

Accurately weigh and transferred 10mg of zidovudine impurity C standard into a 10ml volumetric flask. Add 5ml of methanol and HPLC water to the volumetric flask mix for 5min.

PREPARATION OF WORKING STANDARD SOLUTION:

Preparation of zidovudine working standard solution:

Pipette out 1ml of lamivudine standard stock solution into a 10ml volumetric flask. Diluted up to 10 ml volume with mobile phase and mixed well. From working standard solutions, further dilutions were made up to 5-25µg/ml.

Preparation of zidovudine impurity B standard solution:

Pipette out 1ml of zidovudine impurity B standard stock solution into a 10ml volumetric flask. Diluted up to 10 ml volume with mobile phase and mixed well. From working standard solutions, further dilutions were made up to 1-5µg/m

Preparation of zidovudine impurity C standard solution:

Pipette out 1ml of zidovudine impurity C standard stock solution into a 10ml volumetric flask. Diluted up to 10 ml volume with mobile phase and mixed well. From working standard solutions, further dilutions were made up to 1-5µg/ml

Method optimization:

The chromatographic separation was performed using Shimadzu RP-C_{18} ODS (250×4.6mm, 5µm) column. For selection of mobile phase, various mobile phase compositions were observed for efficient elution and good resolution. The mobile phase consisting of HPLC grade water and Methanol (70:30) (v/v) was found to be the optimum composition for efficient elution of analyte. The mobile phase was injected to the column at a flow rate 1.0ml/min for 20min. Column

temperature was maintained at 30°C. Analyte was monitored at 270 nm using PDA detector. The retention time of Zidovudine (AZT) and related substances was eluted at 9.5, 11.3 and 3.5 min respectively with good resolution. Mobile phase was used as diluents during the preparation of standards and test samples.

RESULTS:

Method validation: [11]

System suitability:

System suitability test should be carried out to verify the analytical system is working properly and can give accurate and precise results. Standard solutions were prepared as per the test method and injected into the system. The system suitability parameters was evaluated from resolution, retention times (RT) and theoretical plates (N) was evaluated for six injections of standard solution at Zidovudine (10µg/ml) and Zidovudine impurities (B and C) (2µg/ml). The results are tabulated in the table no 1 and chromatogram was shown in figures no 4.

Linearity:

The linearity of an analytical method was carried out to check its ability to elute test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The linearity of concentration zidovudine and impurities B and C were 5-25 µg/ml, 1-5 µg/ml. From the linearity studies calibration curve was plotted and concentrations were subjected to the least square regression analysis to calculated regression equation. The regression coefficient was found to be 0.998, zidovudine 0.998 impurity B and 0.997 and impurity C respectively and showing good linearity for two drugs. The results are tabulated in the table no 2 and chromatograms was shown in figures no 5,6,7.

Precision:

The precision of an analytical method is a measure of the random error and are defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variation (%CV) or relative standard deviation (RSD) of the replicate measurements. The results are tabulated in the table no 3 and chromatograms were shown in figures no 8.

Intra-day:

Repeatability expresses the precision under the same operating condition over a short interval of time. Repeatability is also termed as Intra-assay precision. The results are tabulated in the table no 4 and chromatograms were shown in figures no 9.

Intermediate precision (Day_ Day Precision):

Intermediate precision of the analytical method was determined by performing method precision on another day by different analysts under same experimental condition. Assay of all five replicate sample preparations ware determined and mean % assay value, standard deviation & % RSD was calculated. The results are tabulated in the table no 5 and chromatograms were shown in figures no 10.

Accuracy:

The accuracy of an analytical method is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value finds. Recovery of the method was determined by spiking an amount of the pure drug (50%, 100% and 150%) at the three different concentration levels in its solution has been added to the pre analyzed working standard solution of the drug. The results are tabulated in the table no 6 and chromatograms were shown in figures no 11,12 and 13.

Robustness:

The robustness of an analytical method is a 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.

Small de liberate changes in method like Flow rate, mobile phase ratio, and wavelength are made but there were no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like Flow minus (0.8ml/min), Flow plus (1.2ml/min), mobile phase minus (25:65), mobile phase plus (35:75), Wavelength minus (265) and Wavelength plus (275) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed and all are within the limit. The results are tabulated in the table no 7 and chromatograms were shown in figure no 14- 19.

Table 01 System suitability parameters for Zidovudine and related substances.

S.no	Zidovudine API			Zidovudine impurity B			Zidovudine impurity C		
Inj	RT(min)	TP	Asymmetry	RT(min)	TP	Asymmetry	RT(min)	TP	Asymmetry
1	9.581	37432	1.32	10.605	38237	1.25	3.567	32284	1.47
2	9.541	33954	1.39	10.678	34614	1.24	3.578	29652	1.49
3	9.501	32569	1.33	10.652	33289	1.21	3.583	28438	1.48
4	9.495	31127	1.40	10.608	31776	1.26	3.588	27626	1.45
5	9.566	29695	1.36	10.632	30238	1.31	3.586	26314	1.46
6	9.545	31797	1.34	10.616	42629	1.28	3.566	28142	1.44

Table 02 Linearity table for Zidovudine and related substances

S.no	Zidovudine		Zidovudine Impurity B		Zidovudine Impurity C	
	Conc. (25µg/ml)	Peak area	Conc. (5µg/ml)	Peak area	Conc. (5µg/ml)	Peak area
1	5	255957	1	63026	1	101354
2	10	551910	2	132826	2	252870
3	15	822334	3	190062	3	368315
4	20	1106549	4	252198	4	496267
5	25	1337389	5	310576	5	607764
	R^2= 0.998		R^2= 0.998		R^2= 0.997	

Table 3 System precision table of Zidovudine and Zidovudine impurities B and C

S.no	Zidovudine	impurities B	impurities C
1	555962	149698	252797
2	556910	152223	260328
3	556315	151248	252969
4	568826	156798	253644
5	556843	149891	263254
6	565922	149299	253143
Mean	560129.7	151526.2	256022.5
S.D	5696.787	2804.689	4571.836
%RSD	1.01%	1.85%	1.78%

Table 4 Intra-day table of Zidovudine, Zidovudine impurities B and Zidovudine impurities C

S.no	Zidovudine	Impurities B	Impurities C
1	556315	150248	252969
2	567826	147290	256441
3	563157	149278	257254
4	556317	151908	260268
5	562319	151781	253950
6	569826	147798	260644
Mean	562626.7	149717.2	256921
S.D	5637.17	1954.899	3157.517
%RSD	1.00%	1.30%	1.22%

Table 5 Intermediate precision table of Zidovudine,Zidovudine Impurity B and Zidovudine C

S.no	Zidovudine	Impurities B	Impurities C
1	556843	149891	253254
2	568922	149299	253143
3	556315	150248	252969
4	567826	147290	256441
5	562157	149278	257254
6	567317	152908	260268
Mean	563230	149819	255554.8
S.D	5656.642	1827.023	2955.798
%RSD	1.00%	1.19%	1.15%

Table 6 Accuracy table of Zidovudine

%Concentration (at specification Level)	Area			Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
	Sample Area	Average	Standard Area				
50 %	725748	725178.3	210312.4	6.00	6.25	104.16	101.17
	724644						
	725143						
100 %	1112710	1110252		8.0	8.34	104.25	
	1105140						
	1112907						
150 %	1358936	1358557		10.0	9.51	95.1	
	1359345						
	1357389						

Table 7: Robustness data for Zidovudine

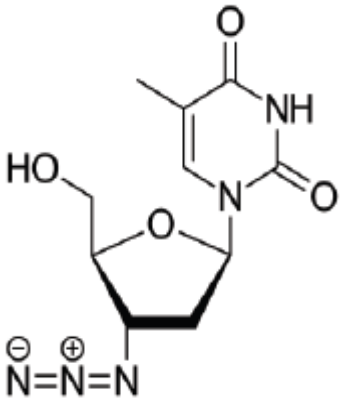
S.No	Parameter	Zidovudine		
		RT (min)	Theoretical plate count	Resolution
1	Standard	9.709	33817	16.612
2	Change in mobile phase ratio(-)25:75	9.781	34198	16.348
3	Change in mobile phase ratio(+)35:65	9.841	33398	16.659
4	Change in flow rate(-) 0.8ml/ min	9.901	33590	16.617
5	Change in flow rate(+)1.2 ml/ min	9.966	34017	16.360
6	Change in wavelength (-)265	9.59	34013	16.073
7	Change in wavelength (+)275	9.60	33693	16.349

Table 8 Robustness data for Zidovudine impurity B

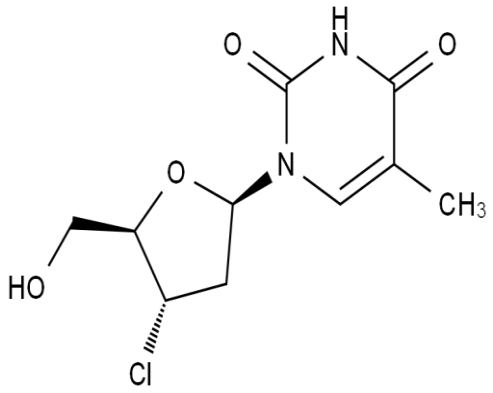
S.No	Parameter	Zidovudine impurity B		
		RT (min)	Theoretical plate count	Resolution
1	Standard	11.138	43938	2.534
2	Change in mobile phase ratio(-)25:75	11.105	42543	2.470
3	Change in mobile phase ratio(+)35:65	11.178	43670	2.488
4	Change in flow rate(-) 0.8ml/ min	11.252	43289	2.557
5	Change in flow rate(+)1.2 ml/ min	11.33	43614	2.510
6	Change in wavelength (-)265	10.918	42911	2.491
7	Change in wavelength (+)275	11.105	43615	2.453

Table 9 Robustness data for Zidovudine impurity C

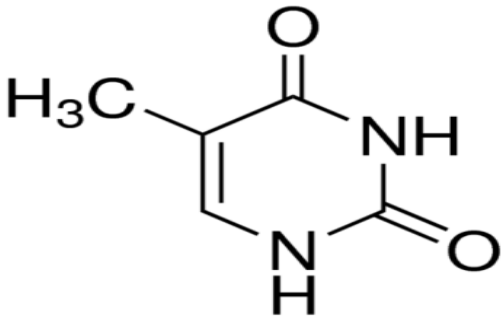
S.No	Parameter	Zidovudine impurity C		
		RT (min)	Theoretical plate count	Resolution
1	Standard	3.578	35922	0000
2	Change in mobile phase ratio(-)25:75	3.569	35954	0000
3	Change in mobile phase ratio(+)35:65	3.578	34390	0000
4	Change in flow rate(-) 0.8ml/ min	3.583	37589	0000
5	Change in flow rate(+)1.2 ml/ min	3.582	35139	0000
6	Change in wavelength (-)265	3.513	35581	0000
7	Change in wavelength (+)275	3.569	34832	0000



STRUCTURE OF ZIDOVUDINE



STRUCTURE OF ZIDOVUDINE B



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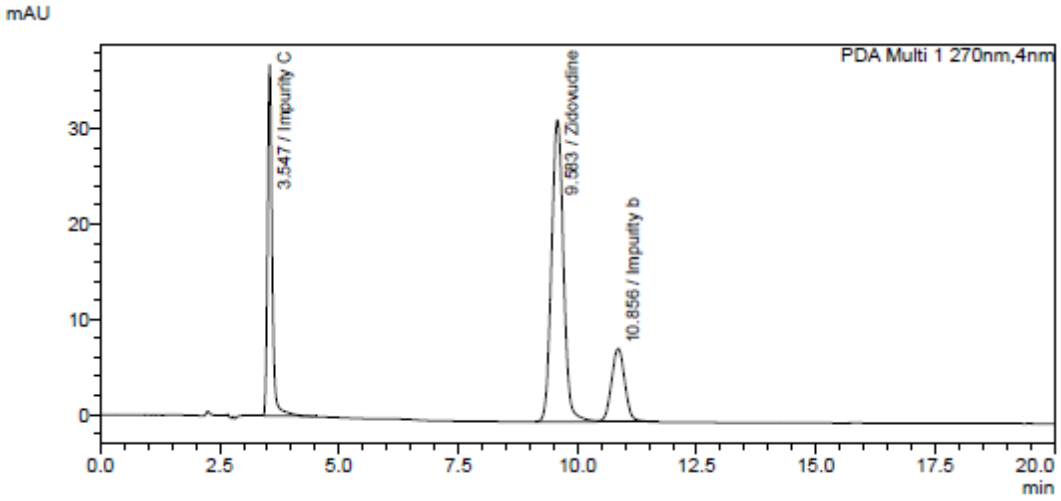

Fig 4 System Suitability Chromatogram

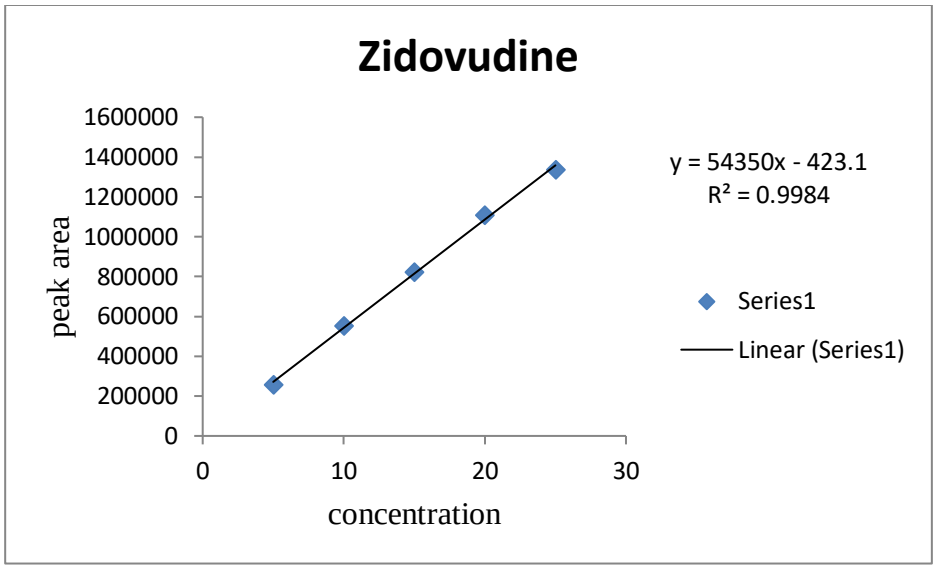

Fig 5 Calibration curve of Zidovudine

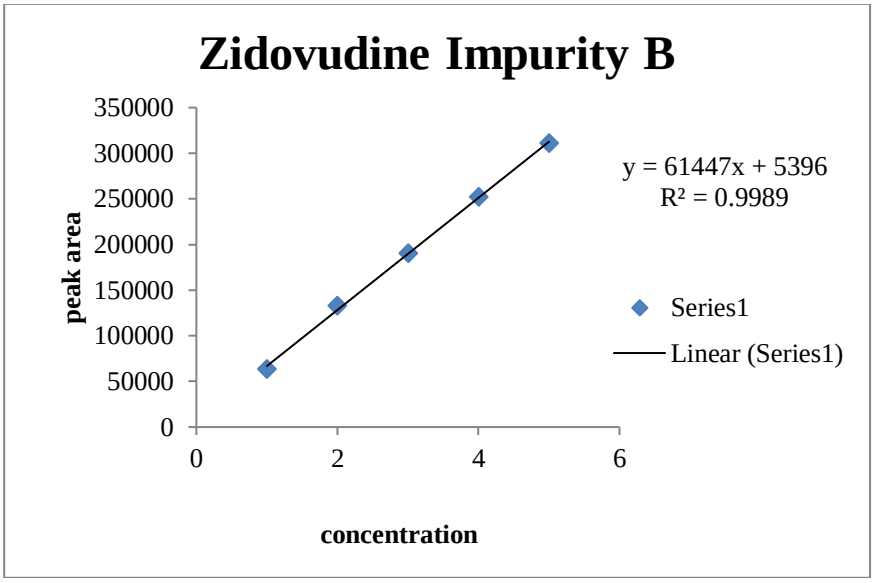

Fig 6 Calibration curve of Zidovudine Impurity B

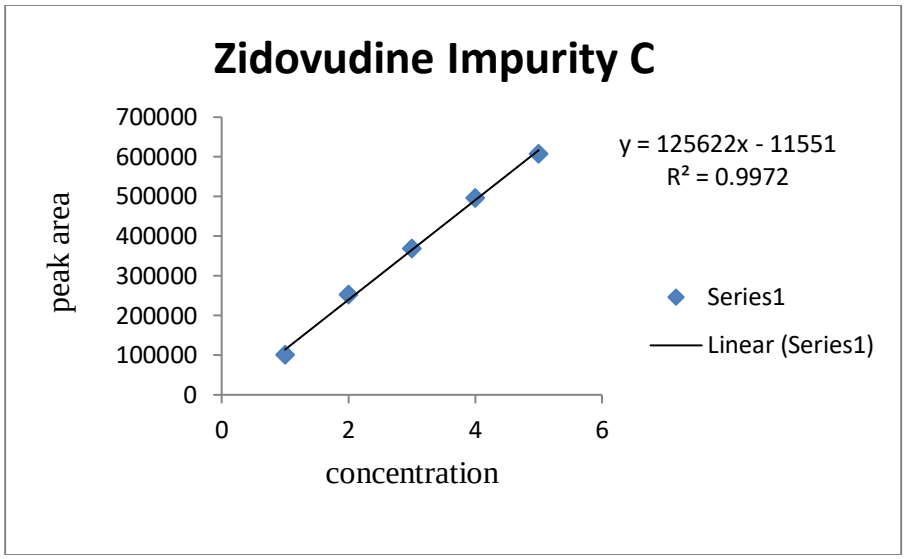

Fig 7 Calibration curve of Zidovudine Impurity C

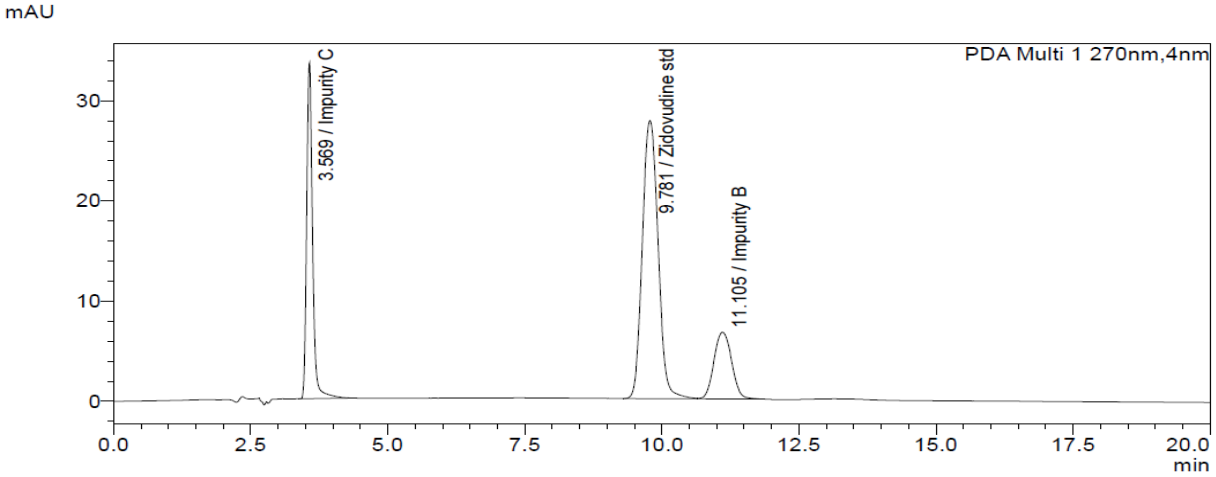

Fig.8 System precision chromatogram injection

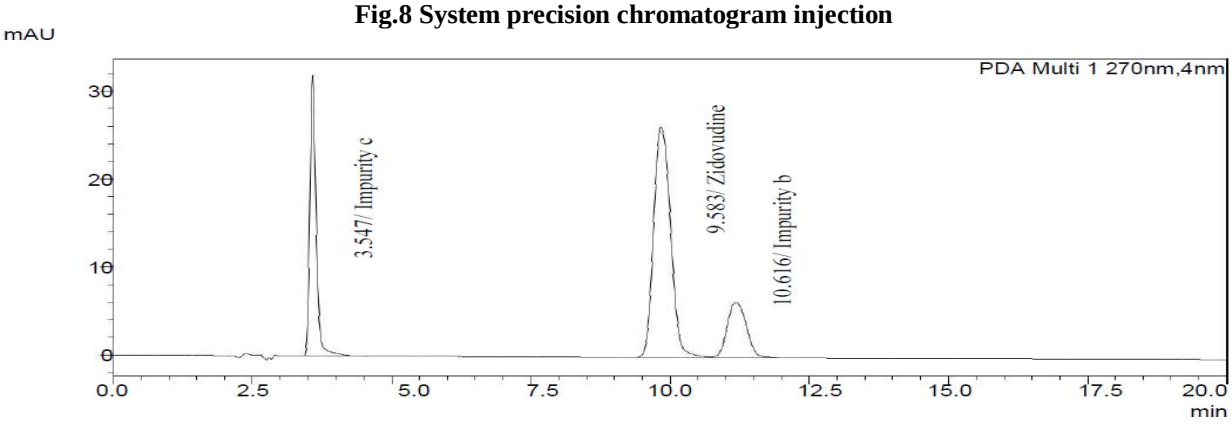

Fig.9 Intra-day chromatogram injection

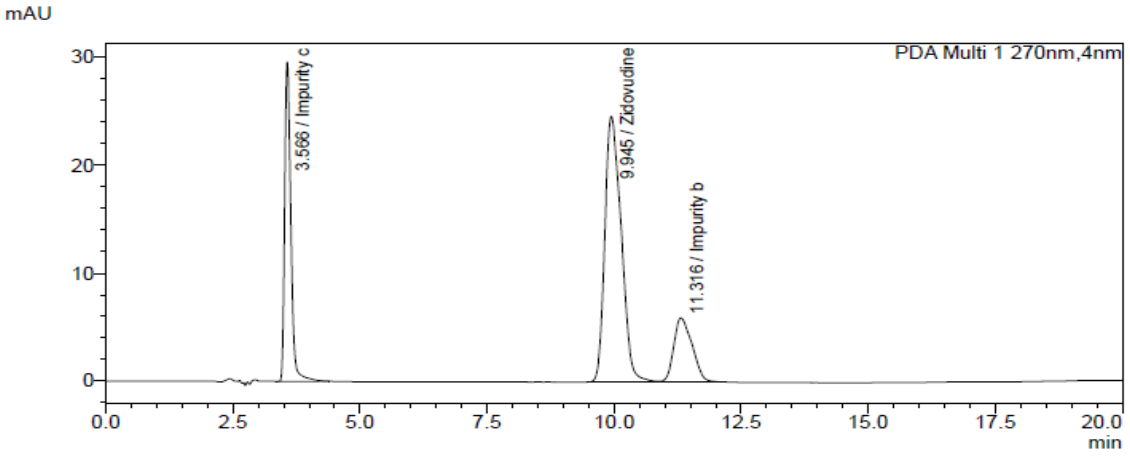

Fig.10 Intermediate precision chromatogram injection

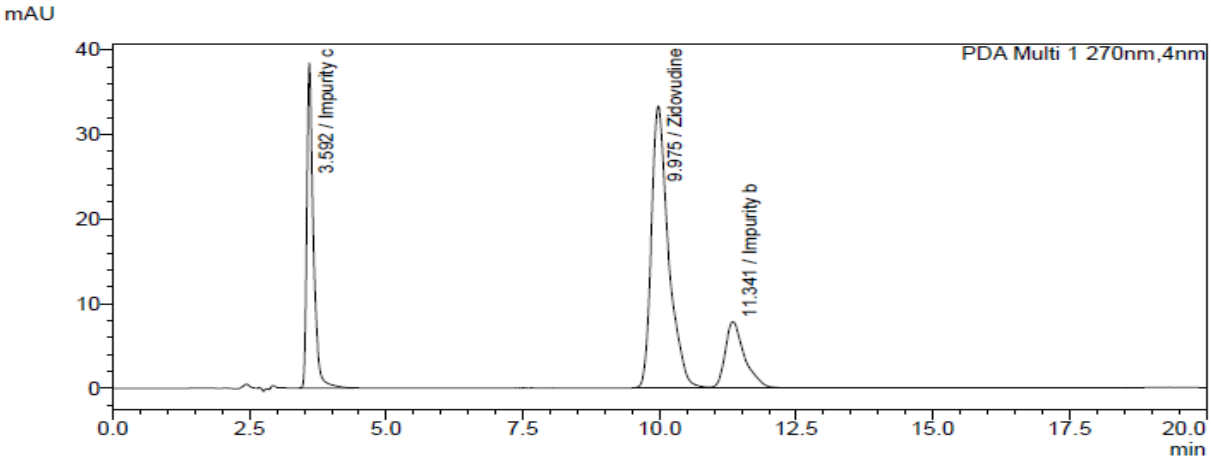

Fig 11 Accuracy 50% chromatogram

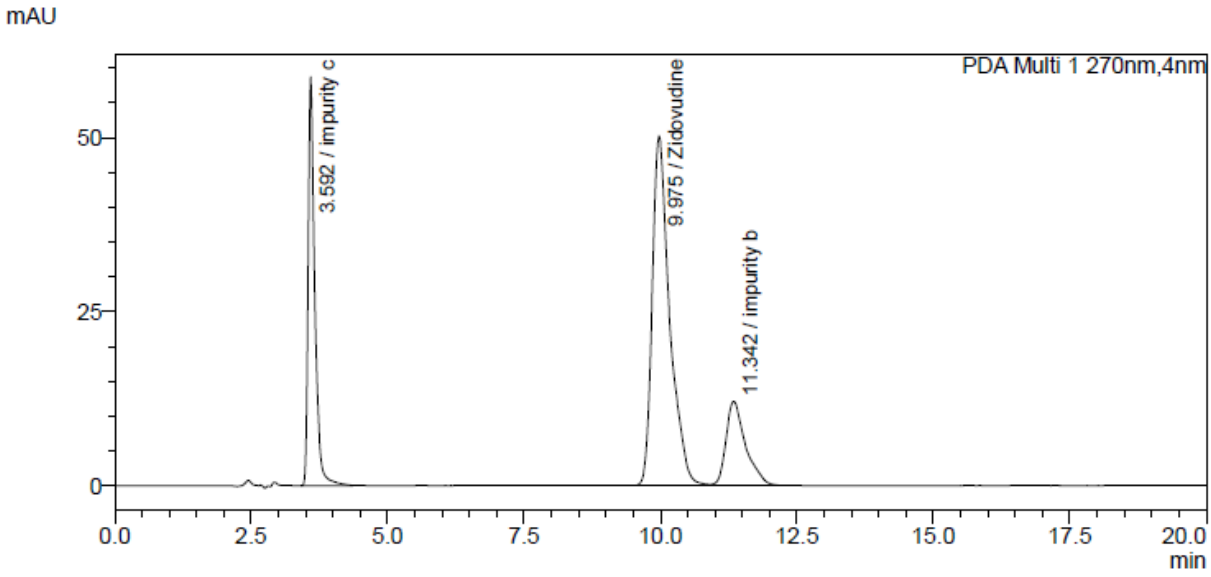

Fig 12 Accuracy 100% chromatogram

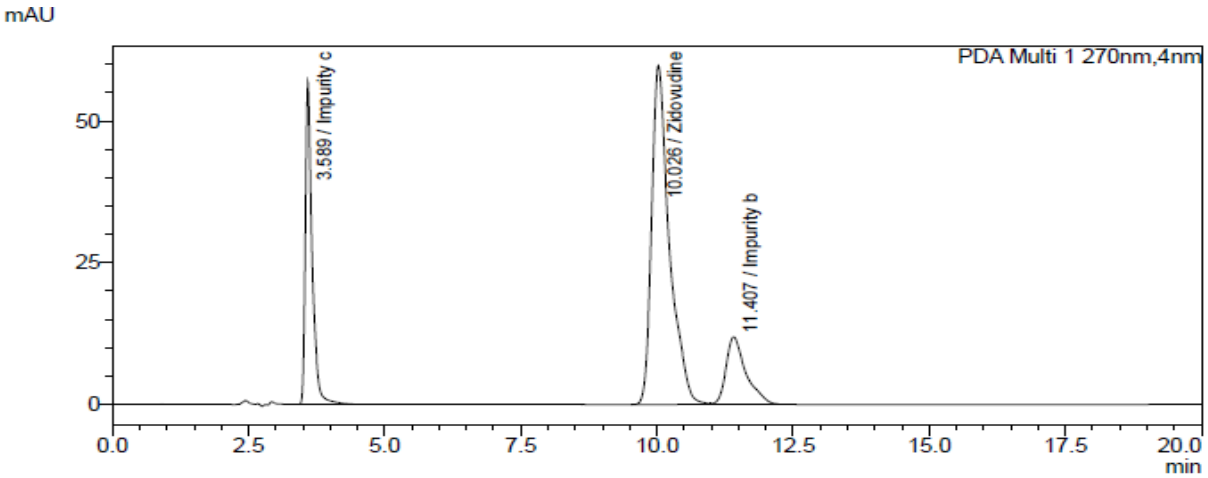

Fig 13 Accuracy 150% chromatogram

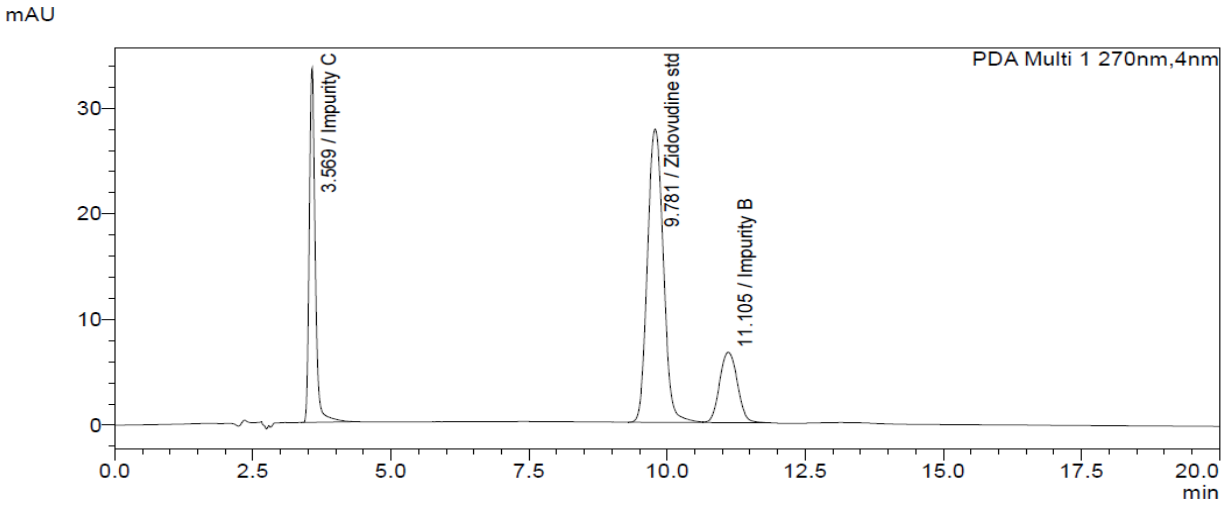

Fig 14 Mobile phase minus chromatogram injection

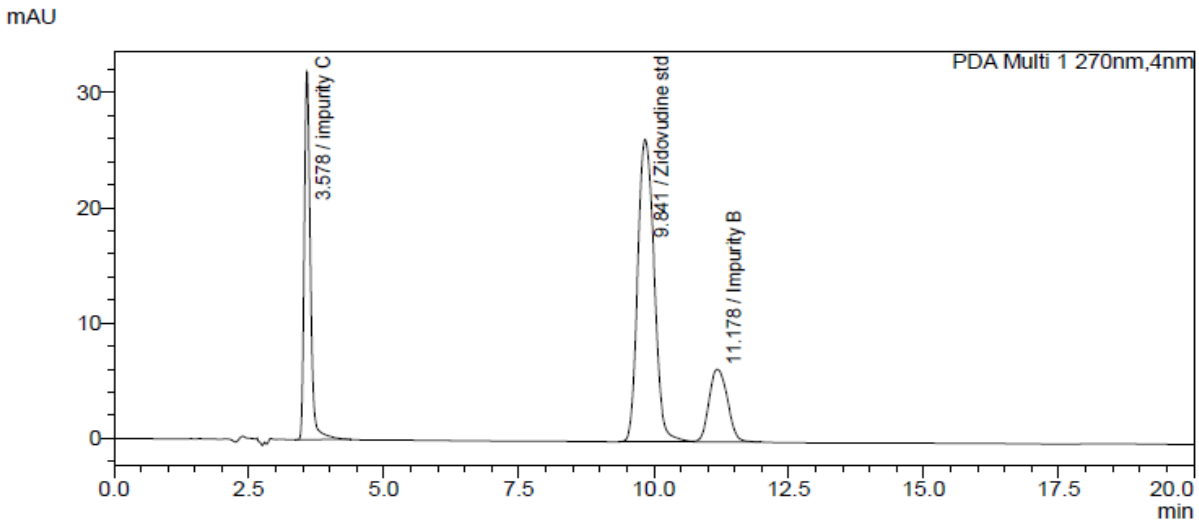

Fig 15 Mobile phase plus chromatogram injection

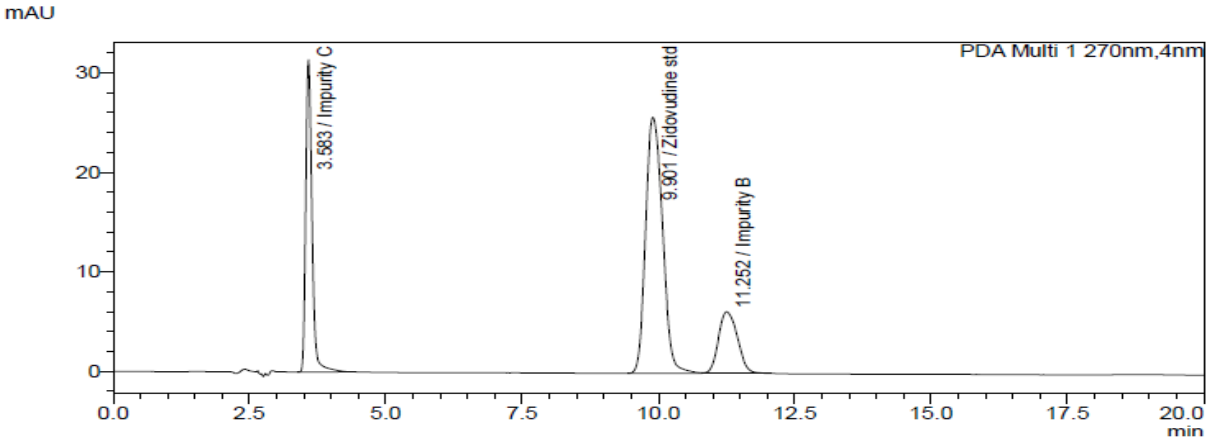

Fig 16 Flow minus chromatogram injection

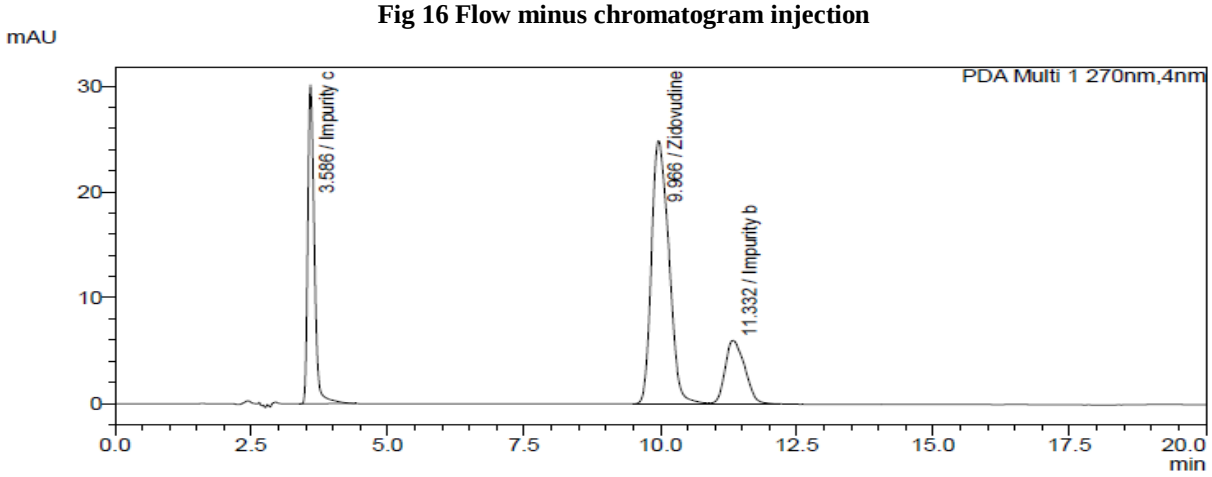

Fig 17 Flow plus chromatogram injection

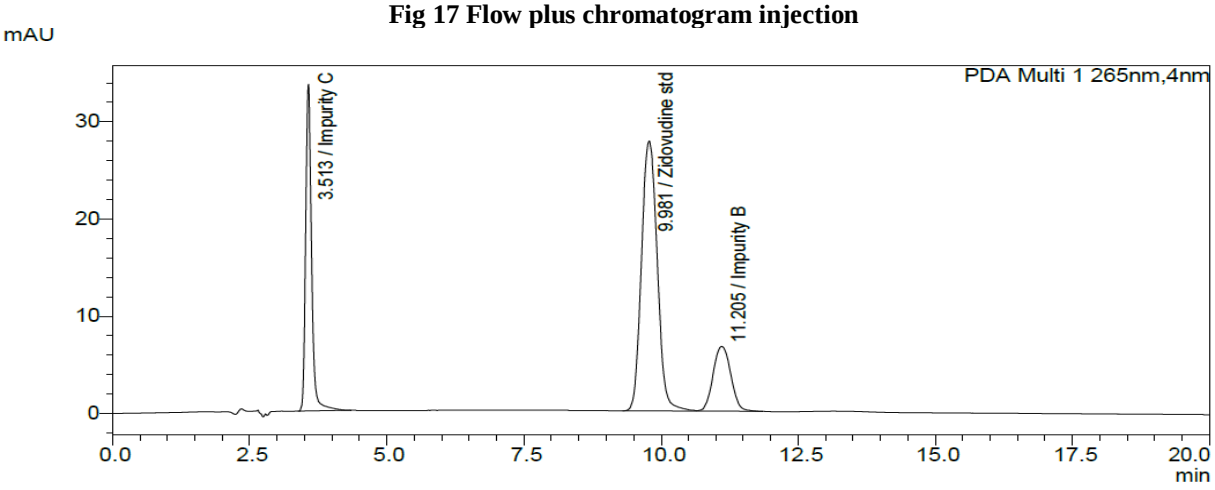

Fig 18 Wavelength minus chromatogram injection

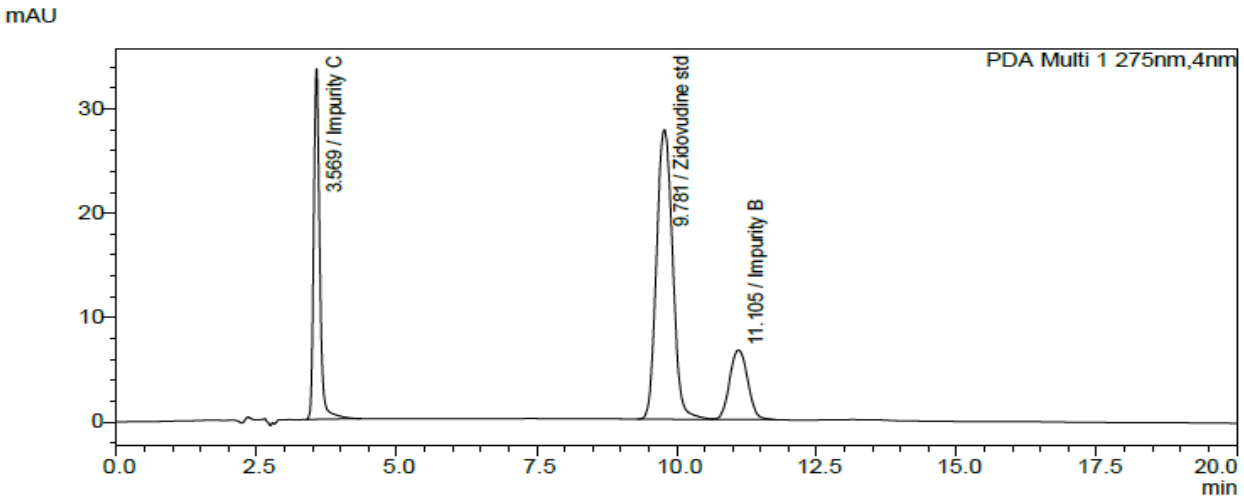

Fig 19 Wavelength plus chromatogram injection

SUMMARY:

A simple, sensitive, precise and specific Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for estimation of accurate quantification of zidovudine (AZT) and related substances in tablet dosage form. The separation was performed on Shimadzu C_{18} (250×4.6mm, 5µm) chromatographic column. The mobile phase was mixture of Water and Methanol (70:30). The flow rate was 1.0 mL/ min and detection was performed at 270 nm. According to guidelines, system suitability parameters constitute integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. The developed method was validated according to ICH guidelines. The linear response was observed in the range of 5-25µg /ml for zidovudine and 1-5µg/ml zidovudine related substances respectively. The proposed method had adequate specificity for estimation of zidovudine (AZT) and related substances in tablet dosage form. The percentage recoveries were found to be within limits of acceptance criteria between the ranges of 98 – 102 %. System precision, method precision and intermediate precision were found to be within limits and method was found to be robust. The results of assay showed good agreement with label claim. The method was validated statistically and was applied successfully for estimation of zidovudine (AZT) and related substances.

CONCLUSION:

High performance liquid chromatography is at present one of the most sophisticated tools of analysis. The estimation of zidovudine (AZT) and related substances was done by Reverse Phase HPLC. The mobile phase used consists of water and Methanol (70:30) (v/v). A Shimadzu (250×4.6mm, 5µm) was used as the stationary phase. The detection was carried out using PDA detector set at 270nm. The solutions are chromatographer at a constant flow rate of 1.0 ml/ min. Retention time for zidovudine, zidovudine B and C impurity was found to be 9.5, 11.3 and 3.5 min.

The method was developed by using RP HPLC. The results obtained are subjected to the statistical validation. The values of RSD are less than 2.0%, indicating the accuracy and precision of the method. The percentage recoveries vary with was 98 – 102 %. for of zidovudine, zidovudine B and C impurity. The results obtained on the validation parameters met the

ICH requirements. It is inferred that the method was found to be simple, specific, precise and linear. The method was found to have suitable applications in routine laboratory analysis with high degree of accuracy and precision.

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