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Validated stability indicating HPTLC methods for the determination of entecavir in bulk and dosage form

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ABSTRACT

The objective of the present work is to develop a simple, precise, accurate, validated stability indicating HPTLC method for the determination of Entecavir in bulk and tablet dosage form. The HPTLC method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of Chloroform:Toluene: methanol: ammonia (7:2:1:0.2v/v) and then scanned. The system was found to give compact spot for Entecavir (R_f value of 0.51 ± 0.02). The linearity was found to be in the concentration range 300-1800 ng/spot. The reliability and analytical performance of the proposed methods, including linearity, range, precision, accuracy, detection and quantitation limits, were statistically validated. When entecavir was subjected to different stress conditions; the proposed methods could effectively separate the drug from its degradation products, and were thus considered as good stability-indicating procedures. It is concluded that this method can be applied for routine quality control of Entecavir in dosage forms as well as in bulk drug.

Keywords: Entecavir, High pressure thin layer chromatography, Method development and validation, Stability indicating.

INTRODUCTION

Entecavir (ETV) is a nucleoside analog [1] with the chemical name 2-Amino-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-6,9-dihydro-3H-purin-6-one (Fig.1) [2]. Entecavir is a guanosine nucleoside analogue with selective activity against hepatitis B virus (HBV). It is designed to

selectively inhibit the Hepatitis B virus, blocking all three steps in the replication process [3, 4].

Literature survey revealed, few analytical methods which include UV-spectrophotometric methods [5-7] liquid chromatographic methods [8, 9] have been reported for estimation of ETV. However, to our knowledge, no information related to the stability-indicating UV and high-performance thin-layer chromatography (HPTLC) determination of ETV in

pharmaceutical dosage forms has ever been mentioned in literature. According to the stability test guidelines issued by ICH [11-12], in the present study the stress induced stability studies were carried out for ETV to establish its stability characteristics. Hence, an attempt has been made to develop an accurate, specific and reproducible method for the determination of ETV in presence of degradation product for the content analysis during stability studies from pharmaceutical dosage form.

EXPERIMENTAL

Materials and Reagents

Pharmaceutical grade of Entecavir kindly supplied as a gift sample by Cipla Ltd., Mumbai, India. All chemicals and reagents used were of HPLC of analytical grade and were purchased from Merck Chemical. India. Analytical grade sodium hydroxide, hydrochloric acid and 30% hydrogen peroxide were used.

Instrumentation and chromatographic conditions

HPTLC was performed on 20 cm x 10 cm aluminium plates coated with silica gel aluminium plate 60F-254 (0.2 mm thickness E. Merck, Germany). ETV was spotted in the form of bands of width 6 mm with Camag microlitre syringe using Camag Linomat V (Switzerland). A constant application rate of 150 nl/sec was employed and space between two bands was 15 mm. The slit dimension was kept 6 mm x 0.45 mm micro, 20 mm/s scanning speed was employed. The mobile phase consisted of Chloroform: Toluene: methanol: ammonia (7:2:1:0.2v/v). The chromatogram was developed in twin trough glass chamber saturated with mobile phase using linear ascending technique. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was approximately 80 mm. The system was found to give compact spot for ETV (R_f value of 0.51 ± 0.02). Subsequent to the development; TLC plates were dried in current of air with the help of an air dryer. Densitometric scanning was performed using Camag TLC scanner III in the absorbance mode at 254 nm. The source of radiation utilized was deuterium lamp emitting a

continuous UV spectrum in the range of 200 - 400 nm.

Preparation of standard solution and calibration graphs

Accurately weighed quantity of ETV (10 mg) was transferred to 10.0 ml volumetric flask. Then small amount methanol was added and ultra sonicated for 5 min and diluted up to the mark with methanol (Concentration: 1000 µg/ml). Different volume of stock solution 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 µl were spotted in six replicates on TLC plates to obtain concentration of 300, 600, 900, 1200, 1500, 1800 ng/spot of ETV respectively

The plate was developed on previously described mobile phase. The peak areas were plotted against corresponding concentrations to obtain the calibration graphs

PREPARATION OF SAMPLE SOLUTION

For HPTLC Densitometry

Twenty tablets were accurately weighed and average weight per tablet was determined. Tablets were ground to fine powdered, and weighed tablet powder equivalent to 10mg of ETV was transferred to 100 mL volumetric flask. The powder was dissolved in 30 mL methanol by intermittent shaking and the volume was made up to the mark with methanol. The solution was then filtered through Whatmann filter paper no.45. 1 µL of above solution was spotted on TLC plate followed by development and scanning as described in Section 2.2.3. The analysis was repeated for six times. ETV gave sharp and well defined peaks at R_f 0.51, when scanned at 254 nm.

METHOD VALIDATION

The method was validated for its linearity range, accuracy, precision, sensitivity and specificity. Method validation is carried out as per ICH guidelines

Precision

Intra- and inter day precisions of the methods were determined by performing replicate (n=3) analyses of standards and samples. This

procedure was replicated on different days (n=3). Recovery studies by standard addition method were performed in view of justifying accuracy of the proposed methods. Previously analysed samples containing ETV was spiked with standard ETV, and the mixtures were analysed in triplicate (n=3) by proposed methods. Precision was calculated from percentage relative standard deviation (RSD %) for repeated measurements, whereas accuracy was expressed as % of recovery.

Robustness and ruggedness of the method

For HPTLC–densitometric method

Robustness was studied in six replicate at the concentration level of 1000 ng/spot. In this study, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography and chromatography to spotting) were studied and the effects on the results were examined.

Ruggedness of the proposed method was studied by two different analysts using the same experimental and environmental conditions. The spots 1000 ng/band of ETV was applied on TLC plates. This procedure was repeated in triplicates.

Limit of detection and limit of quantitation

Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were calculated by the use of the equation $LOD = 3.3 \times ASD/S$ and $LOQ = 10 \times ASD/S$; where, 'ASD' is Average standard deviation of the peak height and area of the drugs, taken as a measure of noise, and 'S' is the slope of the corresponding calibration curve.

Different volume of stock solution in the range 300-600 ng/band was spotted on TLC plate. The procedure was repeated in triplicate.

Specificity

To confirm the specificity of the proposed method, ETV sample was spotted on TLC plate. The mobile phase designed for the method resolved ETV very efficiently. The peak purity of ETV was tested by correlating the spectra of ETV extracted from capsules and standard ETV at the peak start (S), peak apex (A) and at the peak end (E) positions. Correlation between

these spectra indicated purity of ETV peak {correlation $r(S, M) = 0.999$, $r(M, E) = 0.9992$ }.

Recovery studies

Recovery experiments were performed at three different levels i.e. 80, 100 and 120 %. To the pre-analysed sample solutions, a known amount of standard drug solution of ETV was added at three different levels.

Forced degradation studies

A stock solution containing 10 mg for ETV in 10 mL methanol was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating property of the method.

Acid degradation

Methanolic solution of the drug (10 mg) was separately dissolved in 10 mL of 1M HCl and these solutions were kept for 8 h at room temperature in dark in order to exclude the possible degradative effect of light. The solutions (1 mL) were taken and neutralized and then diluted up to 10 mL with methanol. The resultant solutions were applied on TLC plate in triplicate (10 μ L each, i.e. 1000 ng/band). The plate was chromatographed as described above.

Base degradation

Methanolic solution of the drug (10 mg) was separately dissolved in 10 mL of 1 M NaOH solution. These solutions were kept for 8 h at room temperature in dark in order to exclude the possible degradative effect of light. The solutions (1 mL) were taken and neutralized and then diluted up to 10 mL with methanol. The resultant solutions were applied on TLC plate in triplicate (10 μ L each, i.e. 1000 ng/band). The plate was chromatographed as described above.

Oxidative degradation

The drug (10 mg) was dissolved in 10 mL of methanolic solution of hydrogen peroxide (10% v/v) and kept for 8 h at room temperature in the dark, to exclude the possible degradative effect of light. The solution (1 mL) was then diluted to 10 mL with methanol and treated as described for acid and base-induced degradation.

Photochemical degradation

The photochemical stability of the was studied by exposing the methanolic stock solution to direct sunlight for 8h (from 9 AM to 5 PM, at 20°C).

Thermal degradation at different temperature and different time interval

Expose about 2 gm of sample at different time intervals viz. 0, 90, and 180 days and at different temperatures viz. -20°C, 25°C, and 40°C.

A solution of the treated powder was then prepared and 1000 ng/band was applied to a plate in triplicate. The plate was then chromatographed and treated as described above.

No degradants were observed. However, for 180 days and 40°C the chromatographic peak area of Entecavir decreased insignificantly. Hence, the sample was stable at least for 180 days at 25°C.

Thermal stress (Test sample exposed to sunlight)

Transfer about 2 gm of sample into a clean dry watch glass and spread evenly. Expose to sunlight for 10 hours. A solution of the treated powder was then prepared and 1000 ng/band was applied to a plate in triplicate. The plate was then chromatographed and treated as described above.

RESULT AND DISCUSSION

Optimization of procedures

The TLC procedure was optimized with a view to develop a stability indicating assay method. Initially Chloroform and toluene was tried but does not show proper movement. Therefore, methanol was added in various proportions to toluene and chloroform. Finally, chloroform, toluene and methanol in the ratio of 7:2:1 v/v was selected as mobile phase the developed spot was diffused. To the above mobile phase, 0.2 ml ammonia was added. It gave good resolution, sharp and symmetrical peak with R_f value of 0.51 for (Fig.2). Also the spot for was compact and not diffused. It was observed that prewashing of TLC plates with methanol (followed by drying and activation) and pre-saturation of TLC chamber with mobile phase for 20 min ensure good reproducibility and peak shape of.

Linearity

In case of UV linearity was found to be in the range of 4-24 µg/ml. In case of HPLC, linearity was studied by injecting six concentrations of standard ETV (2,4,6,8,10,12 µg/mL) in triplicate. In HPTLC, a series of dilutions and standard curves were prepared over a concentration range from 300-1800 ng/band of ETV from stock solution. The graph of peak area versus concentration data was performed by least square linear regression analysis, whereby slope, intercept, and correlation coefficient were determined.

Precision

An Intra-day variation was assessed by analysing three different concentrations 900, 1200 and 1500 ng/band of ETV for three times within a day.

Inter-day variation was assessed using same concentration of drug (mentioned above) and analysing it for three different days, over a period of week. The results are as shown in **Table 1.**

Robustness and ruggedness of the method

The parameters such as mobile phase composition, volume of mobile phase, development distance, relative humidity, activation of plates, duration of saturation were studied. The standard deviation of peak areas was calculated for each parameter. The low %R.S.D. 0.56-1.34 indicates robustness of the method.

The ruggedness of the proposed method was evaluated by two different analysts. The results for ETV were found to be 99.45% and 99.90% respectively

LOD and LOQ

Detection limit and quantification limit was calculated and the LOD and LOQ were found to be **56.34 ng and 134.56 ng**, respectively.

Recovery studies

To the preanalysed 1000 ng/band solutions, a known amount of standard drug solutions of ETV was over spotted at 80 %,100 % and 120 % levels i.e.900,1200 and 1500 ng/band. The chromatogram was developed and scanned. The result of % recovery was found to be 0.67-1.43 indicated accuracy of the method.

System Suitability

System suitability tests were also carried out to verify reproducibility. The system suitability was assessed in HPTLC by using six replicate analysis of drugs at concentration of 1000 ng/spot of ETV respectively. % RSD was found to be 1.31 which was less than 2 indicated the method is reproducible.

Summary of validation parameter are as shown in **Table 2**.

Stability- indicating property

The chromatogram of samples degraded with acid, base, hydrogen peroxide, thermal photo light and thermal stress showed well separated spots of pure ETV as well as some additional peaks at different Rf values (**Fig. 3**). Content of

ETV remained, and percentage recovery were calculated and listed in **Table 3**.

Analysis of the marketed formulation

Six replicate determinations were performed on the commercially available tablets. For ETV the spots of Rf 0.51 was observed in chromatograms obtained from drug samples extracted from tablets and recovery was found to be $99.87 \pm 0.65\%$. There was No interference was observed from the excipients commonly present in the tablets. It may, therefore, be inferred that degradation of ETV had not occurred in the marketed formulations analysed by this method. The low RSD indicated that the method is suitable for routine estimation of ETV in pharmaceutical dosage forms.

Table 1. Intra-day and Inter-day Precision Studies

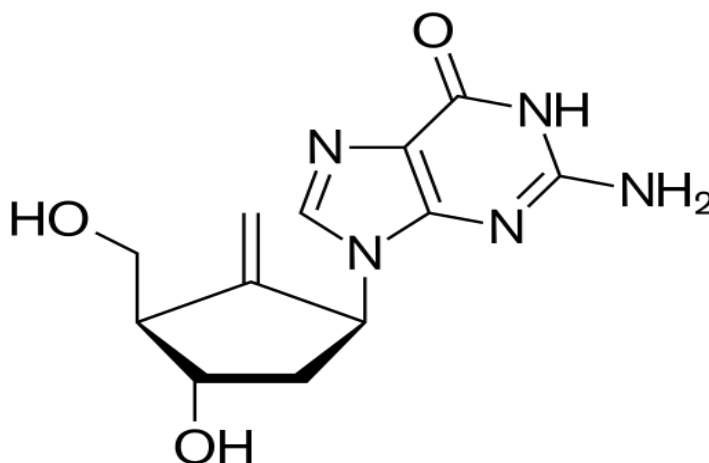
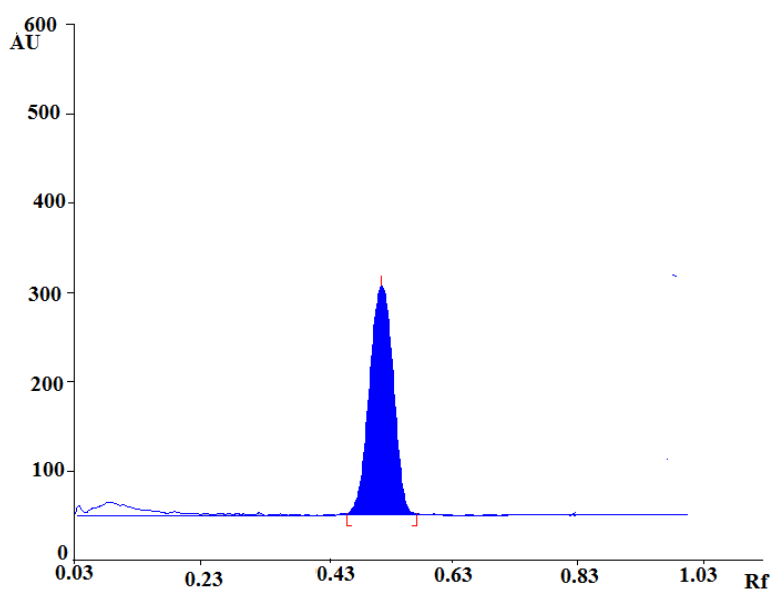
Drug	Conc. [ng/band]	Intra- day		Inter- day	
		Amount found [ng]		Amount found [ng]	
		Mean \pm SD [n = 3]	% RSD	Mean \pm SD [n = 3]	% RSD
ETV	900	896.23 \pm 6.56	0.75	898.09 \pm 27.21	0.89
	1200	1194.47 \pm 32.32	1.45	1203.32 \pm 42.37	1.42
	1500	1498.49 \pm 45.92	0.45	1505.84 \pm 24.57	1.23

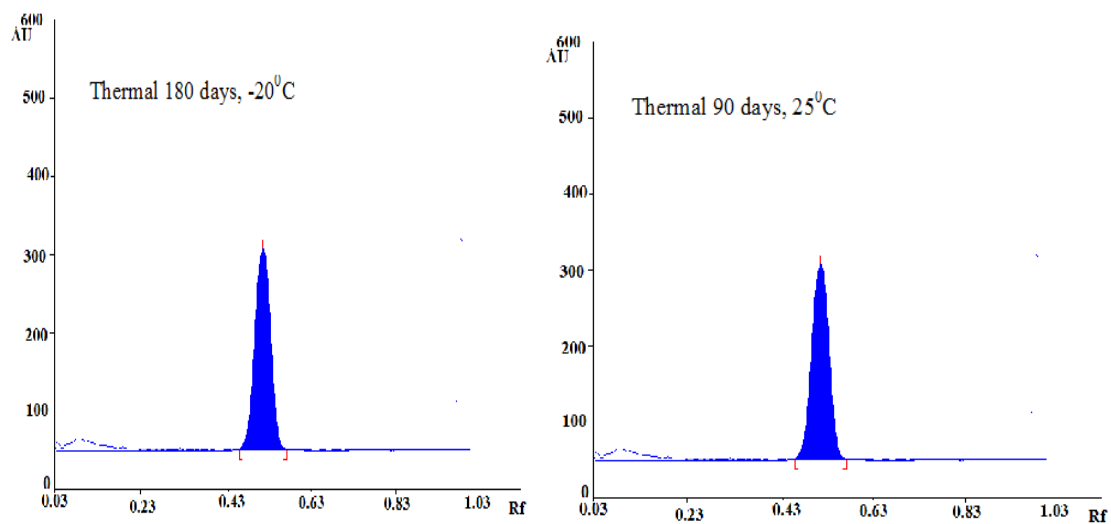
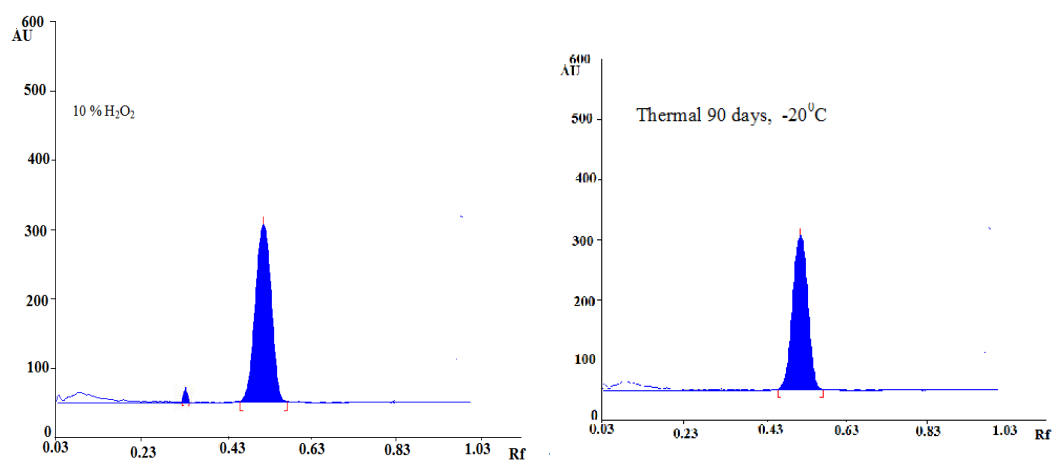
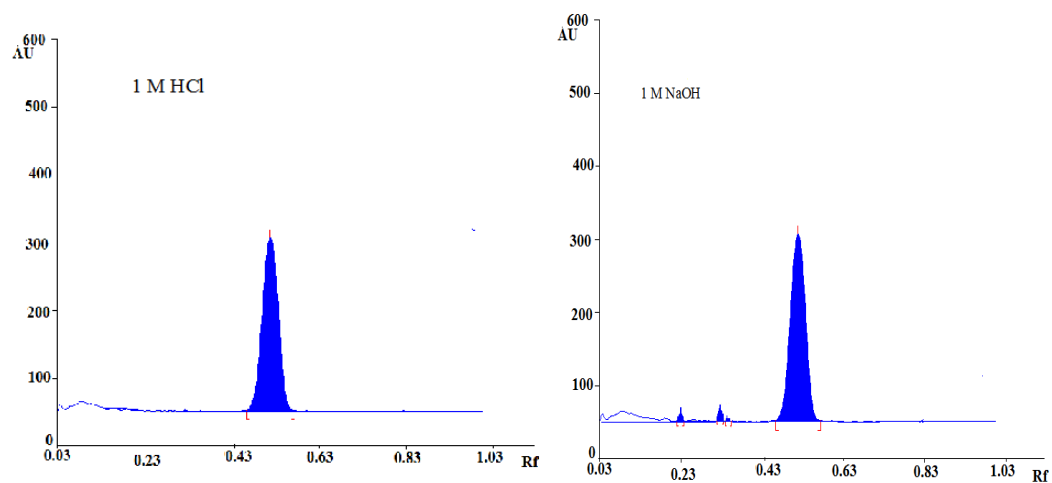
Table 2. Summary of Validation Parameter

Parameter	HPTLC
Linearity range	300-1800[ng /spot]
Regression equation [Y = mX + C]	Y=4.4728X +1755.6
Correlation coefficient	0.998
Limit of detection	56.34ng
Limit of quantitation	134.56 ng
% Recovery [n = 3]	99.78 – 100.23
Ruggedness [%]	
Analyst I [n = 3]	99.45
Analyst II [n = 3]	99.90
Precision [% RSD]	
Repeatability [n = 6]	1.47
Inter-day [n = 3]	0.45-1.45
Intra-day [n = 3]	0.89-1.42
Robustness	Robust
Specificity	Specific

Table 3. Forced Degradation of ETV

Sample exposure condition	Number of degradation products [R_f values]	ETV remained [1000 ng/spot]	SD	Recovery [%]
1 M HCl, 8h, RT	No degradation	998.9	9.68	99.89
1 M NaOH, 8h, RT ^a	3 (0.21, 0.33, 0.35)	821.6	10.40	82.16
10 % H ₂ O ₂ , 8h, RT ^a	1 (0.21)	871.1	5.48	87.11
Thermal 90 days, -20 ⁰ C	No degradation	994.2	12.23	99.42
Thermal 180 days, -20 ⁰ C	No degradation	996.7	6.45	99.67
Thermal 90 days, 25 ⁰ C	No degradation	995.6	9.12	99.56
Thermal 180 days, 25 ⁰ C	No degradation	993.4	7.17	99.34
Thermal 90 days, 40 ⁰ C	No degradation	997.4	9.23	99.74
Thermal 180 days, 40 ⁰ C	2(0.34, 0.42)	863.6	10.12	86.36
Thermal stress	No degradation	998.1	7.12	99.81
Photo, 8 h	No degradation	993.1	4.12	99.31

^aRT = Room Temperature**Fig.1 Chemical structure of Entecavir****Fig.2 HPTLC densitogram of ETV ($R_f=0.51$)**



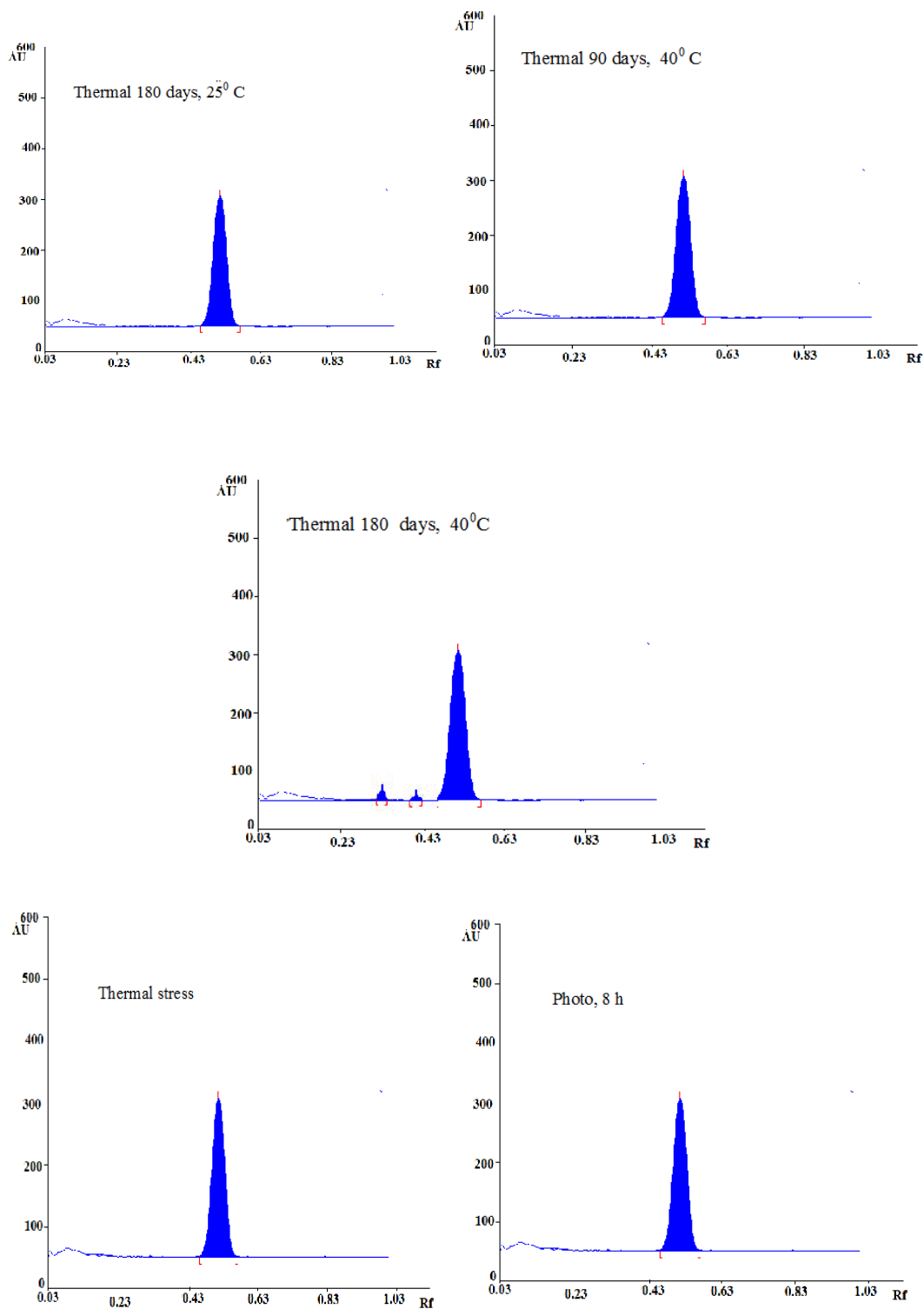


Fig. 3. Forced degradation of ETV by HPTLC: A) 1N HCl + ETV; B) 1N NaOH + ETV; C) 10% H₂O₂ + ETV; D) Thermal 90 days, -20°C; E) Thermal 180 days, -20°C ;F) Thermal 90 days, 25°C;G) Thermal 180 days, 25°C; H) Thermal 90 days, 40°C;I) Thermal 180 days, 40°C; J)Thermal stress K) Photolight,8h.

CONCLUSION

The proposed HPTLC methods provide simple, accurate and reproducible quantitative

analysis for determination of ETV in tablets. The method was validated as per ICH guidelines. As the method could effectively separate the drugs

from their degradation products; therefore, it can be employed as a stability indicating study.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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