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HPTLC method development for bictegravir, emtricitabine and tenofovir in bulk and dosage form in presence of its degradation products

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ABSTRACT

Simple, accurate and stability-indicating high-performance thin- layer chromatographic method for densitometric determination of Bictegravir, Emtricitabine and Tenofovir in bulk and in dosage form was developed and validated as per the ICH guidelines . TLC aluminium plates precoated with silica gel 60F 254 - (Merck, Germany) as the stationary phase were used in the method development. Toluene: Ethyl acetate: methanol: acetic acid (4.0:3.0:3.0:0.2 v/v/v) was used as the development solvent. Bictegravir, Emtricitabine, and Tenofovir were densitometrically measured in absorbance mode at 272 nm. The system was found to give very well resolved spot for Bictegravir, Emtricitabine and Tenofovir at Rf value of 0.26, 0.42 and 0.63 respectively.The calibration curve of the drug 200 to 1600 ng/spot for BIC, 100-800 ng/spot for TAF and concentration range 500 to 4000 ng/spot for ETB was observed. The correlation of coefficient was found to be 0.998 for BIC, 0.9994 for ETB and 0.999 for TAF. The regression line equation for Bictegravir, Emtricitabine and Tenofovir was found to be y = 2.785 x + 2415, y = 1.789 x + 2743 and y = 1.644 x + 2843 respectively. The %RSD less than two indicates method is precise and accurate. In HPTLC, the degradation products were well separated from the pure drug, with significantly different retention factors. The method was demonstrated to be a stability indicating method and can be used in practice to determine shelf life.. Statistical analysis demonstrated that the method is simple, accurate, and selective for estimating Bictegravir, Emtricitabine, and Tenofovir drug concentrations. When subjected to different stress conditions, the proposed method effectively separates the drug from its degradation products and can be used as a stability indicating method.

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KEYWORDS: Bictegravir, Emtricitabine, HPTLC, Stability-indicating, Tenofovir, Validation

1. INTRODUCTION

Bictegravir (BIC) is an integrase strand transfer inhibitor (INSTI). Bictegravir is different from other INSTIs because it contains a bridged bicyclic ring and a distinct benzyl tail with a 2,4,6-trifluorobenzyl group. Chemically it is known as (1S,11R,13R)-5-Hydroxy-3,6-dioxo-N- (2,4,6-trifluorobenzyl)-12-oxa-2,9-diazatetracyclo[11.2.1.02,11.04,9]hexadeca-4,7-diene-7-carboxamide. Bictegravir was found to be less drug resistant than other drugs in the same class.[1] Emtricitabine(ETB) is a nucleoside reverse transcriptase inhibitor used for the treatment of HIV infection in adults. Chemically Emtricitabine is 4-amino5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan5-yl]- pyrimidin-2-one.[2]

Tenofovir Alafenamide (TAF) which is a prodrug of tenofovir act as a nucleotide reverse transcriptase inhibitor (NRTI). In all prodrugs for tenofovir TAF is more efficient at refining HIV-1 therapy. Chemically it is known as propan-2-yl-(2S)-2-{[(S)({[(2R)-1-(6-amino-9H-purin9-yl)propan-2-yl]oxy} methyl) (phenoxy)phosphoryl] amino} propanoate. It converts intracellularly to TFV diphosphate, which is a metabolite in HIV target cells.[3] Tenofovir Alafenamide (TAF) is an oral prodrug of tenofovir, a nucleotide (nucleoside monophosphate) analogue with activity against retroviruses.Tenofovir and emtricitabine are antiviral drugs and act as reverse transcriptase inhibitors.[4]

A review of the literature revealed a few spectrophotometric[5-7] and chromatographic[8-24] methods for estimating BIC,ETB, and TAF in pharmaceutical formulations and biological fluids. However, no high-performance thin-layer chromatography (HPTLC) determination of BIC,ETB, and TAF in pharmaceutical dosage forms has been reported in the literature to our knowledge. Analytical test procedures for stability samples should be fully validated, and the assays should be stability indicating, according to ICH guidelines (Q1A). [25-27] The current paper describes a reliable, accurate, and selective HPTLC method for determining BIC, ETB, and TAF using HPTLC densitometry. As a result, an attempt has been made to develop an accurate, specific, and reproducible method for determining BIC, ETB, and TAF in the presence of degradation product for analysis during pharmaceutical dosage form stability studies.

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2. METHODS

2.1 Reagents and Chemicals

Cipla Ltd., Mumbai, provided active pharmaceutical ingredient samples of Emtricitabine, Bictegravir, and Tenofovir Alafenamide. The solvents and chemicals used were all of HPLC grade. The tablet pharmaceutical dosage of this drug combination was purchased from a local pharmacy..

2.2 Instrumentation

Aluminium plates precoated with silica gel $60F_{254}$ (20 × 10 cm, 250 μm thickness; Merck, Darmstadt, Germany) were used in the study. The equipment used was Linomat 5 applicator (Camag, Switzerland) , twin trough chamber (20 × 10 cm; Camag, Switzerland), TLC scanner IV (Camag, Switzerland), win CATS version 1.4.6 software (Camag, Switzerland), Micro syringe (Linomat syringe 659.0014, Hamilton–Bonaduz Schweiz, Camag, Switzerland), UV cabinet (Camag, Switzerland).

2.3 Preparation of standard solutions

A standard stock solution of BIC, ETB, and TAF was prepared in which precisely 10 mg of drug was weighed and transferred to a 100-mL volumetric flask separately and dissolved in 30 mL methanol. Methanol was used to dilute the solution to the desired concentration of 100 ug/mL. To obtain a working standard solution for BIC,ETB, and TAF, this stock solution was diluted appropriately with the same solvent.

2.4 Chromatographic development and scanning

Using a Linomat V applicator and a micro syringe with a 100 L capacity, the samples were spotted as bands of 6 mm width on an aluminium silica gel-precoated plate 60 F254 (20 cm 10 cm) with a thickness of 250 m (E MERCK, Darmstadt, Germany) (Camag, Switzerland). The plates were activated at 110° C for 5 minutes after being prewashed with methanol before to use in chromatography. Application rate of 150 nl/sec was used, and the distance between the two bands was 15.4 mm. The slit size was maintained at 6 mm 0.45 mm. Toluene, ethyl acetate, methanol, and acetic acid were utilised as the mobile phase in the development (4.0: 3.0: 3.0: 0.2 v/v/v/v). The development was done using the ascending development approach in a twin trough glass chamber. For the mobile phase, the ideal chamber saturation time was 25 minutes at a temperature of 250C 2 and a relative humidity of 60% 5%. The TLC plates were taken out of the chamber and allowed to air dry after the chromatogram run, which lasted up to 8 cm. On a Camag TLC Scanner 3 equipped with winCATS software version 1.3.0 and scanning at 272 nm,

densitometric scanning was carried out. Peak area and a linear regression equation were used for evaluation.

2.5 Preparation of Sample Solution:

Twenty tablets containing 50 mg BIC, 200 mg ETB, and 25 mg TAF were weighed and their average weight was calculated for tablet dosage form analysis. The tablets were finely powdered, and the powder equivalent to one tablet was precisely weighed and transferred to a 100 ml volumetric flask containing 30 ml methanol. The solution was sonicated for 30 min before being diluted with methanol to mark. The final solution was filtered through a 0.45 m filter (Millifilter, MA). 0.4µL of the aforementioned solution was applied to a TLC plate. The development and scanning were carried out in the manner described in section 2.2. The analysis was carried out six times. The Rf values for BIC, ETB, and TAF were discovered to be at Rf 0.26, 0.42, and 0.63, respectively. When scanned at 272 nm, the drug exhibits sharp and well-defined peaks. The amount of drug recovered was calculated using slope and intercept values from the calibration graph.

2.6 Method validation

The method was validated in accordance with the ICH guidelines. The parameters ICH Q2A (R1) 2005) used for dosage form assays such as linearity, precision (repeatability, intraday, interday), accuracy, specificity quantification limit, detection limit, robustness and ruggedness.

2.6.1 Calibration curve and Linearity:

The method's linearity was determined by applying eight different concentrations of the drug to the TLC plates three times each. The plate was then developed with the previously described mobile phase, and a calibration curve was created by plotting the peak areas against the corresponding concentrations.

2.6.2. Precision

The repeatability, intra-day, and inter-day precision of the method were evaluated. The repeatability of the sample solution was determined by spotting the drug solution six times on a TLC at 300 ng/spot for TAF, 600 ng/spot for BIC, and 2400 ng/spot for ETB, followed by plate development and scanning. The intra-day precision was determined by analyzing standard drug solutions three times on the same day within the calibration range of individual drugs. Inter-day precision was determined by analyzing drug solutions over a week on three different days within the calibration range.

2.6.3. Accuracy

Recovery studies by standard addition were used to test the accuracy of the proposed method. The formulation solution (BIC,EMB, and TAF combination tablets) was supplemented with known amounts of BIC, EMB, and TAF standard powder corresponding to 50, 100, and 150 percent of the label claim, and the absolute recovery was calculated by comparing the peak areas obtained from standard BIC,EMB, and TAF solution with the peak areas of samples of different concentrations.

2.6.4 Specificity

The method's specificity was determined by analysing standard drug and sample. As shown in, the mobile phase resolved all of the drugs very efficiently at various Rf values (**Figure 1.**). By comparing the Rf and spectra of the spot to that of the standard, the spot for BIC,ETB, and TAF was confirmed. Peak purity of BIC, TDF, and ETB was determined by comparing drug and sample spectra at the peak start (S), peak apex (M), and peak end (E) positions of the spot..

2.6.5. Limit of detection and limit of quantification

Concentrations in the calibration curve's lower linear range were used to determine the detection and quantification limits. The amount of drugs used versus the average response (peak area) was plotted, and the regression equation was determined. Response standard deviations (S.D.) were computed. The average of standard deviations was calculated from this data (A.S.D.). LOD was calculated using the formula (3.3x A.S.D.)/b, and LOQ was calculated using the formula (10x A.S.D.)/b, where "b" corresponds to the slope obtained in the method's linearity study.

2.6.6. Robustness

The robustness of the system was evaluated by comparing the results obtained for various parameters such as change in mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography, and time from chromatography to spotting. On a TLC plate, the solution was applied in six replicates at concentrations of 600 ng/spot for BIC, 300 ng/spot for TAF, and 2400 ng/spot for ETB. Seven parameters were studied in this study, and their effects on the results were examined.

2.6.7. Ruggedness

The proposed method was evaluated by two different analysts in the ruggedness study.

Forced degradation of BIC, ETB and TAF

Acid and base induced degradation

The 10 mg of BIC, ETB, and TAF were transferred to a 10 ml volumetric flask. It was dissolved in a methanolic solution containing 2 M HCl and 2 M NaOH respectively. In order to rule out any potential degradation caused by light, these solutions were maintained at room temperature in the dark for 8 hours. The 1 ml of the aforementioned solutions were taken, neutralised, and diluted with methanol to a final concentration of 10 ml. The 10 l were applied in triplicates (i.e., 1000 ng/spot) to TLC plates. The chromatograms were performed in accordance with section 2.2.

Hydrogen peroxide – induced degradation

The 10 mg of BIC, ETB and TAF were weighed and transferred to 10 ml volumetric flask and 10%, v/v methanolic solution of hydrogen peroxide was added. The volume was made upto the mark. The solutions were maintained at room temperature for 8 hours in the dark to prevent any potential degradation from light. The 1 ml of above solution were taken and diluted up to 10 ml with methanol. The 10 µl were applied on TLC plates in triplicates (i.e. 1000 ng/spot). The chromatograms were run as described in section 2.2.

Dry heat degradation products

BIC, ETB, and TAF were stored separately in petri dishes at 55^0 C for 3 hours in an oven. BIC 10 mg, ETB 10 mg, and TAF 10 mg were placed in a volumetric flask with a volume of 10 ml. the volume was made upto the mark with Methanol. The 10 µl were applied in triplicate on TLC plates (1000 ng/spot) and the chromatogram was run as described in section 2.2.

Light heat degradation products

10 mg of BIC, ETB, and TAF were weighed and transferred to a volumetric flask of 10 ml. The drugs were dissolved in 10 mL of methanol separately. The solutions were exposed to sunlight for 8 hours. 1 mL of each of the above solutions was taken and diluted with methanol to a volume of 10 mL. The solutions obtained were applied in triplicate on a TLC plate with a concentration of 1000 ng / spot. The chromatograms were obtained in the manner described in section 2.2.

3.RESULTS AND DISCUSSION

Optimization of HPTLC method

Initially, BIC, ETB, and TAF drugs were tried together with toluene: methanol in the ratio of 4: 2 (v/v/v). The spots did not fully develop, and tailing was seen. Then, toluene: ethyl acetate: methanol in the ratio of 6: 4: 3 (v/v/v) was tried. Spots that had developed were scattered. Finally, a mobile phase with the composition (4.0:3.0:3.0:0.2 v/v/v) of toluene, ethyl acetate,

methanol, and acetic acid produced prominent peaks. The peaks on the plate were determined to be symmetrical in form and no tailing was seen when it was examined at 272 nm. The chamber was saturated for 25 minutes. To get clearly defined spots, the plates were activated at 110° C for 5 minutes.

Linearity

The linearity was observed for BIC concentrations ranging from 200 to 1600 ng/spot, TAF concentrations ranging from 100 to 800 ng/spot, and ETB concentrations ranging from 500 to 4000 ng/spot. The coefficients of correlation for BIC, ETB, and TAF were found to be 0.996, 0.9960, and 0.999, respectively. For BIC, ETB, and TAF regression line equations were found to be $y = 2.785 x + 2415$, $y = 1.789 x + 2743$, and $y = 1.644 x + 2843$, respectively..

Precision

The repeatability and intermediate precision (intraday and interday) were expressed as a percentage of RSD. Because of the low percentage RSD value, the proposed method provides an acceptable intraday and interday variation. The results are as shown in **Table 1**.

Accuracy

Recovery studies were carried out to validate the developed method using the standard addition method, which involved addition of standard drug solution to pre-analyzed sample solution. The mixture was analyzed after a known amount of pure drug was added to the previously analysed solution containing pharmaceutical formulation. The recoveries have been computed. The low percentage RSD value indicated that no interference was caused by the excipients used in the formulation. As a result, the method's precision was confirmed (**Table 2**).

Sensitivity

Sensitivity of the drugs was analyzed by Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limits of detection and quantitation were found to be 67.34 and 276.19 for BIC, 41.24 and 785.39 for ETB and 45.14 and 668.49 for TAF, indicating that the method has good sensitivity..

Analysis of marketed formulation

The proposed technique was applied to examine the commercial formulation Bictarvy. The chromatogram of the tablet sample revealed peaks at Rf values of 0.26, 0.42, and 0.63 for BIC, ETB, and TAF, respectively, demonstrating that the excipients used in the tablet formulation do

not interfere with the results. Peak areas of the sample and the standard were compared to determine the content of BIC, ETB, and TAF.

Robustness

The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low %R.S.D. values indicated method is robust. Results are shown in **Table 3**.

Ruggedness

Two different analysts evaluated the robustness of the proposed method. The results for BIC,ETB, and TAF were 99.45 %, 99.65 %, 99.61 %, 99.28 %, and 100.23, 100.15 %, respectively..

Stability- indicating property

The samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure BIC, ETB and TAF and some additional peaks at different Rf values as shown in **Figure 2,Figure 3 and Figure 4.**

The spots of degraded product were well resolved from the drug spot. The number of degradation product with their Rf values, content of BIC,ETB and TAF remained, and percentage recovery were calculated and listed in **Table 4.**

The summary of validation parameters are summarized in **Table 5**

4.CONCLUSION

The suggested HPTLC method offers a straightforward, precise, and repeatable quantitative study for the determination of BIC, ETB, and TAF in bulk drugs. ICH guidelines were followed in the method's validation. The method can be used as a stability indicating research because it was able to isolate the drugs from their degradation products in an efficient manner.

Abbreviations

NaOH: Sodium hydroxide; HCl: Hydrogen chloride; RP-H PLC: Reversed-phase high performance liquid chromatography; HPTLC: High-performance thin-layer chromatography; ICH: International Conference on Harmonization; LOD: Limit of detection; LOQ: Limit of quantitation; BIC: Bictegravir; ETB: Emtricitabine; TAF: Tenofovir Alafenamide; SD: Standard deviation;RSD: Relative standard deviation; RF: Retention factor;UV: Ultraviolet.

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Authors' contributions

CJB contributed for experimental work and manuscript preparation. SNH contributed in hypothesis and finalization of manuscript. Both authors read and approved the final manuscript.

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Table 1. Precision Study

Drugs	Concentration (ng/spot)	Intra-day		Inter-day	
		% Amount found ± SD	(% RSD)	% Amount found ± SD	(% RSD)
BIC	600	98.56±1.26	0.56	98.56±1.36	0.75
	800	99.56±1.45	1.42	99.56±1.25	1.12
	1000	99.45±1.23	0.28	99.45±1.23	0.62
ETB	2400	99.49±1.12	0.78	99.49±1.02	0.48
	3200	99.67±1.14	1.32	99.67±1.04	1.52
	4000	99.23±1.27	0.36	99.23±1.27	0.63
TAF	300	98.98±1.42	0.65	98.98±1.32	0.55
	400	99.16±1.63	0.54	99.16±1.33	0.48
	500	99.67±1.49	1.05	99.67±1.59	1.56

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Table 2: Accuracy study

Drug	% level	Initial amount(ng/band)	Amount added(ng/band)	% recovery
BIC	50	600	300	98.54%
	100	600	600	99.12%
	150	600	900	98.78%
ETB	50	2400	1200	99.15%
	100	2400	2400	98.76%
	150	2400	3600	99.41%
TAF	50	300	150	99.23%
	100	300	300	99.11%
	150	300	450	99.54%

Table 3: Robustness study

Condition	Parameter	BIC[%RSD]	ETB[%RSD]	TAF[%RSD]
Mobile phase Composition	+0.1 ml	1.12	1.23	1.29
	-0.1 ml	1.41	1.47	1.25
Amount of mobile phase	+5%	1.22	1.42	1.56
	-5%	1.49	1.63	1.59
Duration of saturation	30 min	0.89	1.25	1.23
Development distance	75mm	1.22	1.52	1.71
	80mm	1.26	1.56	1.56
Relative humidity	50^0C	0.96	0.89	0.78
	60^0C	1.17	1.11	1.12
Time from spotting to development	+10 min	0.88	1.14	0.96
	-10 min	1.23	1.25	1.56
Time for development To scanning	+10 min	1.23	1.45	1.12
	-10 min	1.89	1.78	1.42

Table 4: Stability study

Sample Exposure condition	Number of degradation products (Rf values)			Drug remained (600 ng/spot)			Recovery (%)		
	BIC	**ETB**	**TAF**	**BIC**	**ETB**	**TAF**	**BIC**	**ETB**	**TAF**
2M HCl, 8h, RT	0.08,0.11	0.22,0.29	0.21,0.35	523	514	541	87.16	85.66	90.16
2M NaOH, 8h, RT	0.05,0.1	0.31,0.36	0.34,0.52	517	529	526	86.16	88.16	87.66
10%H_2O_2,8h, RT	0.12	0.32	0.49	549	542	559	91.5	90.33	93.16
Heat, 3H, 55^0 C	0.07	0.29	0.34	562	588	563	93.66	98	93.83
Photo, 8 h	0.13	0.30	0.39	544	575	571	90.66	95.83	95.16

Table 5:Validation Parameter

Parameter	**BIC**	**ETB**	**TAF**
Linearity range [ug/ml]	200-1600ng/spot	500-4000ng/spot	100-800ng/spot
Regression equation [Y = mX + C]	y = 2.785 x + 2415	y = 1.789 x + 2743	y = 1.644 x + 2843
Correlation coefficient	0.998	0.9994	0.999
Limit of detection [µg]	67.34	41.24	45.14
Limit of quantitation [µg]	276.19	785.39	668.49
% Recovery [n = 3]	98.54-99.12	98.76-99.15	99.11-99.54
Ruggedness [%]			
Analyst I [n = 3]	99.45	99.61	100.23
Analyst II [n = 3]	99.65	99.28	100.15
Precision [% RSD]			
Repeatability [n = 6]	1.41	0.97	1.05

Inter-day [n = 3]	0.28-1.42	0.36-1.32	0.54-1.05
Intra-day [n = 3]	0.62-1.12	0.48-1.52	0.48-1.56
Robustness	Robust	Robust	Robust

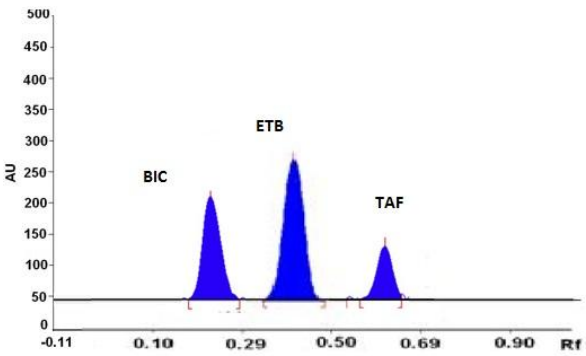

Figure 1. Densitogram of BIC, ETB and TAF

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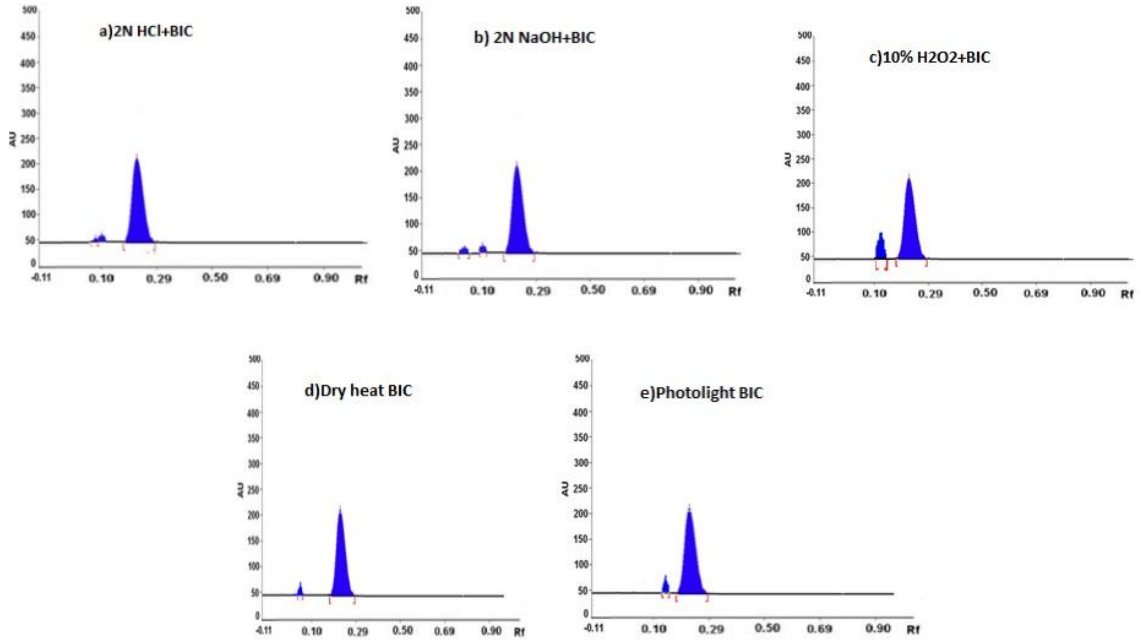

Figure 2 . Forced degradation of BIC :a) 1N HCl + BIC ;b) 1N NaOH + BIC ; c) 10 % H_2O_2 + BIC ; d) Dry heat BIC; e) Light heat BIC

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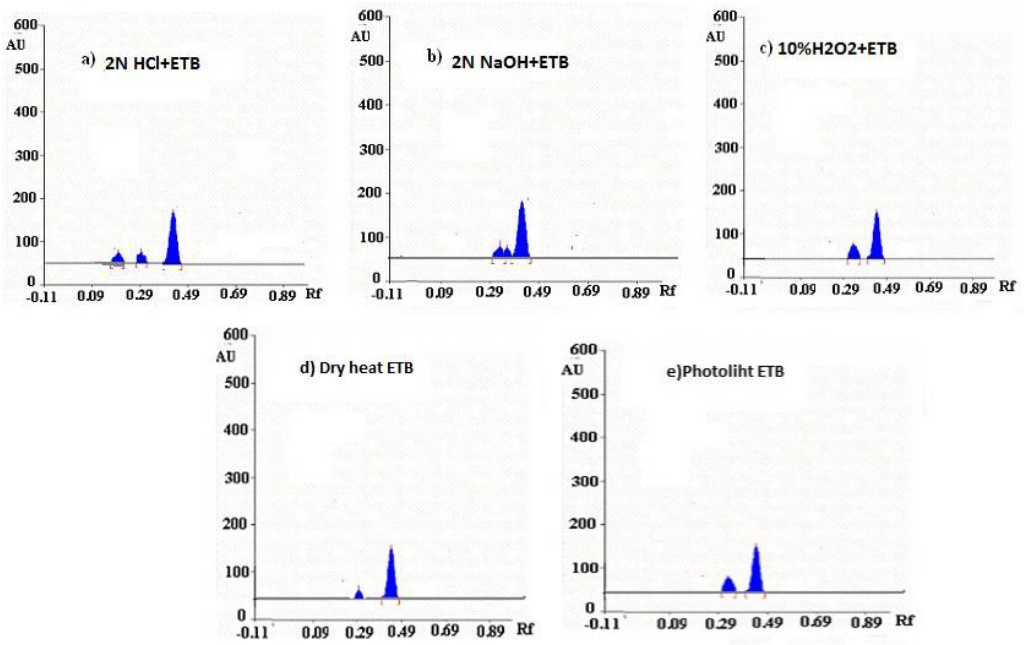

Figure 3. Forced degradation of ETB :a) 1N HCl + ETB ;b) 1N NaOH + ETB ; c) 10 % H_2O_2 + ETB ; d) Dry heat ETB; e) Light heat ETB

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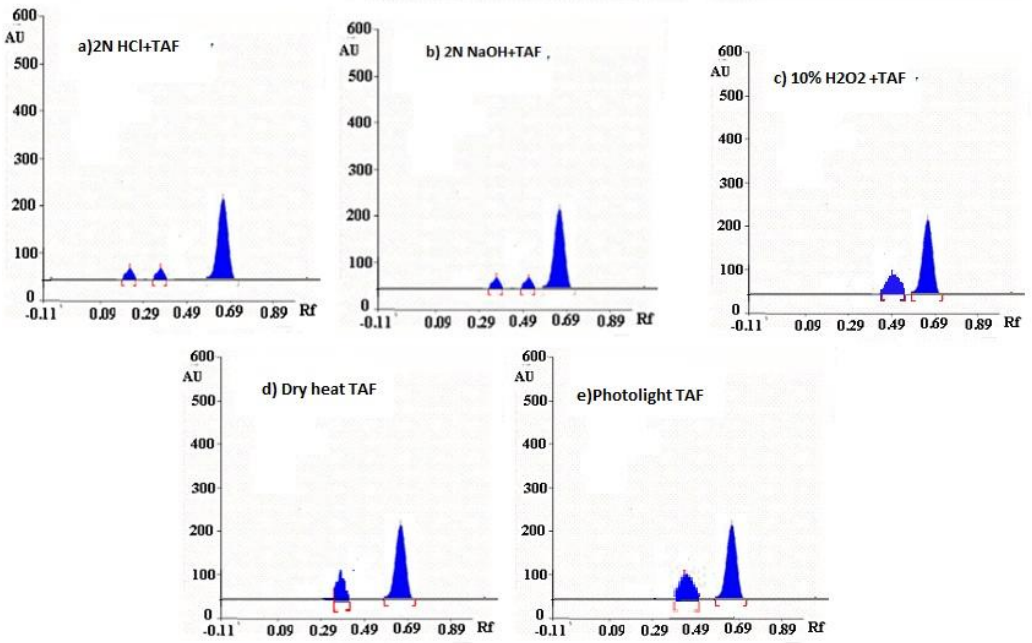

Figure 4. Forced degradation of TAF :a) 2N HCl + TAF ;b) 2N NaOH + TAF ; c) 10 % H_2O_2 + TAF ; d) Dry heat TAF; e) Light heat TAF

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Figure Captions

Figure 1. Densitogram of BIC, ETB and TAF

Figure 2 . Forced degradation of BIC :a) 1N HCl + BIC ;b) 1N NaOH + BIC ; c) 10 % H_2O_2 + BIC ; d) Dry heat BIC; e) Light heat BIC

Figure 3. Forced degradation of ETB :a) 1N HCl + ETB ;b) 1N NaOH + ETB ; c) 10 % H_2O_2 + ETB ; d) Dry heat ETB; e) Light heat ETB

Figure 4. Forced degradation of TAF :a) 2N HCl + TAF ;b) 2N NaOH + TAF ; c) 10 % H_2O_2 + TAF ; d) Dry heat TAF; e) Light heat TAF