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SIMULTANEOUS STABILITY-INDICATING METHOD FOR THE DETERMINATION OF ABACAVIR, DOLUTEGRAVIR AND LAMIVUDINE BY RP-HPLC

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Keywords:

High performance liquid chromatography, abacavir, dolutegravir and lamivudine, Stability-indicating method

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ABSTRACT: A simultaneous stability-indicating reversed-phase high performance liquid chromatography (HPLC) method for analysis of abacavir (ABC), dolutegravir (DTG) and lamivudine (3TC) as the bulk drug and in the formulation was developed. Compounds were separated on Kinetex 5 µ C18 100 A (250 mm x 4.6 mm). A gradient program of mobile phase at different proportions of acetonitrile (ACN) and water was used. The retention times of ABC, DTG and 3TC were 5.2, 8.4 and 3.1 minutes (mins) respectively. The drugs were subjected to the stress conditions of acid, base, oxidative, hydrolytic, humidity, thermal and photolytic degradation. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating ability of the method. The method was linear in the concentration range of 20–100 µg/mL, 2-16µg/mL and 10-80 µg/mL for ABC, DTG and 3TC respectively. The method was accurate and precise with a limit of detection and limit of quantitation of 2.05 and 6.73 µg/ mL, 0.28 and 0.94 µg/ mL and 2.32 and 7.72 µg/ mL for ABC, DTG and 3TC respectively. The method was applied for the analysis of ABC, DTG and 3TC in the presence of its degradation products and commonly used excipients and was found to be specific. The developed method is stability indicating, precise and specific which can be applied for the routine analysis.

INTRODUCTION: Abacavir, (ABC) [1] which is chemically *(1S,cis)* – 4 - [2-amino-6(cyclopropyl amino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol sulfate, is a carbocyclic synthetic nucleoside analogue. Intracellularly, it is converted by cellular enzymes to the active metabolite carbovir triphosphate. Carbovir triphosphate is an analogue of deoxyguanosine-5`-triphosphate (dGTP). Carbovir triphosphate inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA.

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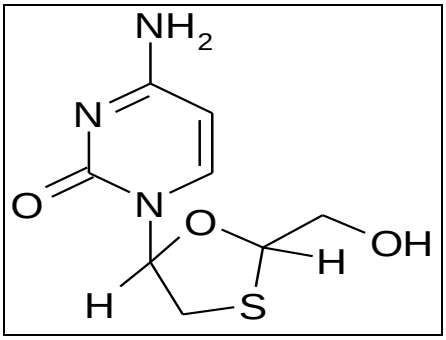

FIG .1: a ABACAVIR

Dolutegravir(DTG) [1] which is chemically (4*R*,12a*S*) - 9 - {[(2,4difluorophenyl) methyl] carbamoyl}-4-methyl - 6,8 -dioxo-3,4,6,8,12,12a-hexahydro-2*H*pyrido[1',2':4,5]pyrazino[2,1-*b*] [1,3] oxazin-7-olate, inhibits HIV integrase by binding to the integrase active site and blocking the strand transfer step of retroviral DNA integration which is essential for the HIV replication cycle.

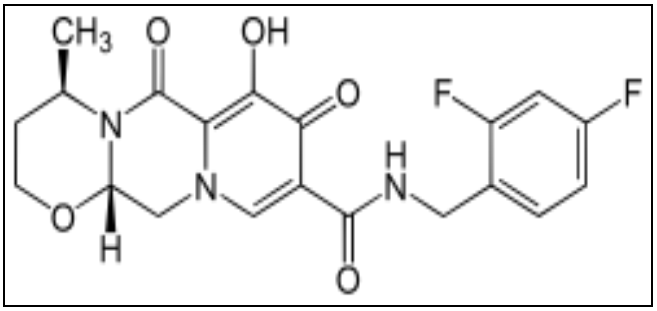

FIG. 1.b DOLUTEGRAVIR

Lamivudine(3TC) [1] which is chemically(2R,cis)-4-amino-1-(2hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one is a synthetic nucleoside analogue. Intracellularly, it is phosphorylated to its active 5′-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 termination after incorporation of the nucleoside analogue into viral DNA.

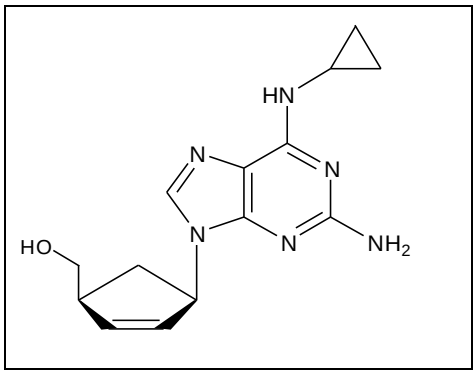

FIG.1. c LAMIVUDUNE

There are several reported spectrophotometric [2-4] and chromatographic [5-7] methods in the literature for analysis of ABC, DTG and 3TC individually but there is no reported method for the simultaneous stability indicating assay of the drugs in combination. Hence LC method that was developed in the present work is advantageous because it enables stability indicating, accurate, specific and reproducible analysis of ABC, DTG and 3TC.

MATERIALS AND METHODS:

Instrumentation and Reagents:

Liquid chromatography was performed with a UFLC Shimadzu LC20 –AD, SPD M20A prominanace DAD detector, Rheodyne universal injector 7725 port and Hamilton 50 µL manual injector. Data processing was performed with shimadzu LC Solutions software version 1.25for LC peak integration. ABC, DTG and 3TC was obtained as a gift sample from Hetero Labs, Hyderabad, India. The components of placebo formulation was D-mannitol, magnesium stearate, microcrystalline cellulose, povidone, and sodium starch glycolate.

Preperation of solutions:

Preparation of diluents:

ACN - water 60:40 v/v mixture was used as diluents which was prepared by mixing 600 mL of ACN and 400 mL of water in a 1000mL volumetric flask. The mixture was filtered through 0.45 µ membrane filter and sonicated before use.

Standard stock solution:

A standard solution was prepared by dissolving 60 mg, 5 mg and 30 mg of ABC, DTG and 3TC with diluent in a 100 mL volumetric flask and was sonicated for 30 min. From this, working standard solutions of 60µg/mL, 5µg/mL and 30 µg/mL of the drugs were prepared by appropriate dilutions using diluent.

Sample stock solution:

20 tablets were weighed and average weight of each tablet was taken and then powder equivalent to 60 mg, 5 mg and 30 mg of abacavir, dolutegravir and lamivudine was transfered into a 100 mL volumetric flask, 30 mL of diluent was added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution, 1.0 mL was pipetted out into a 10 mL volumetric flask and made up to 10 mL with diluent.

Optimized Chromatographic Conditions:

Compounds were separated on a Kinetex 5 µ C18 100 A(250 mm x 4.6 mm) column with gradient program of ACN – water [**Table 1**] as mobile phase at a flow rate of 1 mL/min. Chromatography was performed at room temperature and the detection was carried out at 258 nm.

TABLE 1: GRADIENT PROGRAM

Time	Flow	ACN	Water
0.01	1.00	20.0	80.0
5.00	1.00	60.0	40.0
8.00	1.00	20.0	80.0
15.00	1.00	20.0	80.0

Forced Degradation Studies: [9]

Intentional degradation [6] (n = 3) was attempted by using water, heat, light, acid, base, humidity and oxidizing agent. For acid degradation, 2 mL of working standard solution was refluxed with 3N hydrochloric acid (HCl) at 60°C for 1hour and then neutralized by adjusting pH to 7.0 with 5N sodium hydroxide (NaOH). For alkali degradation, 2 mL of working standard solution was refluxed with 2N NaOH at 60°C for 1hour and then neutralized by adjusting pH to 7.0 with 2N HCl. For oxidative degradation, 2 mL of working standard solution was refluxed with 30% hydrogen peroxide (H_2O_2) by heating on water bath at 60°C for 1hour. For photolytic degradation, 2mL of working standard solution was exposed to ultra violet (UV) (200watthour/m^2) as per ICH Guidelines. For thermal degradation, 2mL of working standard solution was exposed to temperatures at 105°C for 3days. For hydrolytic degradation, 2mL of working standard solution was refluxed with water by heating on water bath at 100°C for 1hour. For humidity degradation, 2mL of working standard solution was exposed to 85% Humidity (Prepared potassium nitrate saturated solution) at 3days.

All these solutions except for photo degradation were prepared in amber volumetric flasks. After completion of the degradation treatments the samples were cooled to room temperature, diluted with the diluent, and injected for chromatographic analysis.

Method Validation:

The method was validated in accordance with recognized guidelines [10, 11].

Specificity:

To demonstrate the specificity of the method, in house placebo formulation containing only excipients was subjected to the methods of sample preparation and analysis described above. Forced degradation samples were also analysied by the above described method to establish its specificity.

Leniarity:

Six solutions containing ABC, DTG and 3TC were prepared in diluents. Peak area and concentration data were treated by least squares linear regression analysis (n = 3).

Precision:

Method precision was evaluated by injecting working standard solution of ABC, DTG and 3TC for 6 times (n=3) on different HPLC system.

Accuracy:

The accuracy of the method was determined by measurement (n = 3) of recovery by spiking the in house placebo with 50, 100, and 150% of the drug.

LOD and LOQ:

LOD and LOQ were determined as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively.

Robustness:

Robustness of the method was assessed by varying the instrumental conditions such as flow rate (±2%), proportion of the organic content in the mobile phase (±2%) and wavelength (±2 units).

Stability of the Analytial Solutions:

The bench top stability of abacavir, dolutegravir and lamivudine in the diluent was assessed by injecting a working standard solution at 0, 6, 8,12 and 24 h after preparation (n = 3).

RESULTS AND DISCUSSION:

The retention times of ABC, DTG and 3TC under the chromatographic conditions described above were 5.2, 8.4 and 3.1 mins respectively [**Fig. 2.a**]. Peaks at 5.2, 8.4 and 3.1 min were observed in chromatograms of the drug samples extracted from the in house formulation [**Fig. 2. b**]. Assay caluculations are given in Table no. 2. System suitability data is given in table no. 3 where it is evaluated by theoretical plates and tailing factor. The peaks of the degradation products were well resolved from that of ABC, DTG and 3TC **[Fig. 2e–j**]. There was no interference from the excipients commonly present in the formulation and from the mobile phase. It may therefore be inferred that no degradation of ABC, DTG and 3TC in the pharmaceutical formulation was detected by using this method.

In validation of the assay, placebo formulation samples and blank, yielded clean chromatograms [**Fig. 2. c, d**]; with no interference from the excipients and mobile phase; this is indicative of

the specificity of the method. The LOD and LOQ was2.02 and 6.73 µg/ mL, 0.28 and 0.94 µg/ mL and 2.32 and 7.72 µg/ mL, for ABC, DTG and 3TC respectively. A plot of drug peak area against concentration [**Fig. 3a 3b 3c**] of ABC, DTG and 3TC was linear over the concentration range 20–100µg/ mL, 2 -16µg/ mL and 10- 80µg/ mL respectively. The regression equation was calculated by the least-square method for ABC, y = 49116x - 30073; correlation coefficient 0.999, for DTG, y = 52644x + 845.57; correlation coefficient 0.999 and for 3TC, y = y = 77990x - 60776; correlation coefficient 0.999. Linearity data is given in **Table 4**. The method was found to be precise as the RSD <2 [**Table 5**].

The recovery data listed in, obtained from a study of the in house placebo formulation, ranged from 99.86-100.00 % for ABC, 99.64-101.04% for DTG and 99.94-100.24 for 3TC with low RSD values for all the drugs. This quantitative recovery of the drugs indicates that there was no interference from excipients present in the formulation and the method is accurate whose results are shown in **Table 6**. ABC, DTG and 3TC were found to be stable in the mobile phase for a period of 24hours, because no peaks corresponding to degradation products were observed and there was no significant change in the peak area of the drug (RSD <1%). The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust. **Table 7**. Results of Degradation Studies are given in **Table 8**.

Chromatograms obtained from drugs and its degradation products:

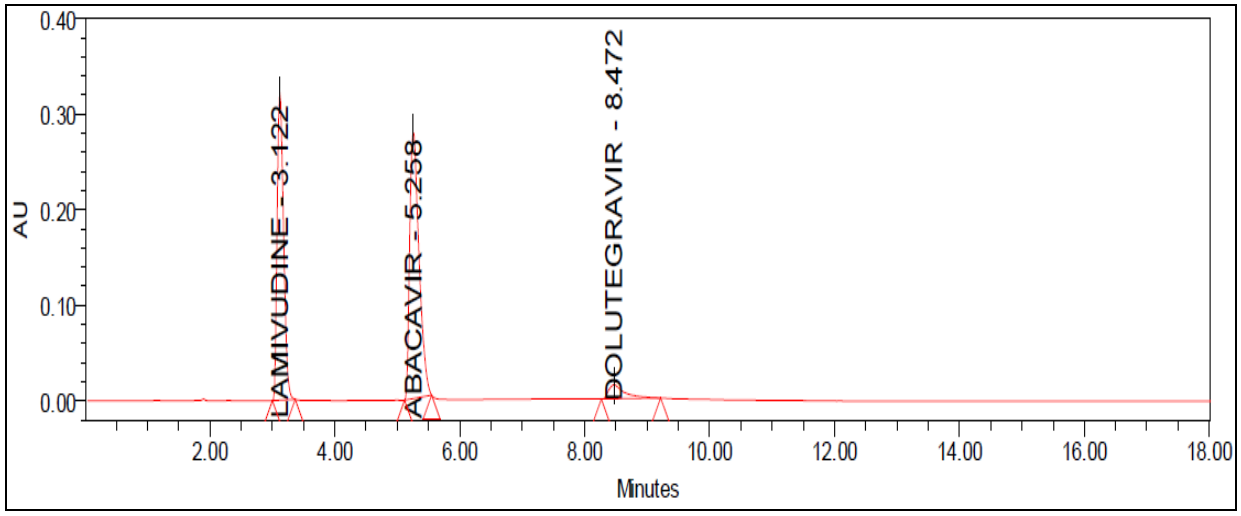

FIG. 2: a CHROMATOGRAM OF STANDARD

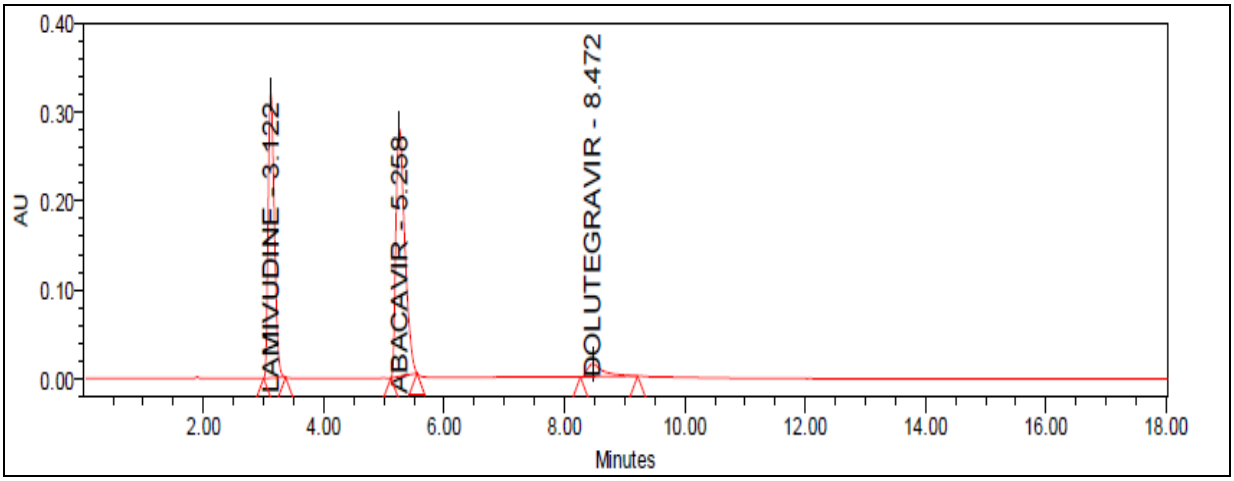

FIG. 2: b CHROMATOGRAM OF FORMULATION

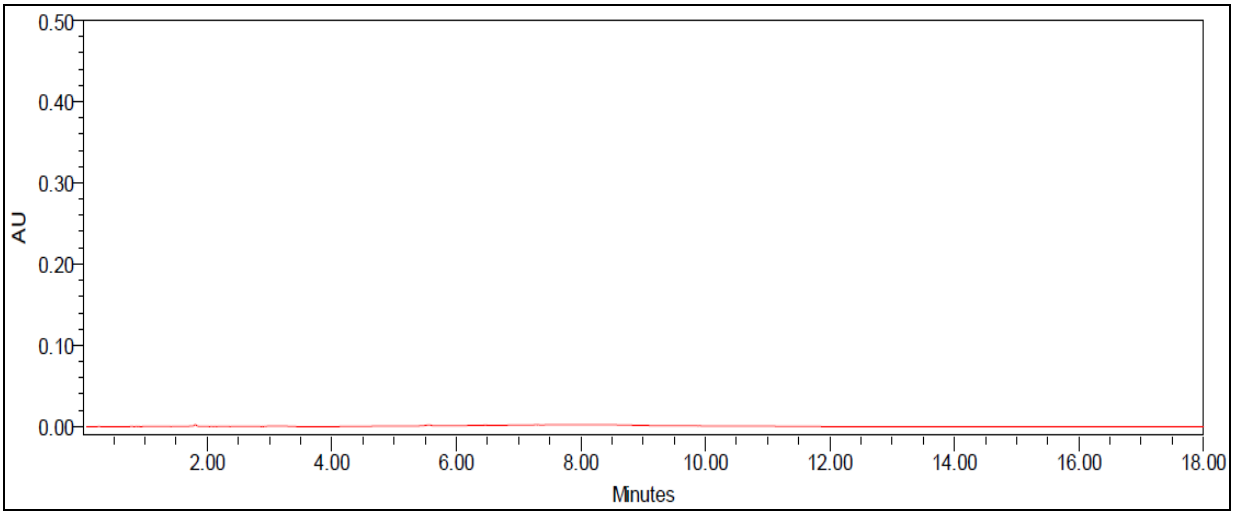

FIG. 2: c CHROMATOGRAM OF BLANK

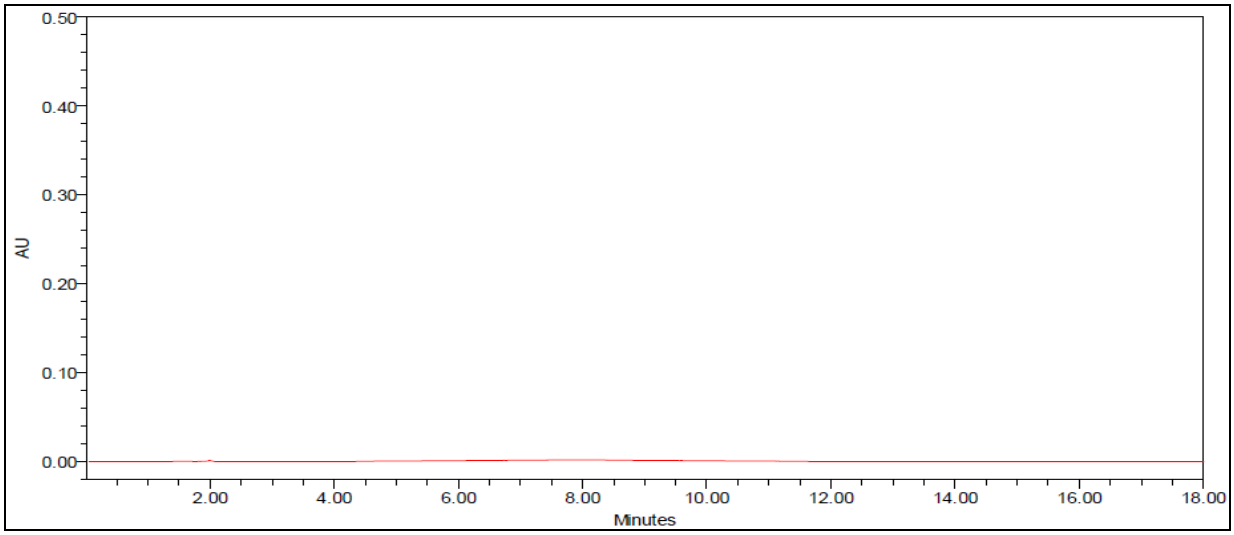

FIG. 2: d CHROMATOGRAM OF PLACEBO

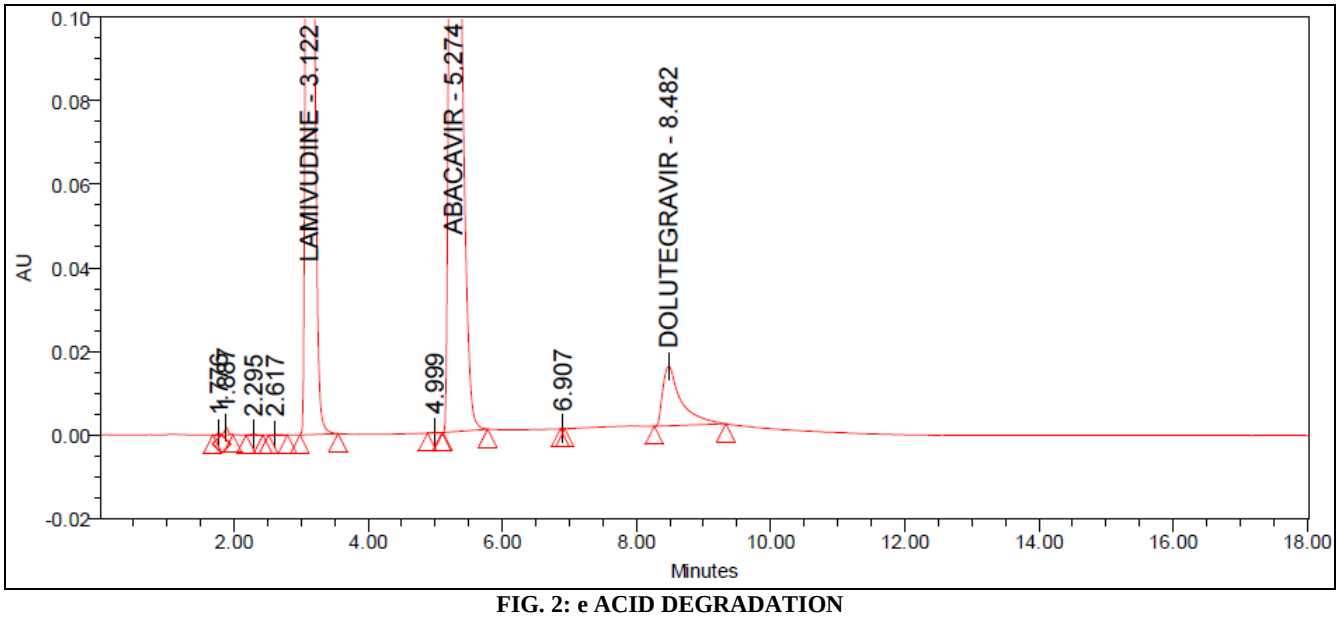

FIG. 2: e ACID DEGRADATION

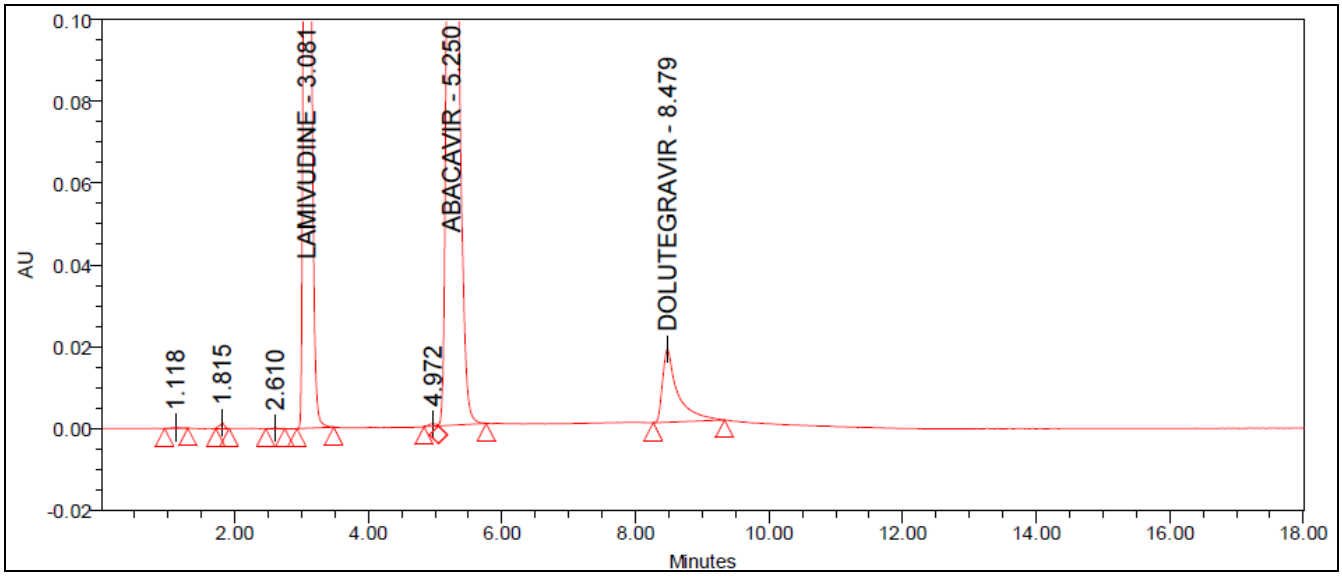

FIG. 2: f ALKALI DEGRADATION

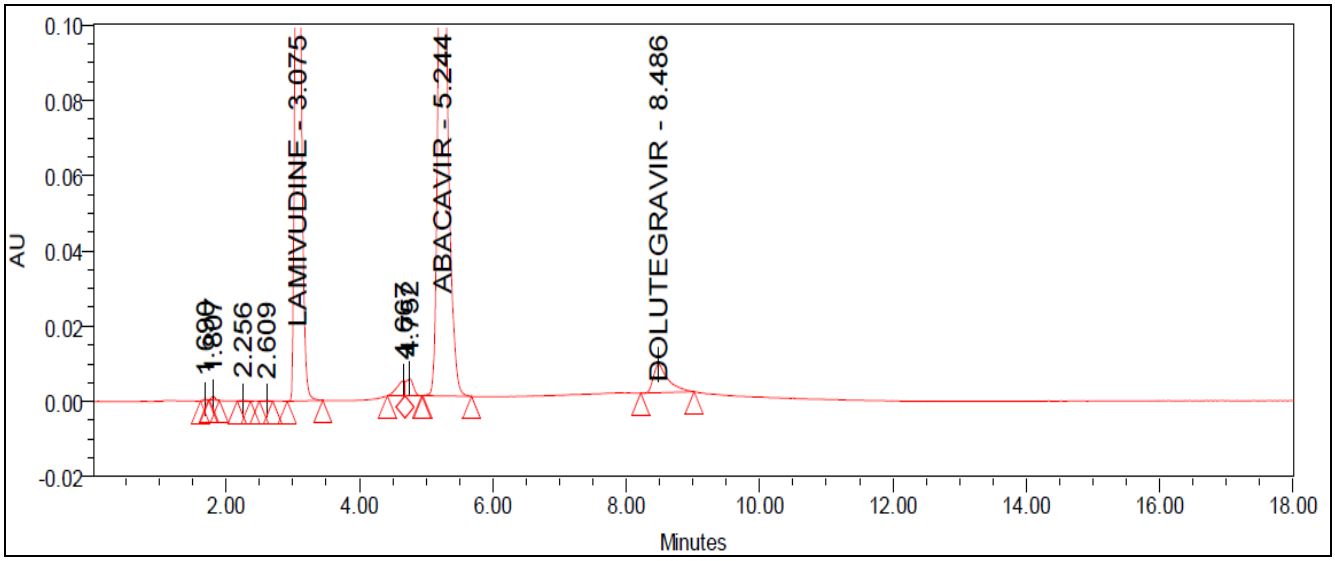

FIG. 2: g PHOTO DEGRADATION

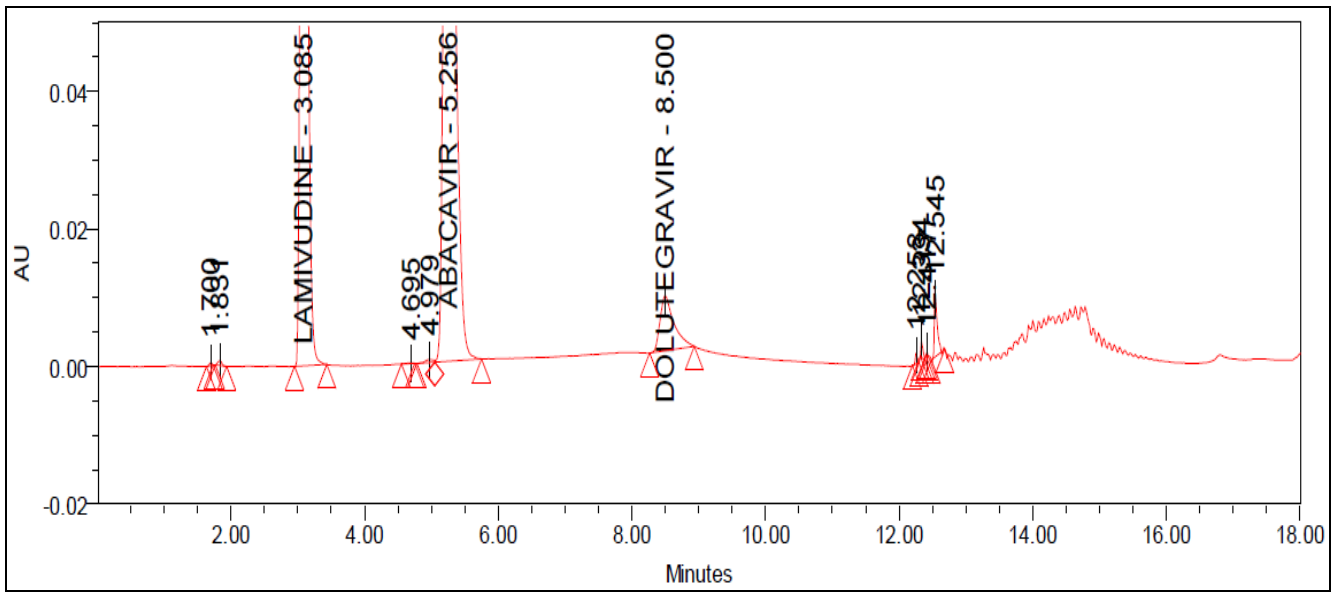

FIG.2: h PEROXIDE DEGRADATION

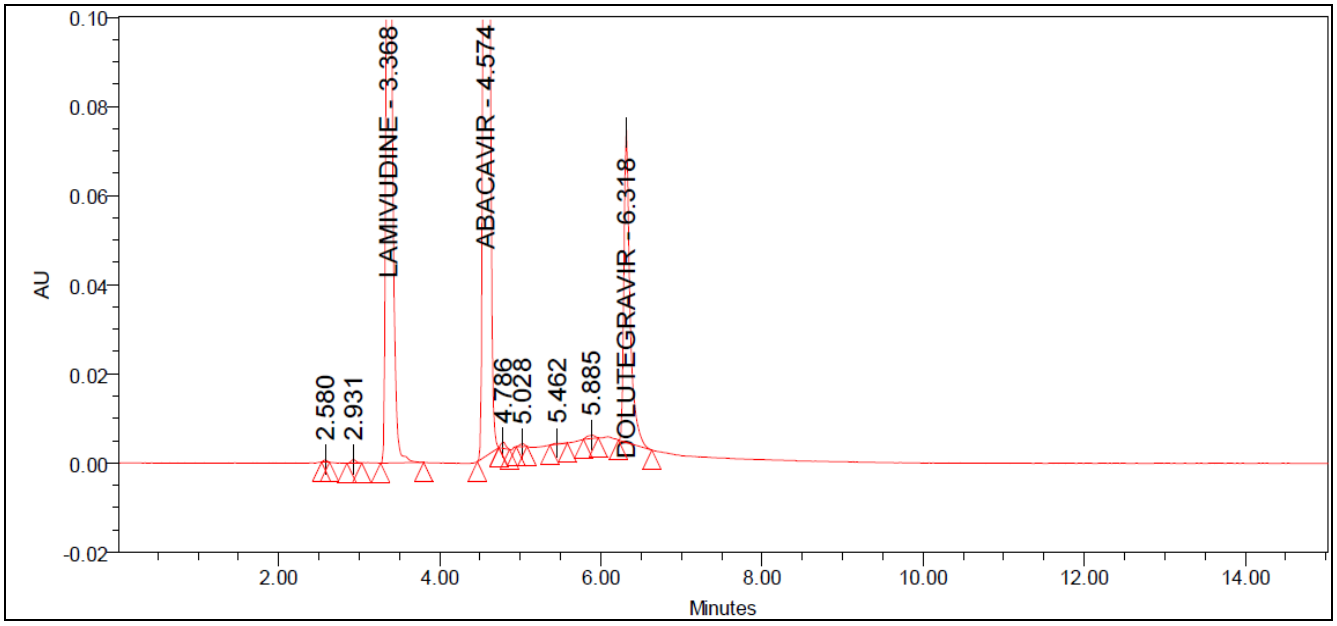

FIG. 2: i HYDROLYTIC DEGRADATION

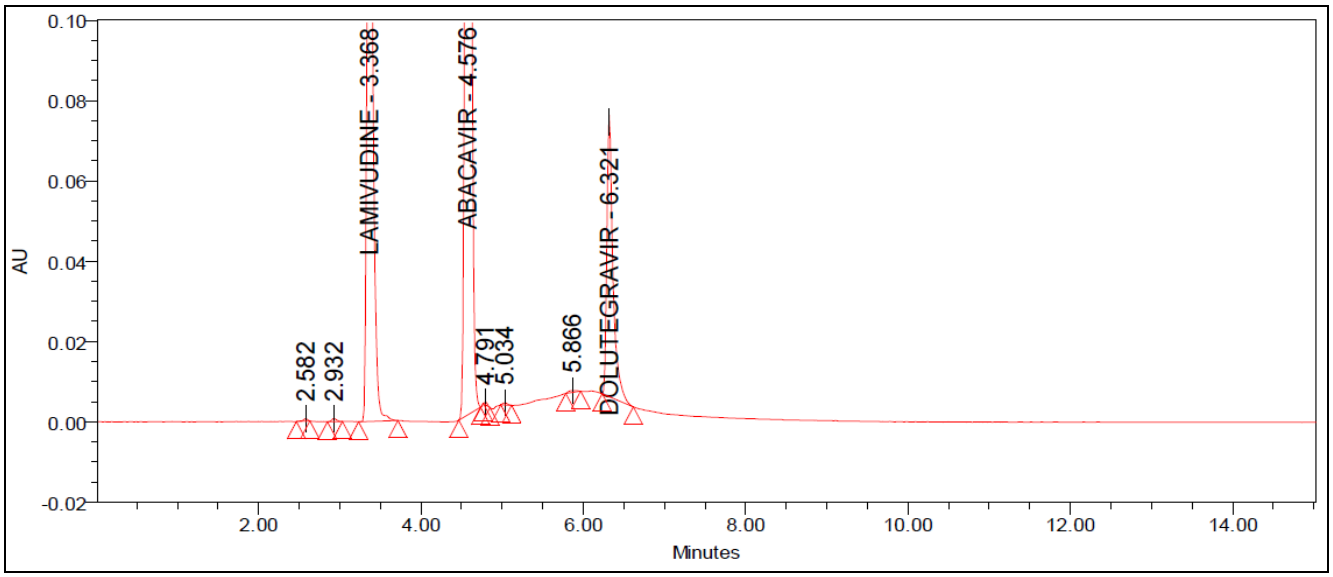

FIG. 2: j HUMIDITY DEGRADATION

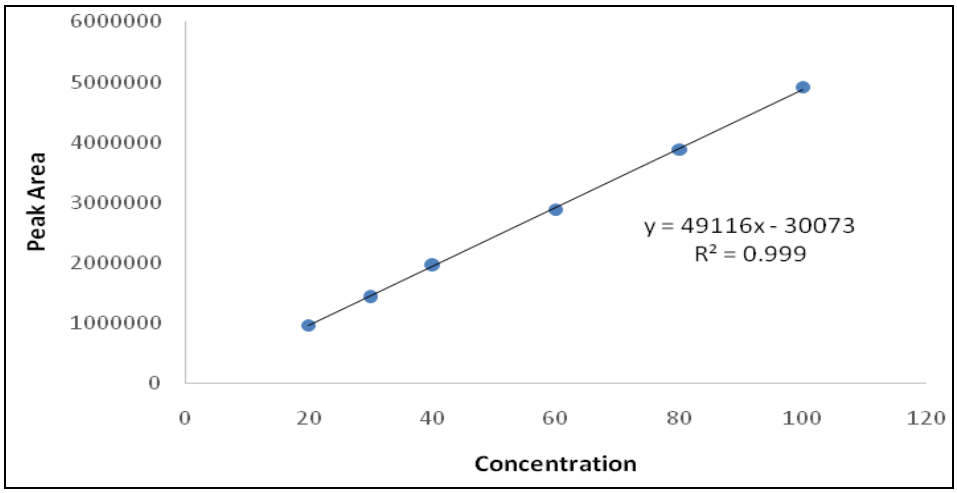

FIG.3: a CALIBRATION PLOT OF ABC

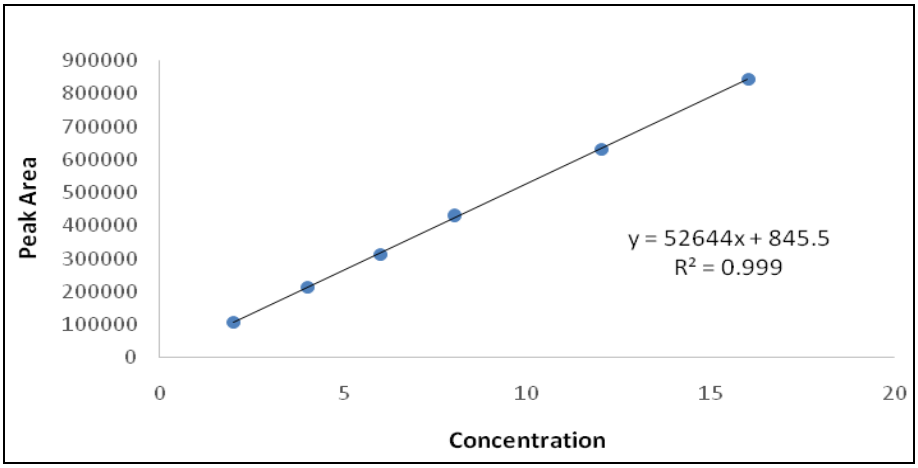

FIG.3: b CALIBRATION PLOT OF DTG

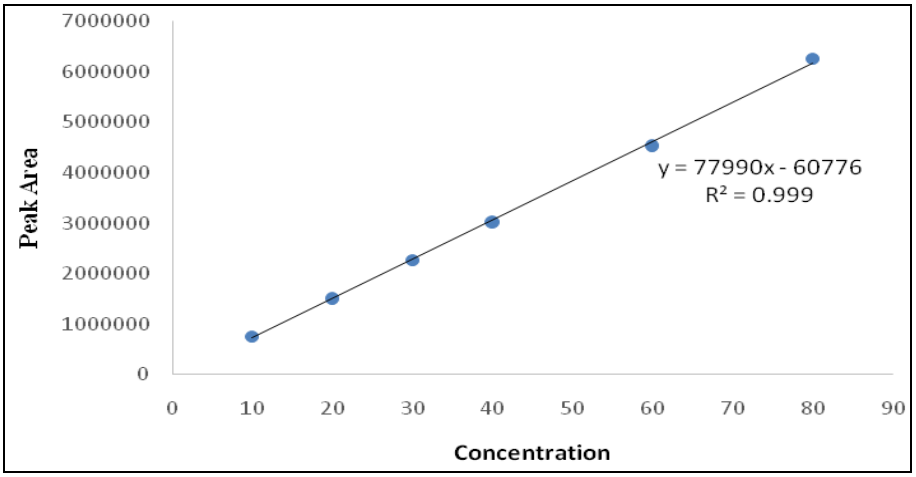

FIG.3: c CALIBRATION PLOT OF 3TC

TABLE 2: ASSAY RESULT OF ABC, DTG AND 3TC

S.No.	Drug	Label Claim	% Amount Found*	% RSD
1	ABC	600	100.0667	0.38
2	DTG	50	100.0667	0.38
3	3TC	300	100.1333	0.60

* Mean of Three Determinations

TABLE 3: SYSTEM SUITABILITY

S.No.	Drug	Peak Area	SD	% RSD
1	ABC	2795214	7916.65	0.26
2	DTG	264173	590.36	0.22
3	3TC	2250381	4964.32	0.22

* Mean of Five Determinations

TABLE 4: LINEARITY OF THE PROPOSED METHOD

ABC		DTG		3TC	
Concentration	**Peak Area**	**Concentration**	**Peak Area**	**Concentration**	**Peak Area**
20	958716	2	106071	10	756373
30	1438089	4	212442	20	1512747
40	1967452	6	310213	30	2269121
60	2876179	8	429285	40	3025494
80	3874904	12	631427	60	4538242
100	4912631	14	842570	80	6250959
Y- Intercept	- 30073	Y- Intercept	845.57	Y- Intercept	- 60776
Slope	49116	Slope	52644	Slope	77990
R^2	0.999	R^2	0.999	R^2	0.999

* Mean of Three Determinations

TABLE 5: PRECISION DATA OF THE PROPOSED METHOD

	ABC		DTG		3TC	
Injection	**Method Precision**	**System Precision**	**Method Precision**	**System Precision**	**Method Precision**	**System Precision**
1	2089342	2197361	92482	92581	1414932	1423842
2	2096298	2171736	92564	92374	1426432	1422181
3	2079668	2213816	92896	91886	1412376	1432283
4	2103296	2182063	92978	92798	1431260	1418650
5	2123354	2183219	92917	92997	1422388	1422608
6	2106281	2209034	92789	91756	1442871	1443245
Mean	2099706.5	2192871.5	92771.0	92398.66	1425043.16	1427134.83
SD	15072.45	16593.72	203.21	495.41	11208.49	9098.44
RSD	0.72%	0.76%	0.22%	0.54%	0.79%	0.64%

TABLE 6: ACCURACY DATA (TRIPLICATE VALUES AT 50, 100 AND 150 PERCENT LEVELS) OF ABC, DTG AND 3TC.

Concentration of spiked level	Amount added	Amount found	% Recovery	Mean Recovery %	%RSD
		ABC			
	30.12	30.04	99.73		
50%	29.94	30.01	100.23	99.86	0.32
	30.04	29.93	99.63		
	60.12	60.16	100.06		
100%	60.08	60.12	100.06	100.00	0.11
	60.09	60.02	99.88		
	90.19	90.21	100.02		
150%	90.10	90.03	99.92	99.96	0.05
	90.22	90.17	99.94		
		DTG			
	2.83	2.8	98.93		
50%	2.68	2.7	100.74	99.64	0.97
	2.71	2.69	99.26		
	5.07	5.13	101.18		
100%	5.13	5.2	101.36	101.04	0.41
	5.18	5.21	100.57		
	7.59	7.65	100.79		
150%	7.64	7.71	100.91	100.30	0.95
	7.58	7.52	99.20		
		3TC			
	15.16	15.18	100.13		
50%	15.20	15.25	100.32		0.10
	15.07	15.11	100.26	100.24	
	29.92	30.01	100.30		
100%	30.32	30.42	100.32	99.95	0.62
	30.10	29.87	99.23		
	45.07	44.89	99.60		
150%	45.32	45.36	100.08	99.94	0.30
	45.14	45.2	100.13		

TABLE 7: ROBUSTNESS DATA OF ABC, DTG AND 3TC

Variation	-2 % of ACN in mobile phase	+2 % of ACN in mobile phase	Flow rate at 0.9ml/min	Flow rate at 1.1ml/min	Wave length at 243nm	Wave length at 247nm
			ABC			
% Assay	100.08	100.25	100.14	99.56	99.81	99.91
Theoretical Plates	8520	4995	7153	4829	5876	5985
Tailing Factor	1.32	1.26	1.32	1.36	1.68	1.62
			DTG			
% Assay	100.10	100.32	100.29	100.43	100.49	100.20
Theoretical Plates	6649	6593	4325	6434	5985	6642

Tailing Factor	1.53	1.57	1.30	1.45	1.35	1.53
3TC						
% Assay	100.21	100.26	100.13	100.30	100.04	100.01
Theoretical Plates	5763	4148	4792	4045	4312	4275
Tailing Factor	1.16	1.19	1.26	1.21	1.37	1.38

* Mean of Three Determinations

TABLE 8: FORCED DEGRADATION DATA

Treatment	% Label Claim	% Degradation	Peak Purity		Pass/Fail
			Purity angle	Purity Threshold	
ABC					
Control	100.5	0	0.270	1.129	
Acid	79.7	21.8	0.420	1.284	Pass
Alkali	79.8	20.7	0.220	1.204	Pass
Peroxide	78.3	22.2	0.245	1.178	Pass
Thermal	76.9	23.6	0.248	1.194	Pass
Photolysis	78.0	22.5	0.251	1.243	Pass
Humidity	80.4	21.1	0.232	1.168	Pass
Hydrolysis	73.0	27.5	0.254	1.196	Pass
DTG					
Control	100	0	1.473	3.756	Pass
Acid	71.5	28.5	3.514	9.286	Pass
Alkali	78.9	21.1	3.262	6.832	Pass
Peroxide	77.9	22.1	2.970	6.443	Pass
Thermal	80.2	19.8	3.411	6.677	Pass
Photolysis	73.1	26.9	3.464	8.901	Pass
Humidity	72.6	27.4	2.683	5.079	Pass
Hydrolysis	76.3	23.7	3.553	6.148	Pass
3TC					
Control	100	0	0.342	1.219	Pass
Acid	81.2	18.8	0.016	1.467	Pass
Alkali	79.4	20.6	0.549	1.387	Pass
Peroxide	81.3	18.7	0.457	1.290	Pass
Thermal	74.5	25.5	0.536	1.363	Pass
Photolysis	76.9	2.1	0.972	1.417	Pass
Humidity	75.5	24.5	0.469	1.278	Pass
Hydrolysis	74.8	25.2	0.570	1.342	Pass

CONCLUSION: This RP-HPLC method for assay of abacavir, dolutegravir and lamivudine is precise, specific, rapid, and stability-indicating. The method may be used to assess the stability of abacavir, dolutegravir and lamivudine as the bulk drug and in its pharmaceutical formulation. Chromatographic analysis time of less than 20 min was advantageous for use of the method in routine analysis. It may be extended to study of abacavir, dolutegravir and lamivudine and also analysis of the drug in plasma and other biological fluids.

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