Research Paper

Simultaneous Quantification of Novel Antiretroviral Drug Combination by Stability-indicating High Performance Liquid Chromatography Method

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Research in the many areas of human immunodeficiency virus treatment, eradication and prevention has necessitated measurement of antiretroviral concentrations in nontraditional specimen types. For HIV infection, drug combinations are typically used as highly active antiretroviral therapy, intended to maximize viral suppression. A novel four drugs combination was used, which contains two nucleoside analog reverse transcriptase inhibitors (lamivudine and tenofovir) and two protease inhibitors (darunavir and ritonavir). A new simple, efficient, and sensitive reverse phase high performance liquid chromatographic method has been developed for simultaneous extraction and determination of the concentrations of lamivudine, tenofovir, darunavir and ritonavir in bulk and in their tablets. Four compounds were separated on a reversed-phase C18 column at 30±2.0° using a gradient mobile phase combination containing potassium dihydrogen phosphate, acetonitrile and methanol. The pH was adjusted to 3.5±0.05 by the addition of orthophosphoric acid. The samples were detected using a UV detector, 260 nm for lamivudine, tenofovir and darunavir and 240 nm for ritonavir. The procedure separated analytes and its potential degradation products such as lamivudine, tenofovir, darunavir and ritonavir eluting at about 2.385, 4.055, 11.353 and 14.010 mins, respectively. The linear range of lamivudine, tenofovir, darunavir and ritonavir was 58.32-174.96 µg/ml, 58.32-174.96 µg/ml, 72.00-216.00 µg/ml and 112.50-337.5 µg/ml, respectively. The relative standard deviation for precision was less than 2.0%. The drug was subjected to acid, alkaline peroxide and photolytic stress conditions and the performance of the method was validated according to the International Conference on Harmonization guidelines for specificity, linearity, accuracy, precision and robustness.

Key words: Stability indicating, RP-HPLC, lamivudine, tenofovir, darunavir and ritonavir

Viruses have been producing an enormous health hazards continuously for the mankind since ages. These challenges were constantly met by the mankind by producing the effective drugs. There are number of new drug molecules that have been developed for the effective treatment of human immunodeficiency virus (HIV) infection or other viral infections. One of the deadliest and unmanageable chronic health catastrophes is HIV/AIDS. It requires lifelong treatment with combination of potent life-saving essential drugs which include, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse-transcriptase inhibitors (NNRTI) and protease inhibitors[1,2]. Amongst these two nucleoside analog NRTI lamivudine (2′,3′-dideoxy-3′-thiacytidine, commonly called 3TC), tenofovir (({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy} methyl)phosphonic acid), two protease inhibitors (PI) like darunavir ([(1R, 5S, 6R)-2,8-dioxabicyclo[3.3.0] oct-6-yl] N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl) amino]-3-hydroxy-1-phenyl-butan-2-yl]carbamate) and ritonavir (1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl}) carbamoyl] amino} butanamido]-1, 6-diphenylhexan-

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2-yl]carbamate) constitute first-line therapy[3]. Fig. 1a-d shows the chemical structures of the drugs. Since, the introduction of highly active antiretroviral combination therapy (HAART) in the late 1990s, the life expectancy and quality of life of HIV-infected patients have improved due to plasma virus load reductions to below detectable levels[4]. Combination of these four drugs into fixed dose combinations (FDCs) has been an essential constituent of the HAART.

Few reports have described bioanalytical methods for simultaneous detection of lopinavir and ritonavir, alone or in combination with additional PI and NNRTI, from plasma and/or cell samples[3,5-9]. Some methods detect tenofovir alone or in combination with other drugs, such as lamivudine[10-12]. Two different analytical assays were employed to determine time course plasma drug concentrations in lopinavir-ritonavir and tenofovir drug interaction studies[13-15]. Liquid chromatography-mass spectrometry (LC-MS) method was reported for the simultaneous detection of lopinavir, ritonavir and tenofovir in plasma[16]. No high performance liquid chromatography (HPLC) method has been reported for the quantification of these four drugs in any of the matrices. Hence, a reproducible stability-indicating RP-HPLC method was developed for the quantitative determination of four drugs. This method was successfully validated according to the International Conference on Harmonization (ICH) guidelines[17,18]. One of the challenges in developing a single assay for these four drugs is the significant differences in hydrophobicity of the nucleoside reverse transcriptase inhibitors (NRTI, lamivudine and tenofovir) and the PIs (darunavir and ritonavir). While darunavir and ritonavir are hydrophobic, and hence insoluble in water, lamivudine and tenofovir is hydrophilic. This difference makes it challenging to extract these drugs effectively and simultaneously from tablets and also to identify a suitable chromatographic column matrix for the separation. Thus, a creative solution is needed. We systematically addressed these issues and developed a single chromatographic assay to detect all four compounds, lamivudine, tenofovir, darunavir and ritonavir, simultaneously by using LC coupled with PDA detector. The optimized method is capable of extracting and quantifying three drugs simultaneously with high efficiency, selectivity, and sensitivity.

MATERIALS AND METHODS

Original standards of lamivudine, tenofovir, darunavir and ritonavir were provided by the Bio-leo laboratories, Hyderabad. Potassium di hydrogen phosphate, orthophosphoric acid, HPLC grade acetonitrile and HPLC grade methanol was purchased from Merck, Mumbai, India. HPLC grade water was prepared in-house by Milli-Q water purifying system. Fixed dosage combination tablets containing 150 mg of lamivudine, 150 mg of tenofovir, 400 mg of darunavir and 50 mg of ritonavir was used for analysis.

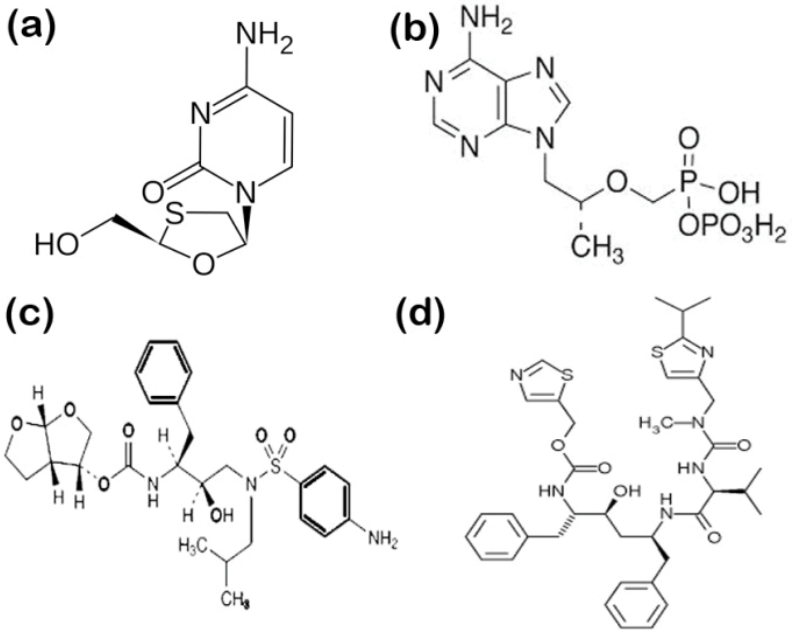

Fig. 1: Chemical structures of analytes.
Chemical structures of (a) lamivudine, (b) tenofovir, (c) darunavir and (d) ritonavir.

Chromatographic conditions:

Waters e 2695 series HPLC consisting pump, auto sampler, auto injector, VWD and photo diode array detector, thermostatic column compartment connected with Empower 2 software connected with an Inertsil ODS-3V, 250×4.6 mm, 5 µ column. Lamivudine, tenofovir and darunavir were determined at 260 nm and ritonavir at 240 nm.

Mobile phase:

Accurately weighed 1.36 g of potassium dihydrogen phosphate in 1000 ml of water, adjusted the pH 3.5±0.05 with orthophosphoric acid. Filtered the solution through 0.22 µ nylon filter and sonicated to degas it. The buffer was used as mobile phase preparation A and acetonitrile:methanol (450:150 v/v) used as mobile phase preparation B. Lamivudine, tenofovir, darunavir and ritonavir were separated and eluted in a gradient program represented in Table 1. The flow rate of the mobile phase was maintained at 1.0 ml/min. The column temperature was maintained at 30° with the injection volume of 10 µl. Methanol was used as diluent-1 and a mixture of 750 ml of water and 250 ml of methanol was used as diluent-2.

Preparation of standard solution:

Weighed accurately and transferred 108 mg of lamivudine, 108 mg of tenofovir, 120 mg of darunavir and 150 mg of ritonavir working standard into 100 ml volumetric flask. Added about 75 ml of diluent-1, sonicated to dissolve and diluted the volume with diluent-1 and mixed well. Pipetted 5 ml of the above solution and transferred into a 50 ml volumetric flask and diluted to volume with diluent-2. Mixed well and filtered the solution through 0.45 µm syringe filter.

Preparation of test stock solution:

Weighed 20 tablets and determined the average weight. Crushed into a fine powder in mortar using pestle and mixed homogeneously. Weighed accurately and transferred tablet powder equivalent to about 450 mg of lamivudine or 450 mg of tenofovir or 1200 mg of darunavir or 150 mg of ritonavir into a 500 ml volumetric flask. About 400 ml of diluent-1 was added and sonicated to disperse the sample completely. The sonication was continued for about 60 min with intermittent shaking. Then, diluted to volume with diluent-1 and mixed well. Centrifuged a portion of the above solution in a centrifuge tube with cap, at 5000 rpm, for about 10 min and transferred the clear supernatant liquid into another centrifuge tube.

TABLE 1: MOBILE PHASE GRADIENT TABLE

Time (min)	Mobile phase A (%v/v)	Mobile phase B (%v/v)
0	90	10
2.0	85	15
3.0	75	25
5.0	40	70
11.0	40	70
12.0	90	10
15.0	90	10

Test solution for lamivudine and tenofovir:

Pipetted 6.0 ml of the clear solution into a 50 ml volumetric flask and dilute to volume with diluent-2 and mixed well and filtered a portion of above solution through 0.22 µ syringe filter.

Test solution for darunavir:

Pipetted 5.0 ml of the clear solution into a 100 ml volumetric flask and diluted to volume with diluent-2 and mixed well and filtered a portion of above solution through 0.22 µ syringe filter.

Test solution for ritonavir:

Pipetted 10.0 ml of the clear solution into a 20 ml volumetric flask and diluted to volume with diluent-2 and mixed well and filtered a portion of above solution through 0.22 µ syringe filter.

Elution pattern:

Fumaric acid peak eluted first at about 2.10 min retention time (Rt), next lamivudine peak eluted at about 2.385 min, next eluted tenofovir at about 4.055 min, next darunavir eluted at about 11.353 min and finally eluted ritonavir peak at 14.010 min. Disregarded the peaks due to fumaric acid (from tenofovir disoproxil fumarate) from test chromatograms.

Force degradation study:

Forced degradation study was performed to evaluate the stability of the developed method using the stress conditions like exposure of sample solution to acid (0.1 N HCl), base (0.1 N NaOH), peroxide (H_2O_2) and heat. Investigation was done for the degradation products. For acid treatment, 10 ml of 1 N HCl was added to the 10 ml stock solution and kept aside at 80° for 12 h. This solution was cooled and neutralized with 10 ml of 1 N NaOH and diluted suitably to a final volume of 100 ml with mobile phase and filtered through 0.22 µ membrane filter. For alkali, 10 ml of 0.5 N NaOH was added to the 10 ml stock solution and

kept aside at 80° for 48 h. This solution was cooled and neutralized with 10 ml of 1 N HCl and diluted suitably to a final volume of 100 ml with mobile phase and filtered through 0.22 µ membrane filter. For Peroxide degradation studies, to the 10 ml stock solution, 5 ml of 3% H_2O_2 was added and kept aside at 80° for 24 h. This solution was cooled and diluted suitably to a final volume of 100 ml with mobile phase and filtered through 0.22 µ membrane filter. To study heat degradation, 10 ml stock solution, kept at 70° for 10 days. This solution was cooled and diluted suitably to a final volume of 100 ml with mobile phase and filtered through 0.22 µ membrane filter.

RESULTS AND DISCUSSION

In order to achieve good separation between all the four components different buffer pH conditions were maintained and different proportions of solvents like methanol, acetonitrile and water tested binary and tertiary eluents were added. However, in potassium dihydrogen phosphate buffer pH 3.5±0.05 adjusted with orthophosphoric acid achieved good satisfactory results at a flow rate of 1.0 ml/min and was measured at a detection of 260 nm for lamivudine, tenofovir and darunavir and 240 nm for ritonavir. Blank, standard and sample chromatograms were shown in figs. 2-4. System suitability is an integral part of the method validation to evaluate the parameters like tailing factor, theoretical plates, resolution and percent relative standard deviation (%RSD) for replicate injections. The results were within the limits and were presented in Table 2.

In the blank chromatograms there were no peaks observed at the retention times of lamivudine, tenofovir,

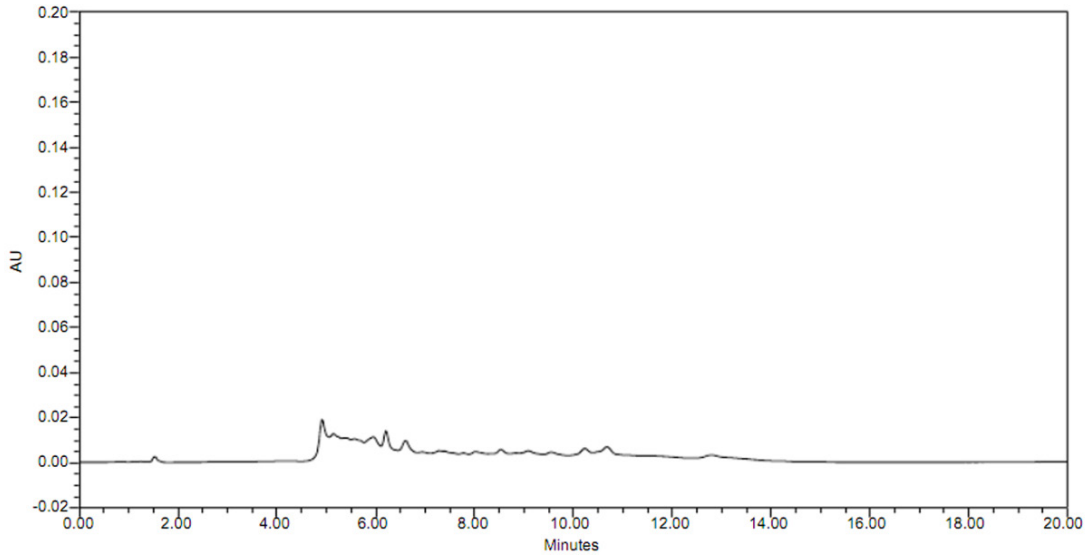

Fig. 2: Blank chromatogram.

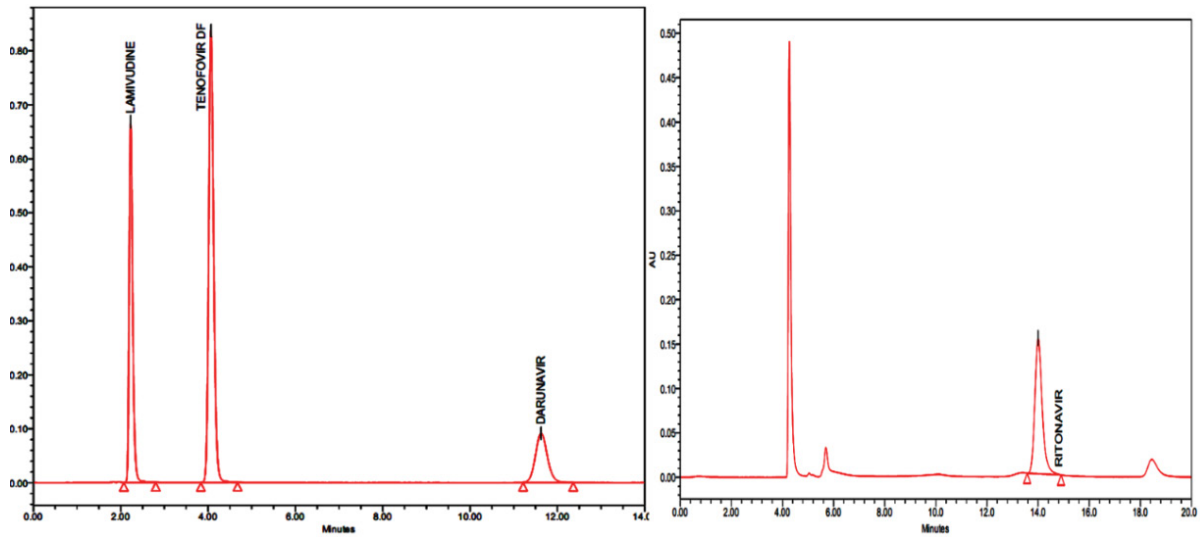

Fig. 3: Chromatogram of standard solution.
Standard chromatogram of lamivudine, tenofovir, darunavir (at 260 nm) and ritonavir (at 240 nm).

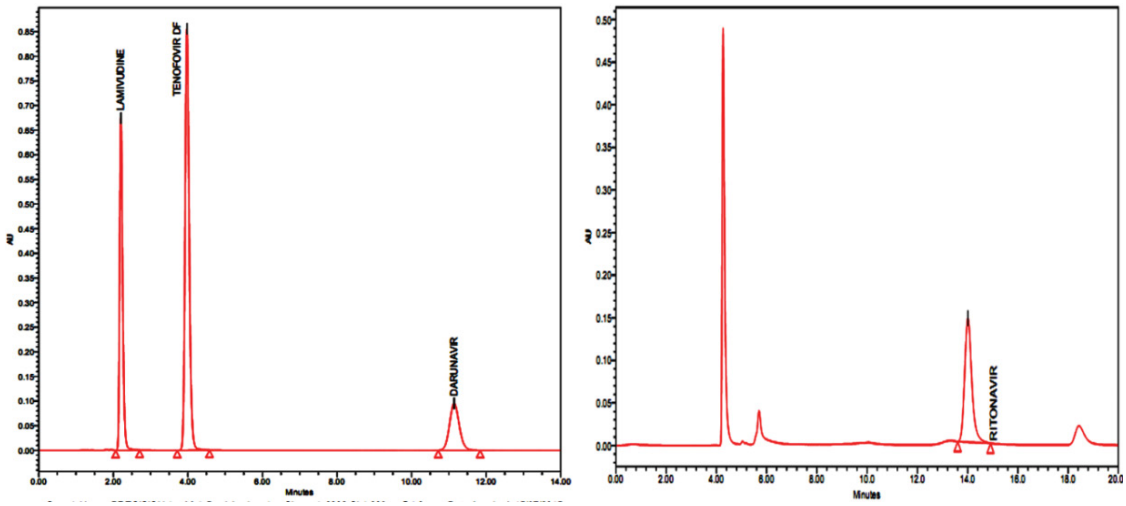

Fig. 4: Chromatogram of sample solution.
Market formulation chromatogram of lamivudine, tenofovir, darunavir (at 260 nm) and ritonavir (at 240 nm).

TABLE 2: SYSTEM SUITABILITY RESULTS

Parameter	Results				Required limits
	Lamivudine	Tenofovir	Darunavir	Ritonavir	
RSD of peak area	0.10	1.01	1.24	0.51	<2.0 for n≥6
RSD of retention time	0.25	0.14	0.52	0.85	<1.0 for n≥6
USP Tailing factor (T)	0.25	1.08	0.65	0.84	T<2
USP Plate Count (N)	4500	5235	5874	6850	>2000
USP Resolution (R)	-	2.04	5.71	45.08	R>2

RSD: Relative standard deviation. USP: United States Pharmacopoeia

darunavir and ritonavir, and also the degradation studies showed that there was no interference with degradants that shows the method is specific (figs. 2-4). To determine the accuracy of the proposed method, recovery studies were conducted; known amount of pure drug concentrations were at three different levels, i.e., 50%, 100% and 150% was calculated. Accuracy was calculated as the percentage of recovery. The results were tabulated in Table 3.

The precision was evaluated at three levels, repeatability, reproducibility and intermediate precision each level of precision was investigated by six replicate injections of 100% concentrations of lamivudine, tenofovir, darunavir and ritonavir. The result of precision was expressed as %RSD and was tabulated in Table 4.

The linearity was evaluated by measuring different concentrations (50 to 150%) of the standard solutions to lamivudine, tenofovir, darunavir and ritonavir. The calibration curve was constructed by plotting concentration of standard solutions against mean peak areas and the regression equation was computed. The summary of the parameters were shown in Table 5.

The robustness of the method was unaffected when small, deliberate changes like, flow change, mobile phase composition, column temperature were performed at 100% test concentration. The ruggedness of the proposed method studied under different columns, analyst, instrument, laboratories analysis of the same sample.

The stability of the standard solution was tested at the intervals of 24 and 48 h at room temperature. There were no significant changes observed in the system suitable parameters like theoretical plates, tailing factors, retention time and resolution. Hence, the standard solution is stable up to 48 h of room temperature.

The proposed method was applied for the analysis of lamivudine, tenofovir, darunavir and ritonavir in tablet dosage forms, the results were found to be between 99.0 and 100.0% and the results were summarized in Table 6. Results of forced degradation were shown in Table 7 and fig. 5 shows the chromatograms of forced degradation studies.

While a number of antiHIV drug assays have been developed to detect multiple HIV PIs or reverse transcriptase inhibitors in one assay, it is more challenging to detect both classes of drugs at the same time. NRTI such as lamivudine and tenofovir are generally more hydrophilic and PIs, such as darunavir

TABLE 3: ACCURACY DATA

Parameter	Amount added (µg)	Amount recovered (µg)	% of recovery	Mean % of recovery
		Lamivudine		
50% level	54.00	53.88	99.65	99.88
100% level	108.00	108.02	100.02	
150% level	162.00	161.95	99.95	
		Tenofovir		
50% level	53.96	53.56	99.52	99.81
100% level	107.93	107.55	99.85	
150% level	161.90	162.01	100.05	
		Darunavir		
50% level	59.96	59.85	99.86	99.89
100% level	119.92	120.05	100.01	
150% level	179.89	180.12	100.08	
		Ritonavir		
50% level	14.99	14.77	99.72	99.50
100% level	29.98	29.27	99.55	
150% level	44.97	44.11	99.25	

Values are mean of triplicate preparation

TABLE 4: PRECISION STUDIES

Parameter	Results			
	Lamivudine	Tenofovir	Darunavir	Ritonavir
Repeatability				
Mean %RSD of retention time	0.25	0.58	0.69	1.24
Mean %RSD of peak area	1.02	1.52	1.68	0.57
Mean % Assay	99.85	99.25	100.01	99.41
Reproducibility/intraday precision				
Mean %RSD of retention time	0.20	1.02	1.85	0.81
Mean %RSD of peak area	0.33	0.38	0.21	0.35
Mean % assay	100.00	100.00	100.00	100.00
Intermediate precision				
Mean %RSD of retention time	0.28	1.57	1.62	1.42
Mean %RSD of peak area	0.31	0.18	0.28	0.06
Mean % assay	100.00	100.00	99.98	99.68

TABLE 5: REGRESSION EQUATION PARAMETERS

Parameter	Lamivudine	Tenofovir	Darunavir	Ritonavir
Linearity range (µg/ml)	58.32-174.96	58.32-174.96	72.0-216.00	112.5-337.50
Correlation co-efficient	0.9995	0.9986	1	1
Slope	140604	174897	15660	80167
Y-intercept	5E+06	7E+06	0.2	24435

TABLE 6: ASSAY RESULTS OF MARKETED TABLETS

Drug	Labeled amount (mg/tab)	Amount found (mg/tab)	% of assay
Lamivudine	150	149.95	99.97
Tenofovir	150	149.82	99.88
Darunavir	400	399.99	99.99
Ritonavir	50	50.01	100.02

and ritonavir, are generally more hydrophobic at physiologic pH. With the ability of a column matrix and PDA technique to separate the two PIs as well as the two NRTIs, we developed and optimized a single-step assay with a simplified extraction procedure. The final assay was validated to be effective and sensitive. Also, the one-step assay method is reliable and reproducible, with a high accuracy, precision and recovery. In our assay, we focused on detecting two NRTIs, lamivudine and tenofovir and two PIs, darunavir and ritonavir

TABLE 7: FORCED DEGRADATION STUDY

Condition	Lamivudine		Tenofovir		Darunavir		Ritonavir	
	%Assay	%Degradation	%Assay	%Degradation	%Assay	%Degradation	%Assay	%Degradation
Acid	88.60	11.40	90.85	9.15	90.87	9.13	94.19	5.81
Base	86.39	13.61	93.08	6.92	86.45	13.55	93.08	6.92
Peroxide	94.18	5.82	89.10	10.90	90.89	9.11	89.10	10.90
Heat	96.96	3.04	95.31	4.69	95.32	4.68	95.31	4.69

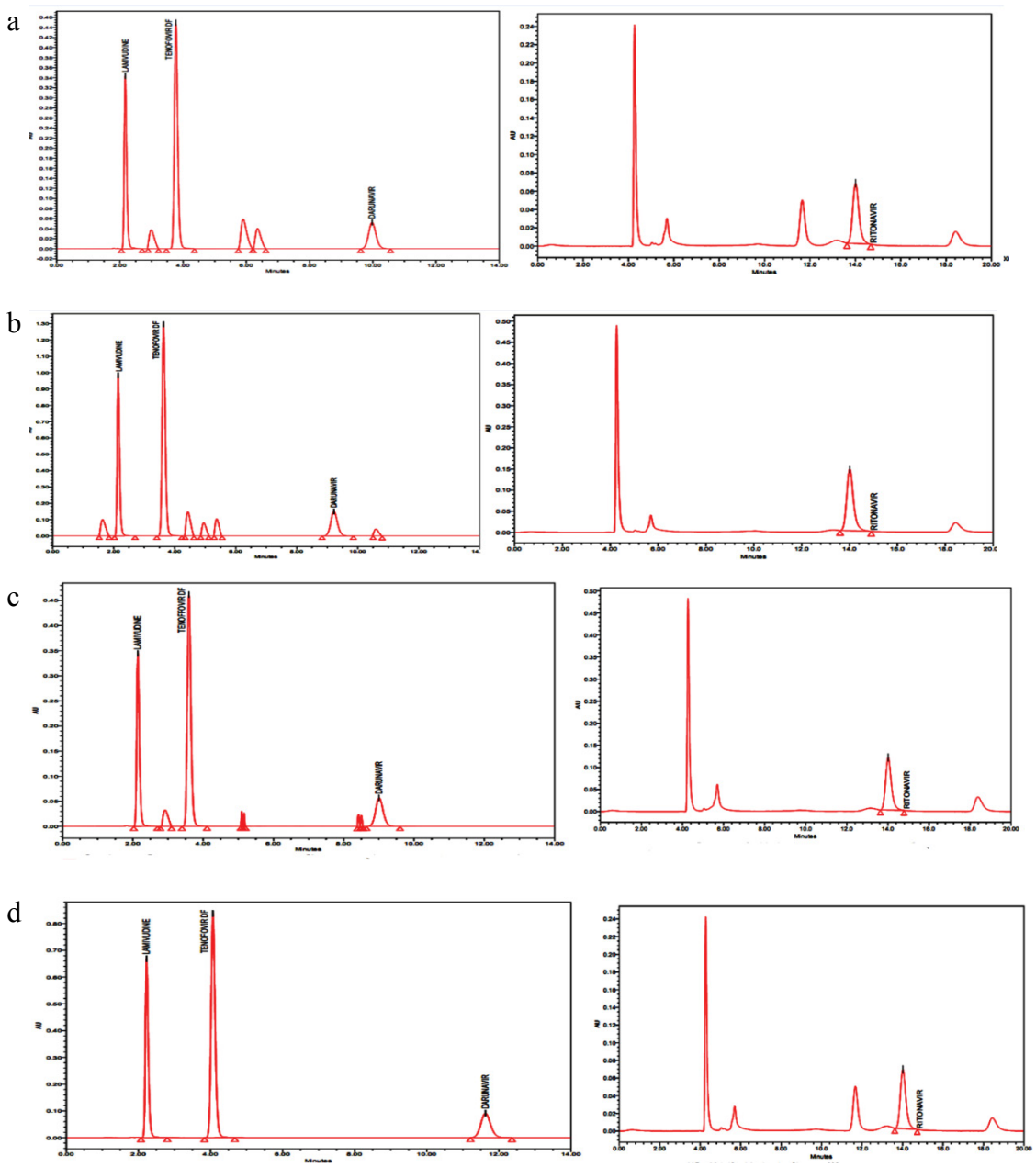

Fig. 5: Force degradation chromatograms.
(a) Acid degradation, (b) alkali degradation, (c) peroxide degradation and (d) heat degradation.

because these four drugs are recommended as a key HAART combination in the most recent HIV/AIDS treatment guidelines. With some modifications, a one-step clinical assay such as that described here, but for other PI and NRTI drug combinations, such as lopinavir or atazanavir with emtricitabine plus tenofovir, could be developed. However, such studies are also beyond the scope of this report.

In summary, using a single column and a combination gradient mobile phase mixture of potassium dihydrogen

phosphate, acetonitrile and methanol, we successfully developed a one-step HPLC-PDA assay to detect four analytes, lamivudine, tenofovir, darunavir and ritonavir. The method can indicate stability and can be used for the routine analysis of production samples and to check the shelf life of the dosage forms.

A simple, specific and reliable gradient HPLC-PAD method was developed for the estimation of lamivudine, tenofovir darunavir and ritonavir in their pharmaceutical formulation. The four compounds were subjected to forced degradation applying several stress conditions. The proposed method was successfully separated all the four compounds with degradants, estimate the active contents. The Proposed method is specific and stability indicating power. Hence, the developed method can be adapted to regular quality control analysis.

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