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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DARUNAVIR, COBICISTAT, EMTRICITABINE AND TENOFOVIR ALAFENAMIDE IN BULK AND PHARMACEUTICAL FORMULATION

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Keywords:

Darunavir (DAR), Cobicistat (COB), Emtricitabine (EMT), Tenofovir alafenamide (TEN), RP-HPLC

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ABSTRACT: A simple, accurate & precise method was developed for the simultaneous estimation of Darunavir (DAR), Cobicistat (COB), Emtricitabine (EMT), and Tenofovir alafenamide (TEN) in their bulk and pharmaceutical formulation. The chromatogram was run through Agilent C18 (150 × 4.6 mm, 5 m) column. Mobile phase containing 0.1% Ortho Phosphoric acid (OPA) (pH 2.4) and Acetonitrile in the ratio of 55:45 was pumped through the column at a flow rate of 0.75 ml/min. While the temperature was maintained at 30°C. The optimized wavelength for the combination was 245 nm. The retention time of DAR, COB, EMT, and TEN were found to be 3.988 min, 3.147 min, 2.205 min, and 2.616 min respectively. The linearity range for the method was found to be 200-1200µg/ml for DAR, 37.5-225 µg/ml for COB, 50-300 µg/ml for EMT and 2.5-15 µg/ml for TEN with regression coefficients of 0.9992, 0.9997, 0.9997 and 0.9994. The method was validated according to ICH guidelines and the drugs were subjected to forced degradation as per ICH Q1A15. Forced Degradation studies were performed on different conditions and the percentage of drug degraded was found to be within limits. The accuracy of the method was indicated by a good recovery in the range of DAR 99.87%, COB 99.41%, EMT 99.74%, and TEN 100%w/v. The developed and validated RP-HPLC method can be routinely used for the estimation of Darunavir (DAR), Cobicistat (COB), Emtricitabine (EMT), and Tenofovir alafenamide (TEN) in their bulk and pharmaceutical formulation.

INTRODUCTION: DAR is chemically [(3aS, 4R, 6aR)-2, 3, 3a, 4, 5, 6a-hexahydrofuro [2,3-b]furan-4yl] N-[(2S, 3R)-4-[(4-aminophenyl) sulfonyl-(2-methylpropyl) amino]-3-hydroxy-1-phenylbutan-2-yl] carbamate- **Fig. 1A**, a Protease Inhibitor (PI), mostly used in combination with Ritonavir (PI) selectively inhibits the cleavage of HIV gag and gag-pol polyproteins, preventing viral maturation and it also inhibits dimerization of HIV-1 protease.

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Inhibiting proteolytic activity and subsequent HIV-1 replication [5]. COB is designated as (1,3-thiazol-5-yl) methylN-[(2R, 5R)-5-[(2S)-2- {[methyl ({[2-(propan - 2 - yl) - 1, 3 – thiazol - 4 - yl] methyl}) carbamoyl amino} - 4 - (morpholin - 4 - yl) butanamido] - 1,6-diphenyl hexan-2-yl] carbamate **Fig. 1B** often used in combination with other PI drugs (Ritonavir & Darunavir) does not have antiviral activity of its own but acts as an pharmacokinetic enhancer by inhibiting cyto-chrome P450 3A (CYP3A) increasing the exposure of PI drugs [2].

EMT is 4-amino-5-fluoro-1- [(2R, 5S)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1, 2dihydropyrimidin-2-one **Fig. 1C**, an NRTI which competitively inhibits blocks the HIV reverse transcriptase

enzyme and blocks the HIV replication thus preventing HIV from multiplying and can reduce the amount of HIV in the body. It is also effective to treat hepatitis virus B infection (HBV) in people with HIV [7]. TEN is chemically propan-2-yl (2S)-2-[[[(2R)-1-(6-aminopurin-9-yl) propan-2-yl] oxy methyl phenoxy phosphoryl] amino] propanoate-**Fig. 1D**, a prodrug of Tenofovir is an NRTI used in the treatment of HBV and often in combinations with other retroviral's used in the treatment of HIV [6]. Combination of DAR, COB, EMT and TEN is a first-ever protease-inhibitor-based single-tablet regimen [8], with DAR being a Protease Inhibitor (PI), COB a pharmacokinetic enhancer of DAR and EMT, TEN belonging to Nucleoside reverse transcriptase inhibitors (NRTI) is recently approved by U.S. Food & Drug Administration [9] and European Medical Agency (EMEA, 2018). As the combination of DAR, COB, EMT, and TEN being recently approved, it has not yet been adopted by any official pharmacopeia. An in-depth literature review has revealed that no analytical method has been published for this combination. The reason for selecting this combination is to provide a validated HPLC method that can separate four different drugs in a single formulation that may be useful in the pharmaceutical industry.

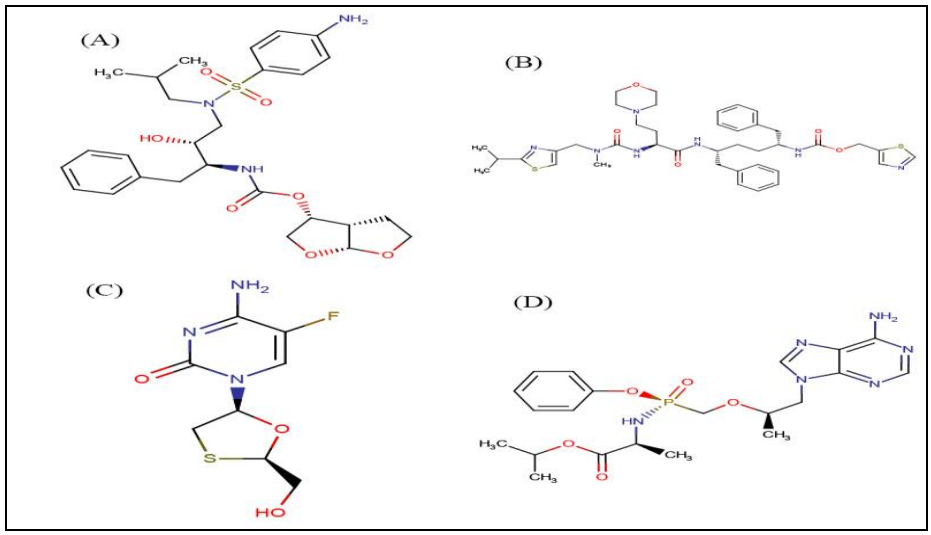


FIG. 1: STRUCTURE OF DARUNAVIR (A), COBICISTAT (B), EMTRICITABINE (C) AND TENOFOVIR ALAFENAMIDE (D)

MATERIALS AND METHODS:

Chemicals and Reagents: The drug samples of DAR, COB, EMT, and TEN were obtained from Spectrum Pharma Research Solutions, Hyderabad, which is analytically pure. The reagents Water, Methanol, and Acetonitrile are HPLC grade while Orthophosphoric is AR grade was procured from Rankem Pvt. Ltd, Hyderabad, India.

Instrument: The liquid chromatographic separation was carried out on WATERS 2690 autosampler, operating with a binary pump and a rheodyne injector, equipped with Waters 2996 Photodiode Array Detector. Experimental conditions were optimised on Agilent C18 (150 x 4.6 mm, 5 m) column. The chromate grams were integrated by EMPOWER software version 2.0. All the standard and sample drugs were weighed on Denver Calibrated electronic balance AY220D. PH meter and Ultrasonicator were purchased from BVK Enterprises India. Proper conditioned Boro-silicate Grade glassware was used for the study.

Selection of Solvent: Solubility studies of drugs were performed, and it was found that all drugs are commonly soluble in Acentonitrile: Water (1:1) and was selected as the solvent for study.

Preparation of Mobile Phase: The mobile phase was prepared by mixing 0.1% OPA (pH 2.4), and Acetonitrile in the ratio of 55:45, and the mixture was sonicated to about 10 min. 0.1% OPA was prepared by diluting 1 ml of orthophosphoric acid to 1000 ml with HPLC grade water.

Preparation of Standard Solution: A standard solution of the four drugs DAR, COB, EMT, and TEN was prepared by accurately weighing 400 mg of Darunavir, 75 mg of Cobicistat, 100 mg of Emtricitabine, and 5 mg of Tenofovir Alafenamide, respectively and transferred into 50 ml volumetric

flasks. The volumetric flask was half-filled with solvent and sonicated for 15 min and later made up to the mark with the same solvent. All the aliquots required for the study were prepared from this standard solution.

Preparation of Sample Solution: In house tablet formulation containing 200 mg DAR, 150 mg COB, 200 mg EMT, and 10 mg TEN were used as a sample. 20 tablets were weighed and average weight was calculated; the weight equivalent to one tablet was transferred to a 100 ml volumetric flask, 50 ml solvent added and sonicated for 25 min and further made up to the mark and finally filtered through HPLC filters.

Chromatographic Condition: Agilent C18 (150 x 4.6 mm, 5 m) column was used for the chromatographic separation at a detection wavelength of 230 nm under 30 ºC temperature. Mobile phase 0.1% OPA: ACN in the ratio 55:45 v/v which was degassed under ultra-sonication, was selected for elution, and the same mixture was used in the preparation of standard and sample solutions.

The flow rate was optimized to 0.75 ml/min, and the injection volume 10 µl was fixed upon the satisfactory results of various system suitability parameters such as retention time, column efficiency, tailing factor, and asymmetry of the peaks.

Method Validation: The proposed method was validated according to Q2 (R1) guidelines [3]. The validation parameters are system suitability, specificity, linearity, accuracy, precision, robustness, ruggedness, LOD, and LOQ.

System Suitability Parameters: Standard solution was injected into the system five times, and the system suitability parameters (Retention time (Rt), Theoretical plates (TP), Tailing factor (t)) were checked.

Specificity: To access the specificity of the method, blank and placebo were injected into the system, and the obtained chromatograms are studied for any responses.

Linearity: The linearity of the method was obtained by preparing suitable aliquots of standard stock solution in different concentrations for DAR (200-1200 µg/ml), COB (37.5-225 µg/ml), EMT (50-300 µg/ml) and TEN (2.5-15 µg/ml). The calibration curve was obtained by plotting the peak area against the respective concentration.

Accuracy: Accuracy of the method was studied at three levels (50%, 100%, 150%) of increasing concentration by adding 1 ml of standard stock solution to 0.5 ml, 1.0 ml, and 1.5 ml sample stock solutions taken individually, injected into system triplicate and % mean recovery was calculated.

Precision:

Method Precision (Repeatability): The method precision/ repeatability was determined by injecting six standard working solutions. The areas of all the injections were taken, and standard deviations, % Relative standard deviation (RSD) were calculated.

Intermediate Precision: The intermediate precision was determined by injecting six standard working solutions and on consecutive days by different personnel. The areas of all the injections were taken, and standard deviations, %RSD was calculated.

LOD & LOQ: Limit of detection (LOD) and Limit of quantification (LOQ) were determined by the calibration curve method. Six sets of Solutions were made from the Standard solution and injected into the system.

$$LOD = 3.3\ SD / S$$

$$LOQ = 10\ SD/ S$$

Where, SD = Standard Deviation of y-intercept of calibration curve**,** S = Mean slope of calibration curves.

Robustness: To study the robustness of the proposed method small deliberate changes in method like flow rate, mobile phase ratio and temperature are made with conditions such as decreasing the flow rate to 0.65 ml/min (FM), increasing the flow rate to 0.85 ml/min (FP), changing mobile phase ratio of OPA: ACN to 60:40 (MM), 50:50 (MP), decreasing the temperature to 25 °C (TM) and increasing the temperature to 35 °C (TP) and the samples were injected into the system and %RSD was calculated.

Forced Degradation Studies: Forced degradation studies were performed according to the ICH Guidelines [4] in different conditions like Acidic, Alkaline, Oxidizing, thermal, Photostability, and Neutral.

For acidic and basic degradation conditions, 1ml of 2N Hydrochloric acid and 1ml of 2N Sodium hydroxide was added respectively and refluxed at 60 °C for 30 min and later neutralized.

For oxidative degradation 1 ml of 20% Hydrogen Peroxide is added and refluxed at 60 °C for 30 min. For thermal degradation, the standard solution was kept at 105 °C for 6 h.

Photostability degradation was carried out by exposing the drug to UV Light for a day. Neutral Degradation Studies were carried out by refluxing the drug in water for 6hrs at 60 °C.

The product obtained after each degradative condition was diluted with a solvent to obtain drug concentration as DAR 800 µg/ml, COB 150 µg/ml, EMT 200 µg/ml, and TEN 10 µg/ml for and the resultant was injected into the HPLC system to assess the stability of sample [10, 14].

RESULTS AND DISCUSSION:

Method Development: The standard stock solution was run through a series of mobile phase combinations with interchanging the columns and method parameters from time to time where no proper separation of compounds took place.

A clear, distinct separation of drugs and symmetric peak shape was observed with 0.1% OPA: ACN in the ratio 55:45 as mobile phase with Agilent C18 (150 × 4.6 mm, 5 m) column at 245 nm wavelength of PDA detector with a constant flow rate of 0.75 ml/min and the column temperature kept at 30 °C. The elution was achieved at 3.98 for DAR, 3.15 for COB, 2.21 for EMT, and 2.62 for TEN **Fig. 2** with good resolution, theoretical plates, and tailing factor.

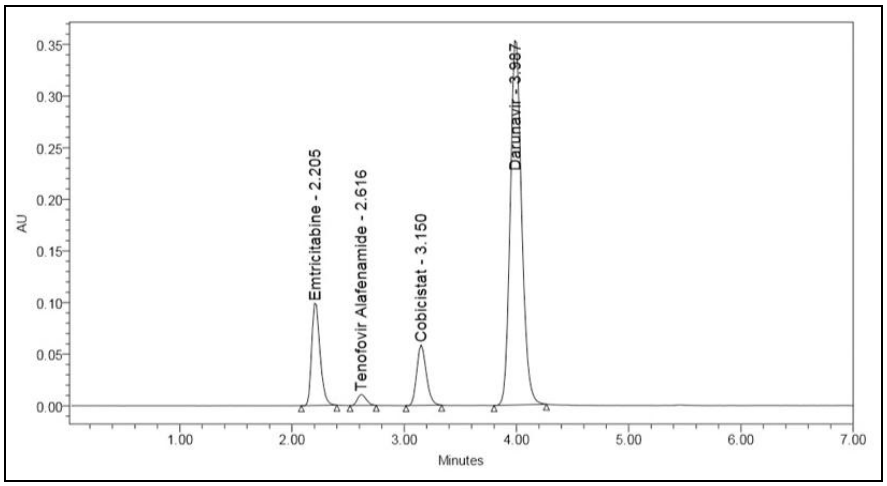


FIG. 2: CHROMATOGRAM WITH OPTIMISED CONDITIONS

Method Validation: [11-14] System Suitability Parameters System suitability parameters are found within acceptable limits. The summary of the parameters was tabulated in **Table 1**.

TABLE 1: SUMMARY OF SYSTEM SUITABILITY PARAMETERS

Parameters	DAR	COB	EMT	TEN	Acceptable Limits
R_t (min)	3.98	3.15	2.21	2.62	≥ 2
TP	7278±200	6248±136	4137±150	5076±200	≥ 2000
T	1.15±0.2	1.16±0.2	1.27±0.3	1.14±0.2	≤ 2

Specificity: Specificity of the method was done by injecting blank and placebo, which showed no interference at retention times of the drugs **Table 2**.

Linearity: A good linearity range was obtained for the method proposed at different concentrations ranging 200-1200 µg/ml for DAR, 37.5-225 µg/ml for COB, 50-300 µg/ml for EMT and 2.5-15 µg/ml for TEN with regression coefficients of 0.9992, 0.9997, 0.9997 and 0.9994 **Fig. 3 Table 2**.

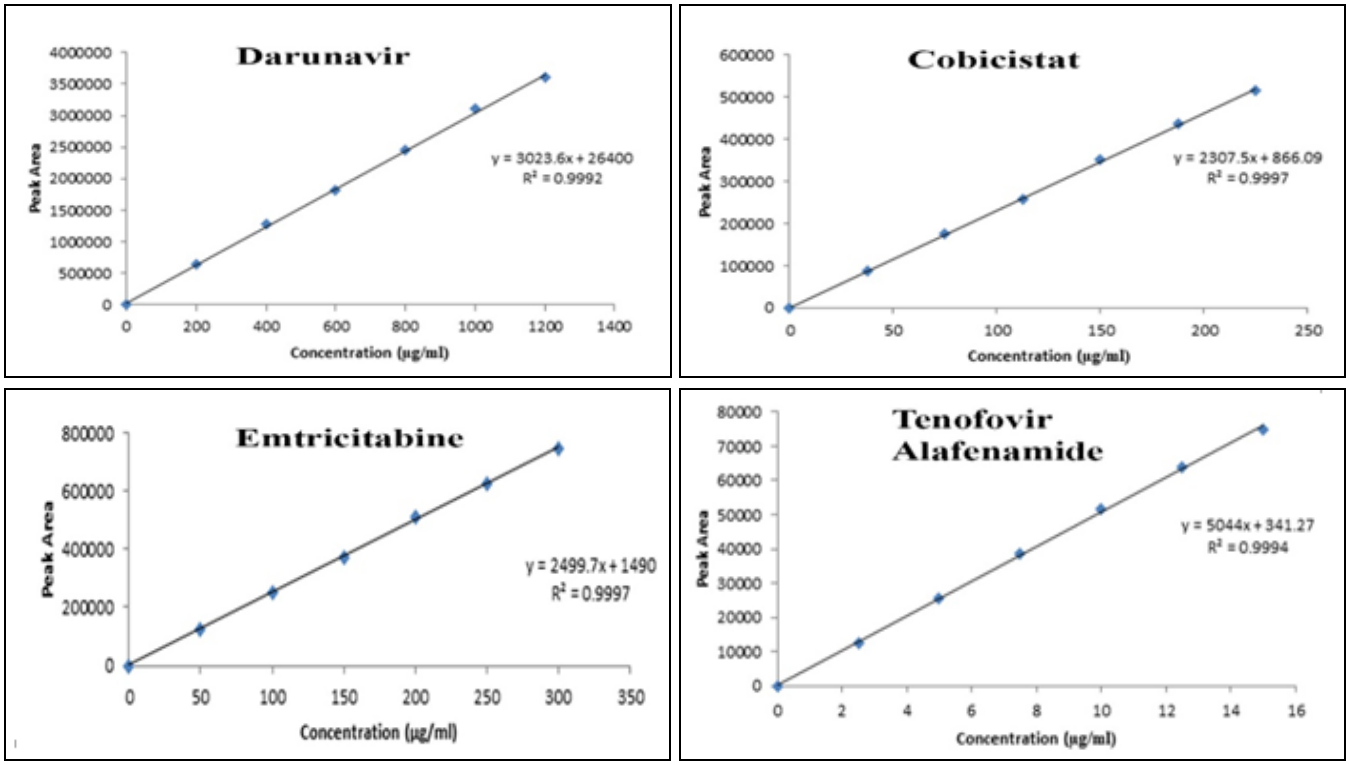
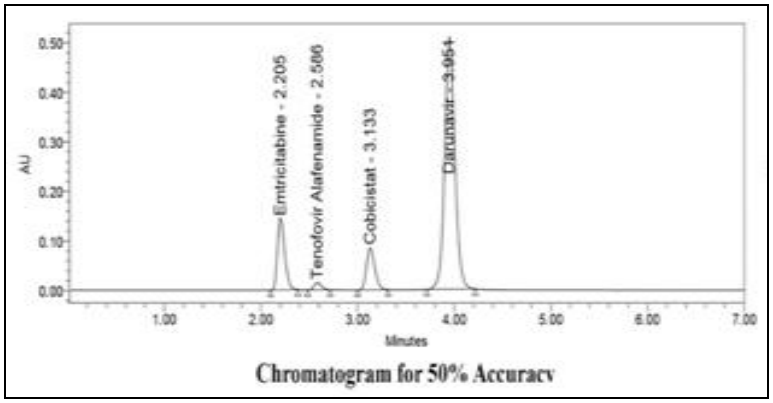
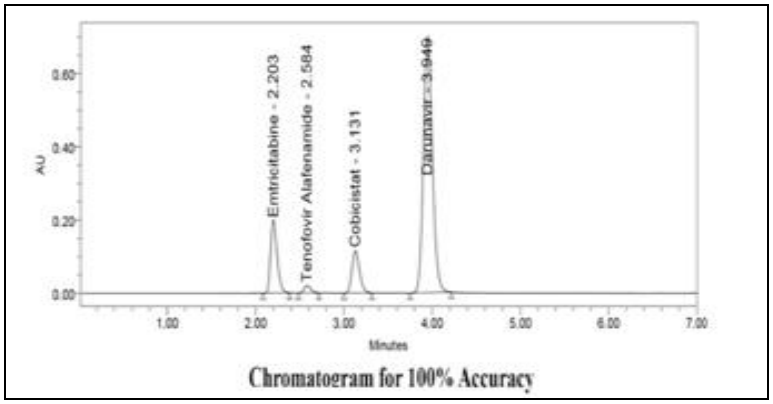


FIG. 3: CALIBRATION CURVE OF DARUNAVIR, COBICISTAT, EMTRICITABINE AND TENOFOVIR ALAFENAMIDE

Precision: The results of both method precision and intermediate precision showed satisfactorily, and % RSD limits were less than 2% **Table 2.**

Accuracy: The accuracy of the method was determined by recovery studies and the Percentage recovery was calculated. The recovery of the drugs was found to be within acceptable limits of 98 - 102% w/v. The Mean recovery of drugs was given in **Table 2.**





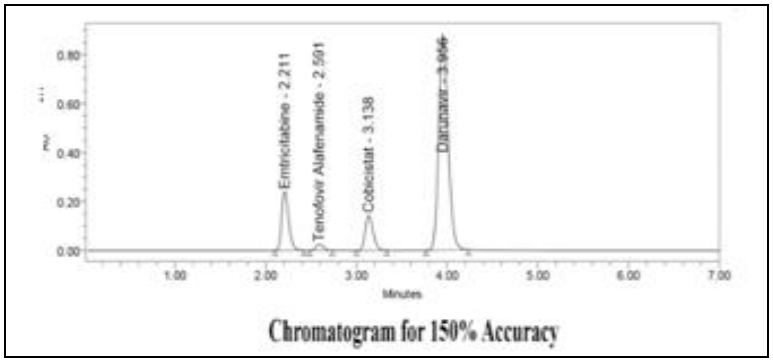


FIG. 4: ACCURACY CHROMATOGRAMS

Assay: The assay of in-house formulation was successfully carried out by the proposed method, and the mean assay of the drugs was calculated in **Table 2,** which are found to be within limits of 90-110%.

LOD and LOQ: The obtained results of LOD & LOQ were presented in **Table 2** are found to be within the acceptable limits.

Robustness: The % RSD of different conditions studied for robustness are given in **Table 3**. The results were obtained satisfactorily as the % RSD is less than 2%.

Forced Degradation Studies: The proposed method was successful in studying the forced degradation of the drugs **Fig. 5**.

Results show that this method was able to separate the drugs successfully and showed that the combination is sensitive to oxidation degradation where two extra peaks are noticed, and it was stable in all the other degradation conditions.

% drug degraded was calculated in **Table 3** and is within limits of 5-20% [1, 16].

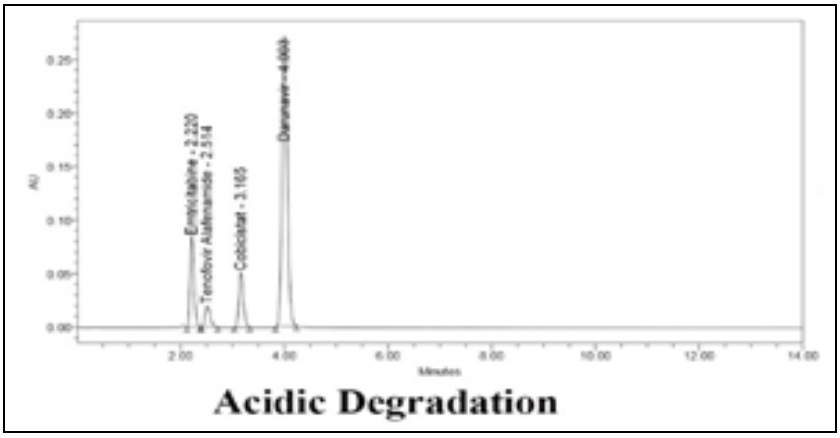
TABLE 2: SUMMARY OF VALIDATION PARAMETERS

Parameters		DAR	COB	EMT	TEN	Acceptable Limits
Specificity		Specific	Specific	Specific	Specific	No interference of any peak
Linearity(µg/ml)		200-1200	37.5-225	50-300	2.5-15	-
Regression equation (Y=mx+c)		y = 3023.x +26400	y = 2307.x + 866.0	y = 2499.x + 1490	y = 5044x + 341.2	-
Regression coefficient (r^2)		0.9992	0.9997	0.9997	0.9994	< 1
Range		1000	187.5	250	12.5	-
Accuracy *(% Mean recovery)		100.2	99.98	99.69	99.90	98 – 102%
Assay *(% Mean assay)		99.87	99.41	99.74	100.00	90 – 110%
System Precision* (%RSD)		0.5	0.5	0.5	0.8	
Method Precision* (%RSD)		0.5	0.2	0.7	0.8	> 2%
LOD(µg/ml)		6.88	1.31	0.33	0.02	> 3 µg/ml
LOQ (µg/ml)		20.84	3.96	1.00	0.06	> 10 µg/ml
Robustness (% RSD)	FM	0.7	0.7	0.7	0.4	
	FP	0.8	0.7	0.3	0.4	
	MM	0.5	0.7	0.3	0.5	> 2%
	MP	0.9	0.7	0.4	0.9	
	TM	0.5	0.9	0.5	0.5	
	TP	0.2	0.9	1.1	0.6	

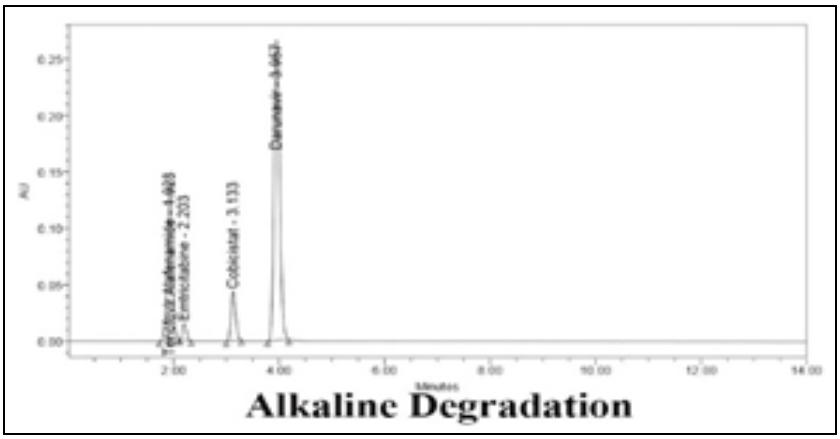
Six determinations.

TABLE 3: PERCENTAGE DRUG DEGRADED IN VARIOUS FORCED DEGRADATION STUDIES

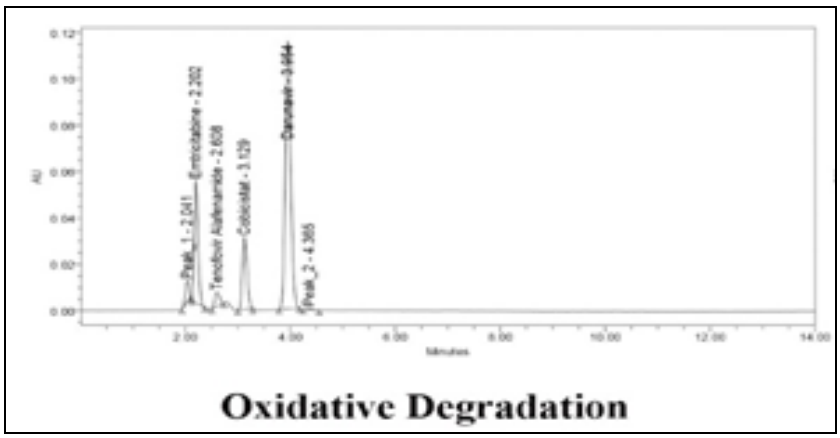
S. no.	Degradation Condition	% Drug Degraded			
		DAR	COB	EMT	TEN
1	Acidic	4.29	6.18	6.28	8.24
2	Alkaline	3.03	5.61	5.32	5.93
3	Oxidizing	8.61	8.78	8.53	9.24
4	Thermal	2.57	2.77	2.77	2.50
5	Photostability	1.81	1.96	1.49	1.22
6	Neutral	0.52	0.58	0.80	0.81



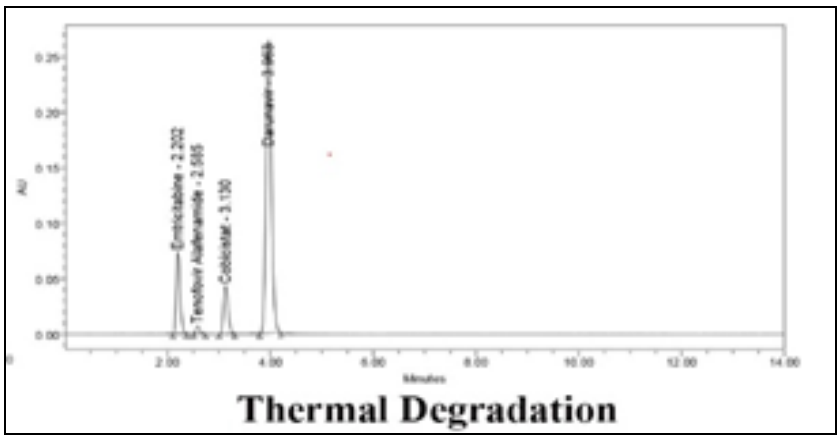
Acidic Degradation



Alkaline Degradation



Oxidative Degradation



Thermal Degradation

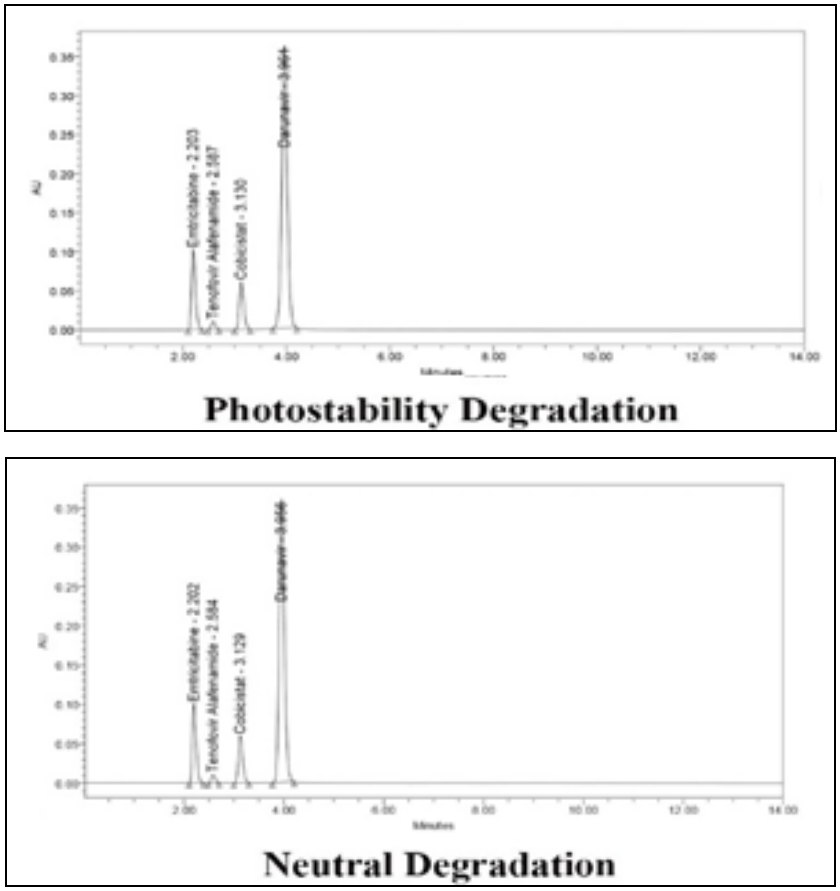


FIG. 5: FORCED DEGRADATION CHROMATOGRAMS OF DARUNAVIR, COBICISTAT, EMTRICITABINE AND TENOFOVIR ALAFENAMIDE

CONCLUSION: In this study, a simple, sensitive, accurate, and precise RP-HPLC method was developed for the simultaneous estimation of DAR, COB, EMT, and TEN in bulk drug and pharmaceutical formulation.

The method was successfully validated according to ICH guidelines with all the parameters found within the acceptable limits. The method was successful in the assay of the in-house formulation and showed good recovery.

The method was good enough to separate the peaks from the degradation products (produced during forced degradation studies). Hence, the proposed method can be conveniently used for routine quality control analysis in industries.

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CONFLICTS OF INTEREST: The author declares there were no conflicts of interest.

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