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Research Article

Stability-Indicating High-Performance Thin-Layer Chromatographic Method for Quantitative Estimation of Emtricitabine in Bulk Drug and Pharmaceutical Dosage Form

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A simple, sensitive, precise, specific and stability indicating high-performance thin-layer chromatographic (HPTLC) method for the determination of emtricitabine both in bulk drug and pharmaceutical dosage form was developed and validated. The method employed aluminium plates precoated with silica gel G60 F_{254} as the stationary phase. The solvent system consisted of toluene : ethyl acetate : methanol (2 : 8 : 1, v/v/v). This solvent system was found to give compact spots for emtricitabine with R_f value 0.26 ± 0.01. Densitometric analysis of emtricitabine was carried out in the absorbance mode at 284 nm. Linear regression analysis showed good linearity ($r^2 = 0.9997$) with respect to peak area in the concentration range of 30–110 ng spot^{-1}. The method was validated for precision, limit of detection (LOD), limit of quantitation (LOQ), robustness, accuracy and specificity. Emtricitabine was subjected to acid and alkali hydrolysis, oxidation, neutral hydrolysis, photodegradation and dry heat treatment. Also the degraded products peaks were well resolved from the pure drug with significantly different R_f values. Statistical analysis proved that the method is repeatable and specific for the estimation of the said drug. As the method could effectively separate the drugs from their degradation products, it can be employed as a stability indicating method.

1. Introduction

Emtricitabine is chemically known as 4-amino-5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] pyrimidin-2-one [1]. The chemical structure of emtricitabine is shown in Figure 1. Emtricitabine, a nucleoside reverse transcriptase inhibitor, is phosphorylated by cellular enzymes to emtricitabine 5′-triphosphate, which, in turn, inhibits the activity of HIV-1 (HIV) reverse transcriptase by competing with the endogenous substrate. Incorporation of the triphosphate into the viral DNA causes chain termination, thereby inhibiting viral replication [2]. Quantification of emtricitabine has been performed in the past using UV spectrophotometry [3–5], HPLC coupled with UV or fluorometric detection [6–11], HPTLC [12], and LC/MS/MS [13, 14] either alone or in combination with other drugs.

To our knowledge, no article related to the stability indicating high-performance thin-layer chromatographic (HPTLC) determination of emtricitabine in pharmaceutical dosage forms has been reported in the literature. The international conference on harmonization (ICH) guideline entitled *Stability Testing of New Drug Substances and Products* requires the testing to be carried out to elucidate the inherent stability characteristics of the active substance [15].

Nowadays HPTLC is becoming a routine analysis technique due to advantages of low operating cost, high sample throughput, and need for minimum sample cleanup. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis [16, 17].

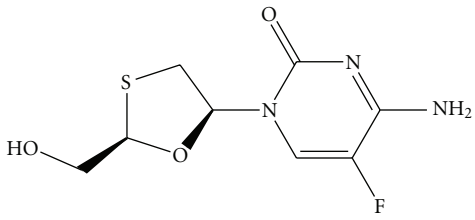

Figure 1: Structure of emtricitabine.

The aim of the present work was to develop an economic, precise, accurate, specific, and stability-indicating HPTLC method using densitometric detection for the determination of emtricitabine in the presence of its degradation products, either in bulk form or in pharmaceutical dosage form as per ICH guidelines [15, 18].

2. Experimental

2.1. Materials. Pharmaceutical grade of emtricitabine (batch no. EM0030606) was kindly supplied as a gift sample by Matrix Laboratories, Hyderabad, India, used without further purification, and certified to contain 99.57% (w/w) on dried basis. Pharmaceutical dosage form (Emtriva Capsules 200 mg; Batch no. 29832AF21) was procured as a gift sample from Gilead Sciences Inc, USA. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, Mumbai, India.

2.2. HPTLC Instrumentation and Chromatographic Conditions. The HPTLC plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. The samples were spotted in the form of bands 6 mm width with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated HPTLC aluminum plate G60 F_{254}, [(20 × 10 cm) with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai] using a Camag Linomat IV applicator (Switzerland). A constant application rate of 0.1 μL s^{-1} was used and the space between two bands was 6 mm. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The mobile phase was consisted of toluene : ethyl acetate : methanol (2 : 8 : 1, v/v/v) and 20 mL were used per chromatography run. The optimized chamber saturation time for mobile phase was 30 min using saturation pads at room temperature (25°C ± 2). The length of chromatogram run was 8 cm. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance-absorbance mode and operated by CATS software (V 3.15, Camag). The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm s^{-1}. The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. All determinations were performed at ambient temperature with a detection wavelength of 284 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression.

2.3. Preparation of Standard Solution. Accurately weighed 100 mg of emtricitabine was transferred to a 100 mL volumetric flask and dissolved in and diluted up to the mark with methanol to obtain a standard solution of emtricitabine (1000 μg mL^{-1}). This solution was further diluted with methanol to obtain working standard solutions of emtricitabine in concentration range of 30–110 μg mL^{-1}.

2.4. Method Validation. The HPTLC method was validated as per the ICH guidelines [18].

2.4.1. Linearity and Range. One microlitre from each working standard solution was spotted on the HPTLC plate to obtain final concentration range of 30–110 ng spot^{-1}. Each concentration was spotted six times on the HPTLC plate. The plate was developed using the previously described mobile phase and scanned. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. Linear calibration curve was generated using least-squares linear-regression analysis. Residual analysis was performed to ascertain linearity.

2.4.2. Precision. Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analyses of three different concentrations (70, 90, 110 ng spot^{-1}) of the drug in hexaplicate on the same day. Intermediate precision of the method was checked by repeating studies on different days.

2.4.3. Limit of Detection and Limit of Quantitation. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted following the same method. The signal-to-noise ratio was determined. An LOD was considered as 3 : 1 and LOQ as 10 : 1. The LOD and LOQ were experimentally verified by diluting known concentrations of standard solution of emtricitabine until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

2.4.4. Robustness of the Method. By introducing small changes in the mobile-phase composition (±0.1 mL for each component), the effects on the results were examined. Mobile phases having different composition like toluene : ethyl acetate : methanol (2.1 : 8.0 : 1.0 v/v/v), (2.0 : 8.1 : 1.0 v/v/v), and (2.0 : 8.0 : 1.1 v/v/v) were tried, and chromatograms were run. Amount of mobile phase was varied by ±5%. Time from spotting to chromatography and from chromatography to scanning was varied by +10 min. Robustness of the method was done at three different concentration levels 70, 90, 110 ng spot^{-1} for emtricitabine.

2.4.5. Accuracy. Accuracy of the method was determined by standard addition method in which the known amount of

standard emtricitabine solutions were added to preanalyzed capsule solution. These amounts corresponded to 50, 100, and 150% of the amounts claimed on the label. The amounts of emtricitabine were estimated by applying these values to the regression equation of the calibration curve. Accuracy study was performed for six times, and % recovery of emtricitabine was calculated.

2.4.6. Specificity. Specificity of the method was by means of complete separation of pure drug from the degradation products. Peak purity of emtricitabine and degradation products was assessed by comparing their respective spectra at peak start (S), peak apex (M), and peak end (E) position of the spots.

2.4.7. Solution Stability. The stability of standard solutions was tested after 0, 6, 12, 24, 48, and 72 h of storage. The stability of the solutions was determined by comparing peak area percentage and peak purity at 1000 ng spot^{-1}.

2.5. Analysis of Marketed Pharmaceutical Dosage Form. To determine the content of emtricitabine in marketed pharmaceutical dosage form (Emtriva Capsules 200 mg; Batch no. 29832AF21), powder of twenty capsules was weighed. An accurate weight of the powder equivalent to 200 mg emtricitabine was weighed and transferred into a 100 mL volumetric flask containing 50 mL methanol, sonicated for 30 min, and diluted to 100 mL with methanol. The resulting solution was centrifuged at 4000 rpm for 5 min, and supernatant was analyzed for drug content. 1 mL of the above supernatant solution was transferred into 20 mL volumetric flask and diluted to volume with methanol. The concentration achieved after the above dilution was 100 μg mL^{-1}. 1 μL volume was spotted for six times to achieve a final concentration of 100 ng spot^{-1}. The plate was developed in the previously described chromatographic conditions. The possibility of excipient interference in the analysis was studied.

2.6. Forced Degradation Studies. Decomposition studies were performed in solutions containing emtricitabine at a concentration of 1000 μg mL^{-1}. Samples were withdrawn at suitable time intervals and subjected to HPTLC analysis. The drug was subjected under different stress conditions as follows.

2.6.1. Acid-Induced Degradation. To 10 mL of methanolic stock solution 10 mL of 0.1 N HCl was added. This mixture was refluxed at 80°C.

2.6.2. Base-Induced Degradation. To 10 mL of methanolic stock solution 10 mL of 0.01 N NaOH was added, and the solution was refluxed at 80°C.

2.6.3. Oxidative Degradation. To 10 mL of methanolic stock solution 10 mL each of hydrogen peroxide 6% (v/v) and 3% (v/v) was added separately. The solution was kept at room temperature and then heated in boiling water bath for 10 min to completely remove the excess of hydrogen peroxide.

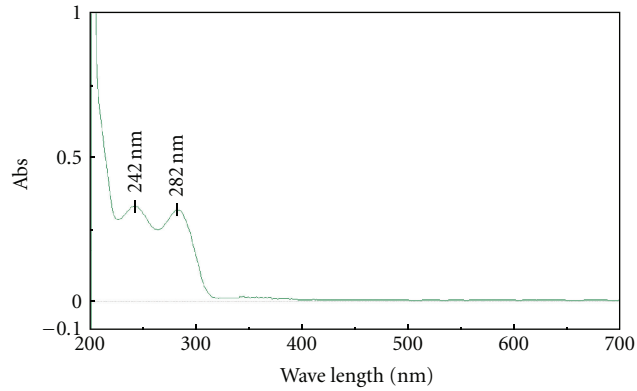

Figure 2: UV spectrum of 10 μg mL^{-1} concentration of emtricitabine.

2.6.4. Wet Heat Degradation. Studies under neutral conditions were performed by dissolving the drug substance in distilled water, and solution was refluxed at 80°C for 5 days.

2.6.5. Dry Heat Degradation. For dry heat degradation, the standard drug was placed in an oven at 80°C for 12 days.

2.6.6. Photochemical Degradation. The photochemical stability of the drug was studied by exposing the solution to direct sunlight for 5 days (~40 h) kept on a terrace.

3. Results and Discussion

3.1. Selection of Analytical Wavelength. Stock solution of emtricitabine was prepared in methanol. UV spectrum of 10 μg mL^{-1} concentration of emtricitabine showed absorbance of less than 1, and two maxima were obtained at 242 and 282 nm (Figure 2). Further, in situ HPTLC spectrum of emtricitabine was taken. λ_{max} was found to be 284 nm and was selected as scanning wavelength (Figure 3).

3.2. Optimization of the Chromatographic Conditions. The HPTLC procedure was optimized with a view to develop stability-indicating assay method. Both the pure drug and degraded drug solutions were spotted on HPTLC plates and run in different solvent systems. Initially, ethyl acetate and methanol were tried in different ratio. Ethyl acetate and methanol in the ratio of 9 : 1.5 v/v was selected, and R_f was found to be 0.51, but in the subsequent run while performing the forced degradation studies, it was found that the degradation peak was eluted out. So, the toluene was added to bring down the R_f of degradation peak. Finally, toluene, ethyl acetate, and methanol were tried in different ratio. The optimum mobile phase was found to be consisted of toluene : ethyl acetate : methanol (2 : 8 : 1 v/v/v). The drug in presence of their degradation products was satisfactorily resolved with R_f value at 0.26 ± 0.02 (Figure 4). In order to

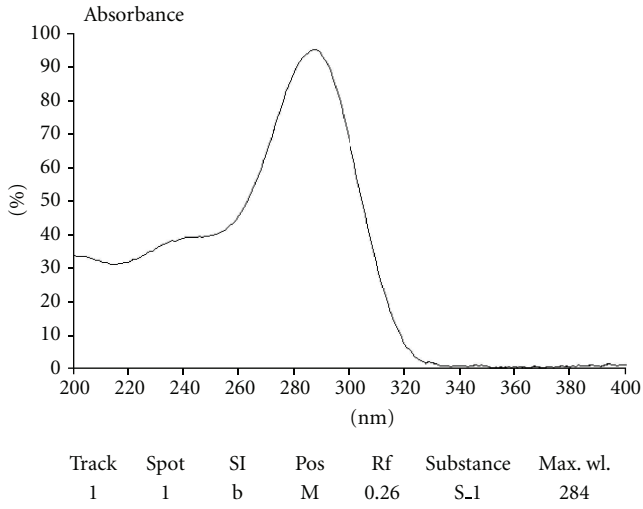

Figure 3: In situ HPTLC spectrum of emtricitabine.

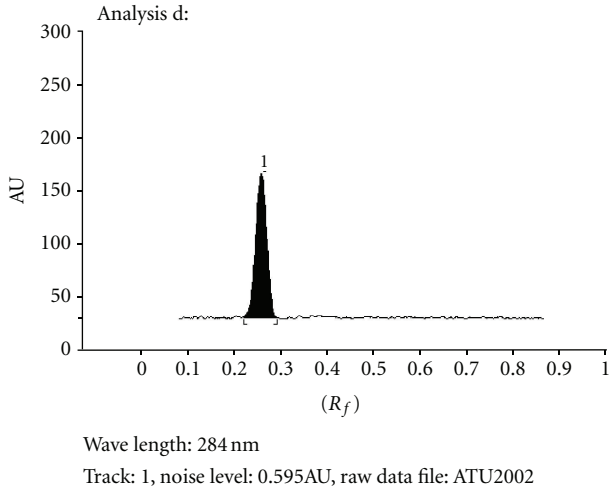

Figure 4: Densitogram of standard emtricitabine 100 ng spot^{-1}; Peak 1 (R_f: 0.26 ± 0.02), mobile phase toluene : ethyl acetate : methanol (2 : 8 : 1 v/v/v).

reduce the neckless effect, the TLC chamber was saturated for 30 min using saturation pads. The mobile phase was run upto distance of 8 cm, which takes approximately 30 min for development of HPTLC plate.

3.3. Validation of the Method

3.3.1. Linearity and Range. Linear relationship was observed by plotting drug concentration against peak areas. Emtricitabine showed linear response in the concentration range of 30–110 ng spot^{-1}. The corresponding linear regression equation was $y = 26.12x - 241.9$ with square of correlation coefficient (r^2) of 0.9997 for emtricitabine. Residual analysis was performed to ascertain linearity (Figure 5). Slope was significantly different from zero (Table 1).

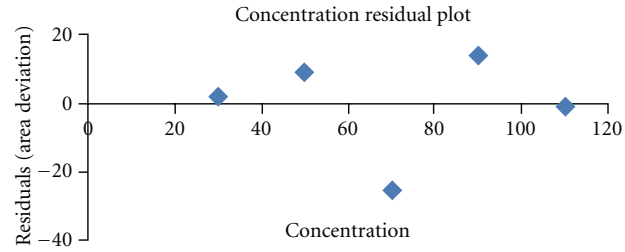

Figure 5: Concentration versus Residual plot of emtricitabine.

Table 1: Linear regression data for the calibration curves ($n = 6$).

Parameters	Emtricitabine
Linearity range	30–110 ng spot^{-1}
r^2	0.9997
Slope ± S.D*	26.12 ± 0.2805
Intercept ± S.D*	−241.9 ± 21.18
Confidence limit of slope[a]	25.23 to 27.01
Confidence limit of intercept[a]	−309.3 to −174.6
Sy.X	17.74

*$P < 0.001$—Slope significantly different from zero.
[a]95% confidence limit.
Sy.X—Standard deviation of residuals from line.

3.3.2. Precision. The results of the repeatability and intermediate precision experiments are shown in Table 2. The developed method was found to be precise as the RSD% values for repeatability and intermediate precision studies were <2%, respectively.

3.3.3. Limit of Detection and Limit of Quantitation. The signal : noise ratios of 3 : 1 and 10 : 1 were considered as LOD and LOQ respectively. The LOD and LOQ were found to be 10 ng spot^{-1} and 30 ng spot^{-1} respectively.

3.3.4. Robustness of the Method. The standard deviation of peak areas was calculated for each parameter, and RSD% was found to be less than 2%. The low values of RSD% as shown in Table 3. indicated robustness of the method.

3.3.5. Accuracy. As shown from the data in Table 4. good recovery % of the drug in the range from 99.84 to 101.46% was obtained at various added concentrations.

3.3.6. Specificity. The peak purity for standard emtricitabine was assessed by comparing spectra acquired at the start (S), apex (M), and end (E) of the peak obtained from the scanning of spot, that is, $r(S, M) = 0.999960$ and $r(M, E) = 0.999917$. Spots obtained of degradation products were well resolved from the active ingredient. Peak purity of spots of emtricitabine resolved during stability study was assessed by comparing the respective spectra at peak start, peak middle, and peak end, that is, $r(S, M)$ and $r(M, E)$. The peak purity

Table 2: Intraday and interday precision of emtricitabine ($n = 6$).

Concentration (ng spot^{-1})	Repeatability			Intermediate precision		
	Measured conc. (ng spot^{-1}) ± S.D	RSD%	S.E.	Measured conc. (ng spot^{-1}) ± S.D	RSD%	S.E.
70	68.97 ± 0.36	0.52	0.15	68.93 ± 0.41	0.59	0.17
90	89.19 ± 0.29	0.32	0.12	88.95 ± 0.38	0.42	0.16
110	108.89 ± 0.34	0.31	0.14	109.08 ± 0.29	0.26	0.12

Table 3: Robustness testing ($n = 6$).

Parameter	SD of peak area[a]	RSD[a]%
Mobile-phase composition (±0.1 mL)	1.34	0.24
Amount of mobile phase (±5%)	2.19	0.49
Time from spotting to chromatography (+10 min.)	1.29	0.18
Time from chromatography to scanning (+10 min.)	1.12	0.09

[a] Average of three concentrations 70, 90, 110 ng spot^{-1} for emtricitabine.

Table 4: Accuracy ($n = 6$).

Actual conc. (ng spot^{-1})	Measured conc. (ng spot^{-1}) ± S.D.	RSD%	SE	Recovery (%)
60	60.88 ± 0.30	0.49	0.12	101.46
80	79.87 ± 0.29	0.36	0.12	99.84
100	101.33 ± 0.53	0.52	0.22	101.33

Table 5: Summary of validation parameters.

Parameter	Data
Linearity range	30–110 ng spot^{-1}
Correlation coefficient	0.9997
Limit of detection	10 ng spot^{-1}
Limit of quantitation	30 ng spot^{-1}
% Recovery ($n = 6$)	100.88 ± 0.89
Precision (RSD%)	
Repeatability ($n = 6$)	0.38
Inter day ($n = 6$)	0.42
Robustness	Robust
Specificity	Specific

data indicated that peaks of emtricitabine resolved after application of stress conditions were pure (Table 6).

The data of summary of validation parameters are listed in Table 5.

3.4. Solution Stability. There was no indication of degradation in sample solutions of emtricitabine as revealed by peak purity data and from the value of RSD% (<2%) for peak areas of bands of solution stored at different times. The solution was found to be stable at ambient temperature for 72 h, and no unknown peaks were observed.

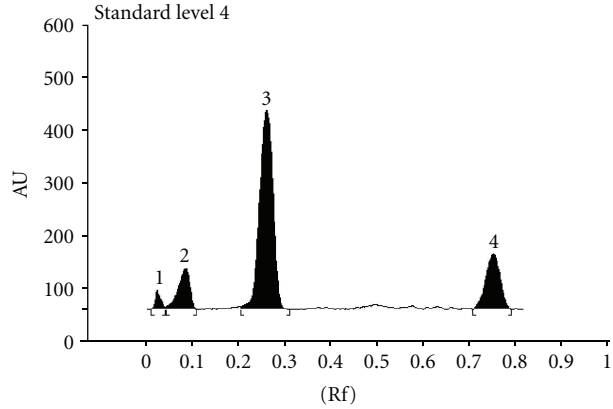

Figure 6: Densitogram of acid degradation peaks 1000 ng spot^{-1}; Condition: 0.1 N HCl at 80°C for 3 h; Peak 1, 2, 4 (degraded, R_f: 0.03, 0.09, 0.75), Peak 3 (emtricitabine, R_f: 0.26).

3.5. Analysis of Marketed Pharmaceutical Dosage Form. A single spot at R_f value of 0.26 was observed in the chromatogram of the drug samples extracted from capsules. There was no interference from the excipients that are commonly present in the formulations. The drug content was found to be 99.87%± 0.56 with a RSD% of 0.56 for six replicate determinations. It may, therefore, be inferred that degradation of emtricitabine had not occurred in the marketed formulations that were analyzed by this method. The good performance of the method indicated the suitability of this method for routine analysis of emtricitabine in pharmaceutical dosage form.

3.6. Forced Degradation Studies. Stress testing of emtricitabine under different conditions using toluene : ethyl acetate : methanol (2 : 8 : 1 v/v/v) as the mobile solvent system suggested the following degradation behaviour.

3.6.1. Acid-Induced Degradation. The rate of degradation in acid was slower as compared to that of alkali. Initially 0.1 N HCl was used at 80°C for 8 h, but more than 80% degradation was observed hence the duration of reaction with acid decreased to 3 h to obtain a reasonable degradation between 20–30%. The degradation peaks were observed at R_f 0.03, 0.09 and 0.75 (Figure 6).

Table 6: Summary of degradation products of emtricitabine and peak purity of spots of emtricitabine resolved.

Stress conditions	R_f values of degradation products	Peak purity data $r(S, M)$, $r(M, E)$
Acid hydrolysis, 0.1 N HCl, refluxed for 3 h at 80°C	0.03, 0.09, 0.75	0.999868, 0.999808
Alkaline hydrolysis, 0.01 N NaOH, refluxed for 3.5 h at 80°C	0.03, 0.07, 0.71	0.998896, 0.998778
Oxidation (H_2O_2, 3% v/v), refluxed for 8 h at RT	0.07, 0.43	0.999982, 0.999852
Wet heat, refluxed for 5 days at 80°C	Not detected	0.999959, 0.999957
Dry heat, heated in oven at 80°C for 12 days	Not detected	0.999966, 0.999944
Day light, exposed to sunlight for 5 days	Not detected	0.999811, 0.999806

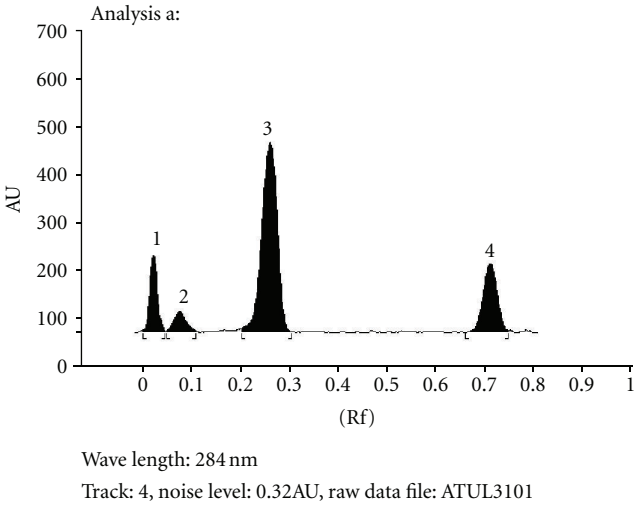

Figure 7: Densitogram of base-treated emtricitabine 1000 ng spot^{-1}; condition: 0.01 N NaOH 80°C for 3.5 h; Peak 1, 2, 4 (degraded, R_f: 0.03, 0.07, 0.71), Peak 3 (emtricitabine, R_f: 0.26).

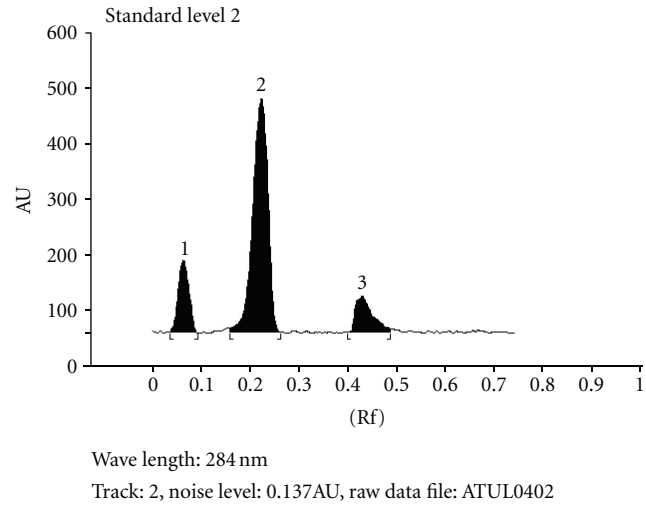

Figure 8: Densitogram of H_2O_2-treated emtricitabine 1000 ng spot^{-1} condition: 3% H_2O_2 at room temperature for 8 h; Peak 1, 3 (degraded, R_f: 0.07, 0.43), Peak 2 (emtricitabine, R_f: 0.23).

3.6.2. Base-Induced Degradation. The drug was found to be highly labile to alkaline degradation. The reaction in 0.1 N NaOH at 80°C was so fast that around 80% of the drug was degraded in 1 h. Subsequently, studies were performed by reducing the alkali strength to 0.01 N NaOH. Drug showed degradation around 30% within 3.5 h at 80°C associated with rise in a two major and one minor degradation peaks at R_f 0.03, 0.71, and 0.07, respectively (Figure 7).

3.6.3. Oxidative Degradation. The drug was found to be highly labile under oxidative conditions. Reaction in 6% peroxide at room temperature shown complete degradation in 1 h. Hence drug was exposed to 3% hydrogen peroxide for 8 h at room temperature. The degradation peaks were observed at R_f 0.07 and 0.43 (Figure 8).

3.6.4. Wet Heat Degradation. The drug was found to be stable when refluxed with water at 80°C for 12 h. No significant degradation was observed, reflux time was then increased for 5 days. No degradation peaks were observed.

3.6.5. Dry Heat Degradation. Drug was found to be stable when subjected to thermal degradation at 80°C for 12 days. No degradation peaks were observed.

3.6.6. Photochemical Degradation. Emtricitabine was found to be stable to photochemical degradation as no degradation peaks were observed after exposing drug to sunlight for 5 days.

Degradation products obtained under different stress conditions are summarized in Table 6.

4. Conclusion

The developed HPTLC technique is precise, specific, accurate, and stability indicating. Statistical analysis proves that the method is repeatable and selective for the analysis of emtricitabine as bulk drug and in pharmaceutical dosage form. As the method separates the drug from its degradation products, it can be employed as a stability-indicating one. The proposed HPTLC method reduces the duration of analysis and is suitable for routine determination of

emtricitabine in pharmaceutical formulation in quality-control laboratories, where economy and time are essential. This study is a typical example of development of a stability-indicating assay, established following the recommendations of ICH guidelines.

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