World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers © All Rights Reserved
Available online at: http://www.wjpsonline.org/
Original Article

Validated specific HPTLC method for simultaneous estimation of zidovudine and lamivudine in its combined dosage form

Anup Giridhar Barsagade and Rajendra Baliram Kakde*

Department of Pharmaceutical Sciences, R. T. M. Nagpur University, Amravati Road, Nagpur – 440033, Maharashtra, India

Received: 20-06-2015 / Revised: 27-07-2015 / Accepted: 28-07-2015

ABSTRACT

The objective of current study was to develop a validated, specific stability indicating normal-phase high performance thin layer chromatographic method for simultaneous estimation of zidovudine and lamivudine in their combined dosage form. The forced degradation studies were performed on pure zidovudine and lamivudine and also on their combined dosage form using acid, base, neutral, oxidation, thermal and photo stress to show the stability indicating capability of the developed method. Significant degradation products of zidovudine and lamivudine were observed in acidic, basic, neutral, oxidation and photo stress. No degradation products were obtained after thermal stress condition. The chromatographic method was optimized using samples generated in forced degradation studies. Good separation between the peaks corresponding to the zidovudine, lamivudine and degradation products from the analyte were achieved on silica gel $60F_{254}$ TLC plate using toluene: ethyl acetate: methanol: formic acid 6.5:2.5:1.5:1.5 (v/v) as mobile phase. Densitometric quantification was performed at 276 nm by reflectance scanning. Validation of the developed method was conducted as per ICH requirements. The limit of detection and limit of quantitation of zidovudine and lamivudine were established. The developed HPTLC method was found to suitable to check the quality of zidovudine and lamivudine in combined dosage form.

Key Words: HPTLC, Zidovudine, Lamivudine, Degradant, Validation

INTRODUCTION

Zidovudine (ZIDO) is chemically 1-[(2R,4S,5S)-4-azido-5-(hydroxymethyl)oxolan-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione [Figure 1]. It is a nucleoside analog reverse transcriptase inhibitor, a type of antiretroviral drug used for the treatment of HIV/AIDS infection. Lamivudine (LAMI) is chemically 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydro pyrimidin-2-one [Figure 2]. It is a potent nucleoside analog reverse transcriptase inhibitor, it has been used for treatment of HIV/AIDS and chronic Hepatitis B at a lower dose than for treatment of HIV. [1-4]

Literature survey revealed estimation of ZIDO and LAMI by UV Spectroscopy in tablet alone [5-8] and in combination with other drugs [9,10], HPLC in alone [11-14] and in combination with other drugs [15,16] and HPTLC in combination with other drugs [17,18] has been reported. The reported HPTLC method is only suitable for of simultaneous estimation of ZIDO and LAMI in the bulk drug and dosage form in presence of their degradation products.

In this manuscript we describe a simple, specific, rapid, precise and accurate stability-indicating HPTLC method which is useful for analysis of ZIDO and LAMI and its degradation products in pharmaceutical preparations on the basis of peak area.

MATERIAL AND METHODS

Chemicals and reagents: Pharmaceutical grade Zidovudine and Lamivudine were procured as a gift samples from Cipla Ltd., Mumbai (India), DUOVIR a tablet formulation, obtained commercially. Toluene, ethyl acetate, formic acid, methanol, hydrochloric acid, sodium hydroxide and hydrogen peroxide 30% of analytical grade were used throughout the work.

***Corresponding Author Address:** Dr. Rajendra Baliram Kakde, Department of Pharmaceutical Sciences, R. T. M. Nagpur University, Amravati Road, Nagpur – 440033, Maharashtra, India*

Preparation of standard solution: For ZIDO, an accurately weighed 20.0 mg of ZIDO was transferred to 10.0 ml volumetric flask and dissolved in 5.0 ml of methanol. The volume was completed to 10.0 ml with methanol. One milliliter of resulting solution was pipetted in 10.0 ml volumetric flask and the volume was made up to 10.0 ml with methanol to furnish a solution of concentration 200 µg/ml of ZIDO.

For LAMI, an accurately weighed 10.0 mg of LAMI was transferred to 10.0 ml volumetric flask and dissolved in 5.0 ml of methanol. The volume was completed to 10.0 ml with methanol. One milliliter of resulting solution was pipetted in 10.0 ml volumetric flask and the volume was made up to 10.0 ml with methanol to furnish a solution of concentration 100 µg/ml of LAMI.

For the working mixed standard solution, an accurately weighed 20.0 mg of ZIDO and 10.0 mg of LAMI were transferred to 10.0 ml volumetric flask and dissolved in 5.0 ml of methanol. The volume was completed to 10.0 ml with methanol. One milliliter of resulting solution was pipetted in 10.0 ml volumetric flask and the volume was made up to 10.0 ml with methanol to furnish a solution of concentration 200 µg/ml and 100 µg/ml of ZIDO and LAMI respectively.

Preparation of sample solution: Twenty tablets were weighed and finely powdered. An accurately weighed amount of powder equivalent to 20.0 mg of ZIDO and 10.0 mg of LAMI was transferred into a 10.0 ml volumetric flask. Then 5.0 ml of methanol was added in it. The flask contents were sonicated for 10 min to make the contents homogeneous. This solution was then diluted up to the mark with methanol. The resultant solution was filtered through Whatman Grade I filter paper. One milliliter of filtrate was transferred to a 10 ml volumetric flask and then volume was made up to the mark with methanol to furnish a sample solution containing 200 µg/ml of ZIDO and 100 µg/ml of LAMI.

Six replicate of tablet powder equivalent to 20.0 mg of ZIDO and 10.0 mg of LAMI was transferred into six 10.0 ml volumetric flask and homogenous sample solutions were prepared in a similar manner.

Chromatography: Chromatography was performed on 10 cm × 10 cm HPTLC plates coated with silica gel 60 F_{254}. Before use plates were washed with AR-grade methanol and activated at 115°C for 30 min. Samples (5 µl) were applied to the plates as bands 4 mm wide and 3 mm apart by use of a CAMAG Linomat IV automatic sample applicator equipped with a Hamilton syringe. The application rate was 5 s/µl.

Linear ascending development to a distance of 80 mm was performed in a 10 cm × 10 cm CAMAG twin-trough chamber using toluene: ethyl acetate: methanol: ammonia 6.5:2.5:1.5:1.5 (v/v) as mobile phase. Before the insertion of the plate, the chamber was saturated with mobile phase vapour for 10 min at room temperature and after the insertion of plate again saturated for 10 min. After development the plate was removed and dried with hot air drier. Densitometric scanning was performed at 276 nm with a CAMAG TLC Scanner III in reflectance–absorbance mode controlled by CATS 4 software (version 1.4.1; CAMAG) resident in the system. The slit dimensions were 3.00 mm × 0.45 mm and the scanning speed 20 mm/s. The radiation source was a deuterium lamp emitting continuous UV radiation between 190 and 360 nm. The amounts of the compounds chromatographed were determined from the intensity of diffusely reflected light.

Method Validation:

Stress studies and specificity: Stress testing of drug substances can help to identify the likely degradation products, which can, in turn, help to establish the degradation pathways and the intrinsic stability of the drug substances. Specificity is the ability of the method to measure the responses of the analyte in the presence of its related substances. All stress degradation studies were performed at initial drug concentrations of 2.0 and 1.0 mg/ml for ZIDO and LAMI, respectively. Acid hydrolysis was performed in 0.1N HCl at 80˚C for 4 hrs. The study in basic solution was conducted in 0.1N NaOH at 80˚C for 2 hrs. Neutral hydrolysis was performed at 80°C for 1 hr. Oxidation studies were conducted at room temperature in 3 % hydrogen peroxide for 1 hr. For photo degradation studies, the drug sample was exposed to sun light for 30 days. The drug sample was exposed to dry heat at 60°C for 30 days. Samples were withdrawn at appropriate times and subjected to HPTLC analysis after suitable dilution to evaluate the ability of the proposed method to separate ZIDO and LAMI from their degradation products. Assessment of the mass balance in the degraded samples was conducted to confirm that the amount of degraded product detected in stressed samples matched with the amount present before the stress was applied. Quantitative determination of ZIDO and LAMI was conducted in all stressed samples against qualified working standards, which is tabulated in Table 1.

Limit of detection (LOD) and Limit of quantitation (LOQ): The LOD is the lowest analyte concentration that can be detected. LOQ is the lowest analyte concentration that can be

quantified with acceptable accuracy and precision. The limits of detection (LOD) and quantification (LOQ) were calculated from the standard deviation of the response and the slope of calibration plot. LOD and LOQ were established, in accordance with ICH definitions [19], by use of the equations LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the regression line and S is the slope of the calibration plot.

Linearity: Linearity test solutions of ZIDO and LAMI were prepared at concentration levels of 50 to 400 µg/ml and 25 to 200 µg/ml respectively. Linearity test solutions were prepared by diluting the stock solution to the required concentrations. Linearity was established by least-squares linear regression analysis of the calibration data. Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves.

Precision: The system precision was evaluated by measuring area of six bands of qualified working standard for ZIDO and LAMI and calculating the percentage of relative standard deviation (RSD). The assay method precision was evaluated by conducting six independent assays of test samples of ZIDO and LAMI against qualified working standards and calculating the percentage of relative standard deviation (RSD). The intermediate precision of the method was also verified using different analysts and different days.

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 accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e., 80, 100 and 120% of the label claim. Standard addition and recovery experiments were conducted to determine the accuracy of ZIDO and LAMI for the quantification of drug in the samples.

Robustness: To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between ZIDO and LAMI was evaluated. To study the effect of wavelength on the estimation, the wavelength was altered by ± 2 nm, i.e., 274 and 278 nm from the actual wavelength, 276 nm. To study the effect of mobile phase composition on estimation, methanol composition was altered by ± 0.2 ml i.e., 1.3 and 1.7 ml from the actual volume, 1.5 ml. To study the effect of saturation time on estimation, saturation time was altered by ± 5 min i.e., 15 and 25 ml from the actual time, 20 min.

RESULTS AND DISCUSSION

HPTLC method development and optimization:
Initially, pure drugs solution was chromatographed using single solvents to ascertain the movement of the drug. Use of toluene: ethyl acetate: methanol: formic acid 6.5:2.5:1.5:1.5 (v/v) as mobile phase gives well separated peaks of drugs and separation of degradation products from drugs as well. The R_F value of LAMI and ZIDO were found to be 0.27 ± 0.03 and 0.78 ± 0.03 respectively. Typical HPTLC densitogram (276 nm) was obtained from standard solution is shown in Figure 3.

Then samples obtained from forced degradation were then chromatographed with the same mobile phase and it was found that densitogram obtained after acidic hydrolysis gave two degradation products of LAMI at R_F value 0.20 ± 0.03 (LDP-I) and 0.72 ± 0.03 (LDP-II) and one degradation product of ZIDO at R_F value 0.68 ± 0.03 (ZDP-I), alkaline hydrolysis gave two degradation products of LAMI at R_F value 0.20 ± 0.03 (LDP-III) and 0.72 ± 0.03 (LDP-IV), neutral hydrolysis gave one degradation product of ZIDO at R_F values 0.69 ± 0.03 (ZDP-II), oxidation gave degradation product of LAMI at R_F value 0.11 ± 0.03 (LDP-V) and photo degradation gave degradation product of ZIDO at R_F value 0.15 ± 0.03 (ZDP-III) (Figure 4). No degradation products were obtained after heat stress condition. Toluene: ethyl acetate: methanol: formic acid 6.5:2.5:1.5:1.5 (v/v) was therefore used as mobile phase and resulted in sharp, well defined, symmetrical peaks with no fronting when scanning was performed at 276 nm. The assay of ZIDO and LAMI was unaffected by the presence of degradation products, which confirms that the HPTLC method is stability-indicating. There was no interference from common excipients present in the tablet. Linear ascending development to a distance of 80 mm was performed in a 10 cm × 10 cm CAMAG twin-trough chamber. Before the insertion of the plate, the chamber was saturated with mobile phase vapour for 10 min at room temperature and after the insertion of plate again saturated for 10 min. After development the plate was removed and dried with hot air drier. Densitometric scanning was performed at 276 nm with a CAMAG TLC Scanner III in reflectance–absorbance mode controlled by CATS 4 software (version 1.4.1; CAMAG) resident in the system. The slit dimensions were 3.00 mm × 0.45 mm and the scanning speed 20 mm/s. The radiation source was a deuterium lamp emitting continuous UV radiation between 190 and 360 nm. The amounts of the compounds chromatographed were determined from the intensity of diffusely reflected light.

Validation of the method:

LOD and LOQ: The LOD of ZIDO and LAMI were 3.05 and 1.46 ng per band for peak area respectively. The LOQ of ZIDO and LAMI were 9.24 and 4.43 ng per band for peak area respectively.

Linearity: Linearity was established by least-squares linear regression analysis of the calibration data. Calibration plots were linear over the concentration range 250-2000 ng/band by area for ZIDO and 125-1000 ng/band by area for LAMI. Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves. Equation for the calibration plots of ZIDO was Y= 1081.820 + 5.505X, for peak area. Correlation coefficient was 0.9972 for peak area. Equation for the calibration plots of LAMI was Y= 537.123 + 10.500X, for peak area. Correlation coefficient was 0.9980 for peak area.

Precision: The percentage RSD of system, method and intermediate precision study was well within ± 2.0%. Results of system, method and intermediate precision are summarized in Table 2.

Accuracy: The percentage recoveries were 100.23 ± 0.9685 % and 100.55 ± 0.9227 % by peak area for ZIDO and LAMI respectively. The RSD value was found to be less than 2% (Table 3).

Robustness: Results of robustness studies are summarized in Table 4. The method enables simple, specific and accurate analysis of zidovudine and lamivudine and its degradation products in combined dosage form. This method was validated as per ICH guidelines. The method can therefore be used for routine quality-control analysis of zidovudine and lamivudine in combined dosage forms.

ACKNOWLEDGMENTS

The authors extend their sincere thanks to Cipla Ltd, Mumbai (India), for providing gift sample of pure zidovudine and lamivudine. We are thankful to University Grant Commission for providing Rajiv Gandhi National Fellowship to carry out our research work. We also extend our thanks to Head of Department, Department of Pharmaceutical Sciences; RTM Nagpur University for providing the necessary facilities.

Table 1: Result of Degradation study

Formulation DUOVIR	**Normal**	**Acid**	**Alkali**	**Neutral**	**Oxide**	**Heat**	**Photo**
ZIDO [%]	99.82	96.59	99.58	97.35	99.70	99.42	96.30
LAMI [%]	100.30	94.23	93.74	100.26	94.55	99.90	100.46

Table 2: System, method, and intermediate precision data

Validation Parameters		**ZIDO**			**LAMI**		
		Mean	**SD [±]**	**RSD [%]**	**Mean**	**SD [±]**	**RSD [%]**
System Precision [a]		6579.09	30.8222	0.4684	5488.01	18.5635	0.3382
Method Precision [a]		99.46%	0.4767	0.4792	100.44%	0.3640	0.3623
Intermediate precision	**Interday** [b]	99.59%	0.3218	0.3231	99.51%	0.3100	0.3115
	Intraday [b]	100.16%	0.3189	0.3184	99.85%	0.3330	0.3344
	Different Analyst [b]	99.31%	0.4315	0.4345	99.90%	0.3487	0.3490

a) Mean from six analyses (n =6)
b) Mean from 3 analyses (n =3)
n = Number of samples, SD = standard deviation; RSD = relative standard deviation

Table 3: Accuracy data

	Level [%]	Wt. of sample (mg)	Amount of standard added(mg)	Calculated Wt. Of drug (mg)	[%] Recovery
LAMI	80	25.3	4.1	4.07	99.26
		25.2	4.0	4.05	101.25
		25.5	4.0	4.06	101.50
	100	25.1	5.1	5.08	99.60
		25.4	5.0	5.03	100.60
		25.5	5.0	5.10	102.00
	120	25.4	6.1	6.09	99.83
		25.2	6.0	6.05	100.83
		25.2	6.0	6.01	100.16
	Mean ±SD				100.55±0.9227
	RSD [%]				0.9176
	Level [%]	**Wt. Of sample (mg)**	**Amount of standard added(mg)**	**Calculated Wt. Of drug (mg)**	**[%] Recovery**
ZIDO	80	25.3	8.0	8.00	100.00
		25.2	7.8	7.86	100.76
		25.5	8.1	8.04	99.25
	100	25.1	10.3	10.20	99.02
		25.4	10.1	10.25	101.48
		25.5	10.0	10.15	101.50
	120	25.4	12.1	11.99	99.09
		25.2	12.0	12.03	100.25
		25.2	11.9	11.99	100.75
	Mean ±SD				100.23±0.9685
	RSD [%]				0.9662

Table 4: Robustness

Condition		ZIDO By peak area* Amount estimated [%] ± SD	RSD [%]	LAMI By peak area* Amount estimated [%] ± SD	RSD [%]
Change in wavelength (276±2 nm)	**274 nm**	99.82 ± 0.2267	0.2271	99.89 ± 0.3411	0.3415
	278 nm	99.38 ± 0.6539	0.6579	99.92 ± 0.4080	0.4083
Change in mobile phase composition (±0.2 ml)	**Toluene: ethyl acetate: acetonitrile: formic acid 6.5:2.5:1.3:1.5 (v/v)**	99.22 ± 0.2121	0.2137	99.75 ± 0.3330	0.3338
	Toluene: ethyl acetate: acetonitrile: formic acid 6.5:2.5:1.7:1.5 (v/v)	99.24 ± 0.2627	0.2647	100.11 ± 0.1301	0.1299
Change in saturation time (20±5min)	**15 min**	100.46 ± 0.3534	0.3517	100.61 ± 0.2514	0.2499
	25 min	100.50 ± 0.1137	0.1131	100.47 ± 0.2707	0.2694

* Each value is a mean of three observations.

Figure 1: Chemical structure of Zidovudine

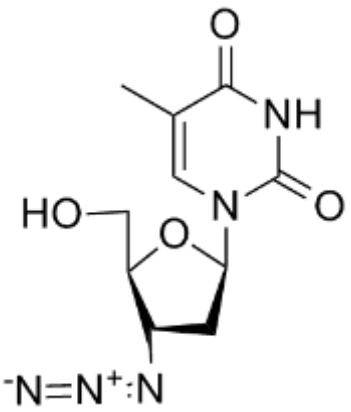


Figure 2: Chemical structure of Lamivudine

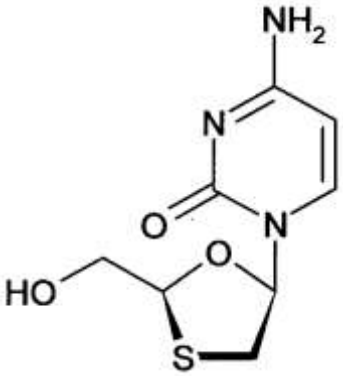
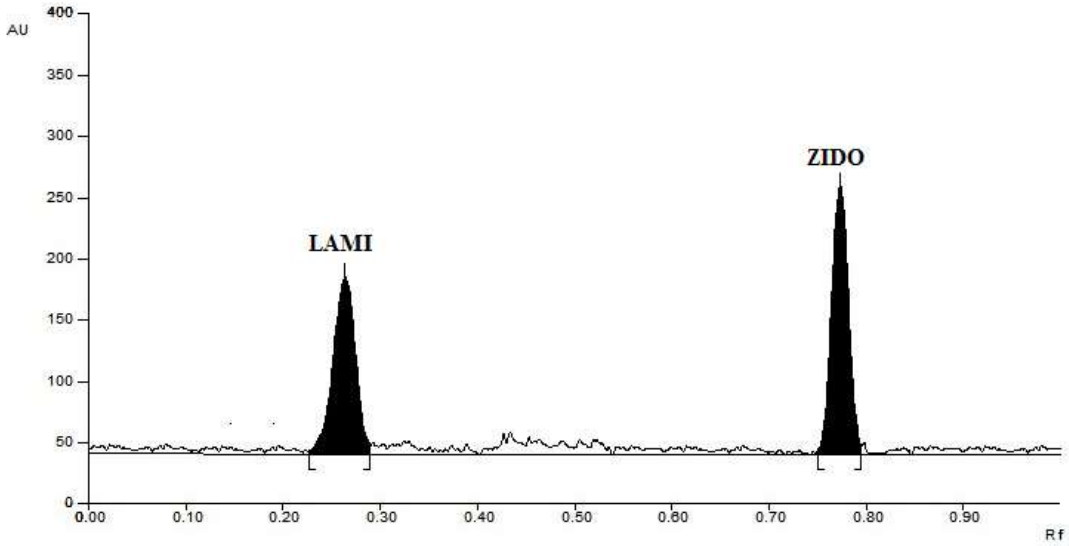


Figure 3: Densitogram of Zidovudine and Lamivudine combination



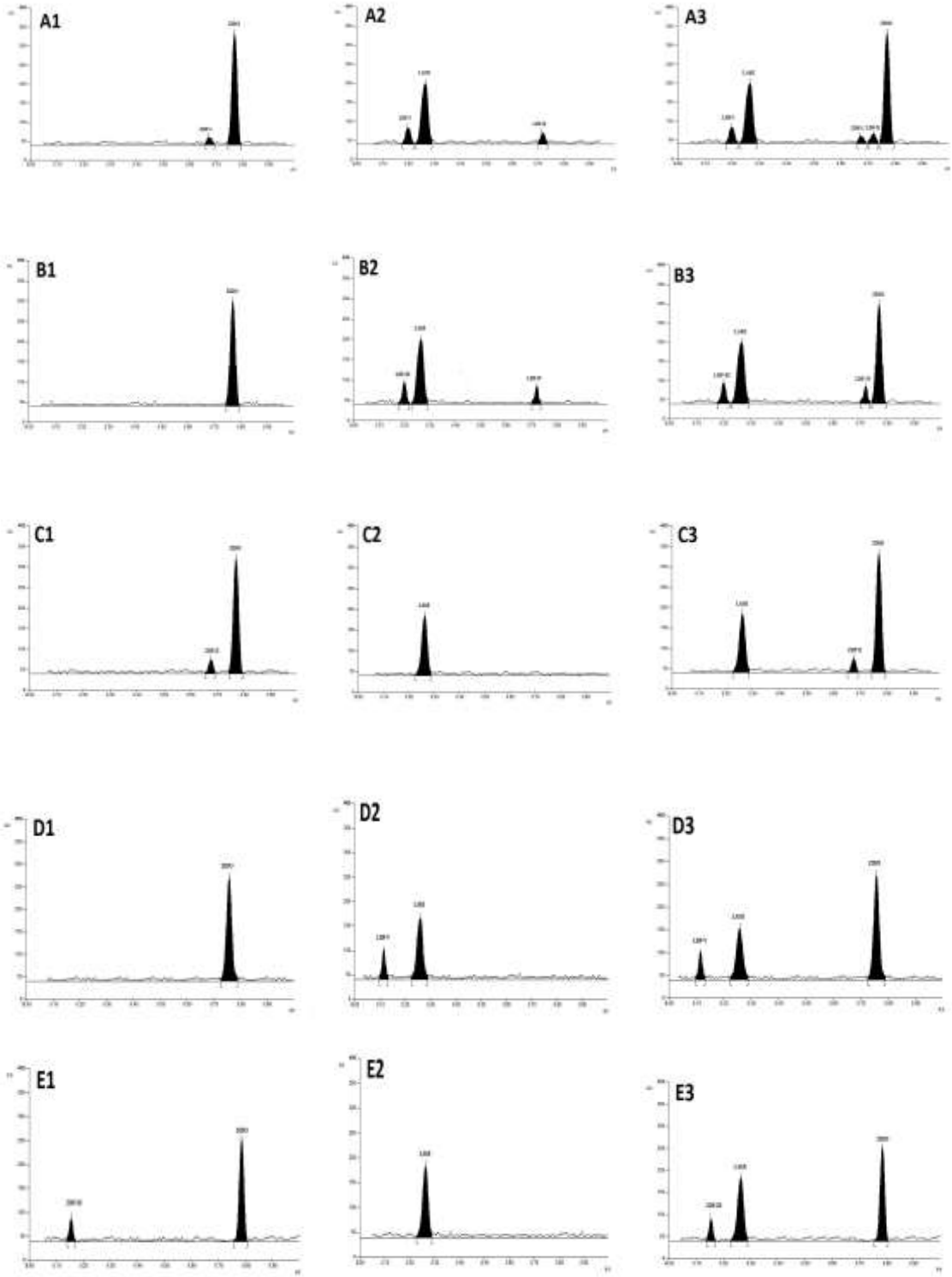


Figure 4. Results from forced degradation of 1) Zidovidune, 2) Lamivudine and 3) Tablet powder in (A) 0.1 N HCl, 4 hrs. at 80ºC (B) 0.1 N NaOH, 2 hrs. at 80ºC (C) Neutral, 1hr. at 80ºC (D) 3 % H_2O_2, 1 hr. at room temp. (E) Sunlight, 30 days

REFERENCES

1. https://en.wikipedia.org/wiki/Zidovudine
2. https://en.wikipedia.org/wiki/Lamivudine
3. United States Pharmacopeia 32; National Formulary 27. United States Pharmacopeal Convention 2009; 2747.
4. United States Pharmacopeia 32; National Formulary 27. United States Pharmacopeal Convention 2009; 3887.
5. Sharada CH et al. Development of a spectrophotometric method for the quantitative estimation of zidovudine concentration in bulk and pharmaceutical dosage forms. KMITL Sci Tech J 2010; 10: 1-8.
6. Saravanan C et al. Method development and validation for determination of zidovudine by UV-spectrophotometer. Int Res J Pharm 2010; 1: 314-23.
7. Basavaiah K, Somashekar BC. Sensitive titrimetric and spectrophotometric methods for the assay of lamivudine in pharmaceuticals. J Sci Ind Res 2006; 65: 349-354.
8. Deepali G, Elvis M, UV spectrophotometric method for assay of the anti-retroviral agent lamivudine in active pharmaceutical ingredient and in its tablet formulation. J Young Pharm 2010; 2: 417–19.
9. Reddy DR et al. Validated spectrophotometric method for simultaneous estimation of zidovudine and lamivudine in combined pharmaceutical dosage form. Int J PharmTech Res 2012; 4: 311-14.
10. Rekha et al. UV spectrophotometric absorption correction method for the simultaneous estimation of efavirenz, lamivudine and zidovudine in tablet dosage forms. The Pharma Innovation – J 2013; 2: 174-79.
11. Kusuma SL et al. method development and validation of RP-HPLC method for determination of zidovudine. Int J Res Pharm Chem 2011; 1: 677-80.
12. dos Santos JV et al. Development and validation of a RP-HPLC method for the determination of zidovudine and its related substances in sustained-release tablets. Anal Sci 2011; 27: 283- 89.
13. Dunge A et al. Validated specific HPLC method for determination of zidovudine during stability studies. J Pharm Biomed Anal 2005; 37: 1109-14.
14. Krishnareddy NV et al. New RP -HPLC method development for analysis and assay of lamivudine in formulation. Int J Res Pharm Biomed Sci 2011; 2: 220-23.
15. Nijamdeen J et al. Method development and validation of RP-HPLC method for simultaneous determination of lamivudine and zidovudine. J Chem Pharm Res 2010; 2: 92-6.
16. Venkatesh P. Simultaneous estimation of zidovudine and lamivudine tablets by RP-HPLC method. Int J ChemTech Res 2011; 3: 376-80.
17. Venkatesh P, Daggumati M. Development and validation of a normal-phase HPTLC method for simultaneous analysis of lamivudine and zidovudine in fixed dose combination tablets. J Pharm Anal 2012; 2: 152-5.
18. Sagar B et al. A validated HPTLC method for simultaneous estimation of lamivudine and zidovudine in tablets. Asian J Chem 2006; 18: 2669-72.
19. ICH. Q2 (R1) Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonization, Geneva; 2005 November.