Stability-Indicating Green RP-HPLC and HPTLC Methods for Emtricitabine Using AQbD Approach

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Abstract
Emtricitabine, a BCS class III anti-retroviral drug, is highly hydrophilic with low gastrointestinal permeability, necessitating sensitive and reliable analytical methods for quantification at low concentrations. The work aimed to develop and validate an Analytical Quality by Design (AQbD) guided green RP-HPLC and HPTLC methods for estimation of emtricitabine in bulk and tablet dosage form and to assess its degradation behavior under stress conditions. The HPLC separation was carried out using a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm). The mobile phase comprising acetate buffer and acetonitrile (70:30, v/v) adjusted to pH 3.5, at a flow rate of 1.0 mL/min with UV detection at 272 nm. The HPTLC method was optimized and validated on silica gel 60 F_{254} plates using toluene: methanol: n-hexane (6:1:3 v/v/v) as the mobile phase. Method validation performed in accordance with ICH guidelines for linearity, accuracy, precision, ruggedness, robustness, LOD, and LOQ. RP-HPLC method exhibited excellent linearity over 10–60 µg/mL (R^2 = 0.9989) with a retention time of 2.56 ± 0.01 min. Precision and robustness were within acceptable limits (≤2% RSD) with LOD and LOQ of 0.299 and 0.906 µg/mL, respectively. The HPTLC method showed linearity over 200–1200 ng/spot (R^2 = 0.9904) with a well-resolved peak at Rf 0.48 and recovery between 99.90–100.07%. Comparative evaluation indicated that RP-HPLC provides higher sensitivity and resolution, while HPTLC offers cost-effectiveness and higher sample throughput, making both methods suitable for routine quality control and stability studies of emtricitabine.

Keywords: Emtricitabine, QbD, RP-HPLC, HPTLC, method development, validation, Stress degradation
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INTRODUCTION

Emtricitabine (EMCB) is an antiretroviral agent classified as a nucleoside reverse transcriptase inhibitor (NRTI) used for the treatment of HIV infection in adults. The drug works by inhibiting reverse transcriptase, preventing conversion of viral RNA into DNA[1]. It is an organic compound and chemically known as 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine, the (-) enantiomer of a cytidine thio analog[2]. It is also active against the Hepatitis B virus[3,4]. It is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate[2], the active metabolite responsible for enzyme inhibition[5]. EMCB is observed as white to off-white powder, indicating molecular weight of 247.24 $g \cdot mol^{-1}$, and log P (octanol/water) of 0.43 (hydrophilic) The reported pKa was 2.65 (primary pKa reported for the cytosine moiety), Its aqueous solubility found to be 112 mg/mL (25 °C), which is highly water-soluble as per FDA reports. The structure of EMCB is shown in Figure 1. EMCB has a plasma half-life of 10 hours (plasma elimination half-life). It is a BCS Class III drug, characterized by high solubility and low permeability, low protein binding (<4%), and predominant renal elimination and limited passive membrane permeation, which shape both pharmacokinetics and formulation strategies[1,6]. Throughout the literature study, a literature gap was indicated, which showed that several analytical techniques, (UV, HPTLC, HPLC, LC–MS/MS) have been reported for EMCB, most rely on trial-and-error optimization and focus only on ICH Q2(R2) validation. Comprehensive Quality by Design (QbD) implementation involving risk assessment, Design of Experiments (DoE), identification of critical method parameters (CMPs), analytical lifecycle management, and sustainability considerations remains limited. Moreover, limited efforts have been made to develop a stability-indicating, robust, and environmentally sustainable RP-HPLC method suitable for routine analysis. There is also an insufficiency of studies correlating analytical robustness with method lifecycle management (MLCM) principles. The research gap recognized that EMCB has been widely studied for its antiviral efficacy and formulation development, and analytical studies employing QbD-based RP-HPLC methods remain infrequent. The absence of organized documentation and control of variability sources disturbs reproducibility and regulatory compliance. Besides, very few studies integrate QbD principles with ICH Q8–Q14 guidelines, which accentuate method understanding, control strategy, and continual improvement. Hence, there is a significant gap in developing a QbD-guided, validated and stability-indicating RP-HPLC method was required for accurate and routine determination of EMCB in pharmaceutical dosage forms. Therefore, this study focused on development and validation of simple, rapid, precise, and stability-indicating RP-HPLC method for EMCB using an AQbD strategy. The method development process incorporated systematic risk assessment and Design of Experiments

(DoE) to establish an optimized design space by correlating critical method parameters with analytical performance. In addition, a simple, cost-effective and stability-indicating HPTLC method was developed and validated in accordance with ICH guidelines to facilitate routine quality control analysis[7-12].

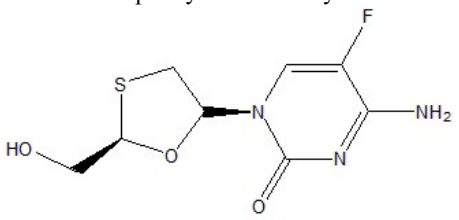


Figure 1: Chemical structure of emtricitabine

MATERIALS AND METHOD

EMCB was procured from Macleods Pharmaceuticals Ltd., Daman and Diu, India. Tablets were purchased from a local pharmacy. All other chemicals were purchased from Loba Chemical, Mumbai, India.

Chromatographic System Selection

The detection wavelength for EMCB by HPLC analysis was selected based on UV–visible spectrophotometric analysis using a Shimadzu UV-1800 spectrophotometer, which showed maximum absorbance at 272 nm Figure 2. This wavelength was therefore employed for subsequent HPLC analysis. Various mobile phase compositions were investigated to achieve optimum sensitivity, resolution, and acceptable analysis time. Based on overall chromatographic performance, a mixture of acetate buffer and acetonitrile in a 70:30% v/v selected as the optimal mobile phase. The aqueous phase was adjusted to pH 3.5 ± 0.05 using glacial acetic acid (1% or 0.1 M) and monitored with an ELICO pH meter (LI-120). The mobile phase was sonicated for 10 minutes to remove dissolved gases followed by filtration through a 0.45 µm membrane filter before use, to ensure clarity and reproducibility of the chromatographic system. This preparation resulted in consistent chromatograms with satisfactory system suitability characteristics. Chromatographic separation was performed using a Shimadzu LC Prominence system equipped with an LC-20AD binary pump, SPD-M20A PDA detector, Rheodyne injector with a 20 µL sample loop and a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm). Thermal and photodegradation experiments carried out using a hot air oven and UV chamber manufactured by Labhosp. The optimized chromatographic parameters are summarized in Table 1.

For High-Performance Thin-Layer Chromatography analysis, pre-coated silica gel 60 F_{254} plates were employed as the stationary phase. A mobile phase composed of toluene: methanol: n-hexane (6:1:3, v/v/v) was optimized to achieve sharp and well-resolved bands. Before development, the twin-trough glass chamber was saturated with the mobile phase for 10 minutes to ensure stable chromatographic conditions. Development of the plates was performed using the ascending technique. Samples were applied as bands with the help of a CAMAG Linomat applicator equipped with a 100 µL syringe to obtain uniform and reproducible application. Densitometric evaluation was subsequently carried out at 287 nm using a CAMAG TLC Scanner, and band visualization was achieved under UV light. Chromatographic condition shown in Table 2.

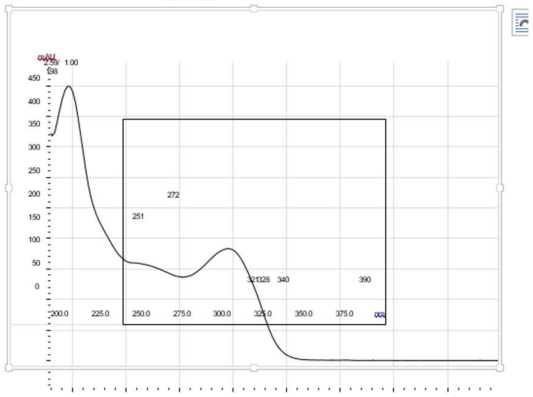
Table 1: **Optimized Chromatographic conditions for EMCB -HPLC**

HPLC system parameters	**Conditions**
Column	Hypersil BDS C18 column (250 × 4.6 mm) with a packing particle size of 5 µm
Mobile phase and diluents	Acetate Buffer (70mL) and Acetonitrile (30mL), with pH adjusted to 3.5 using glacial acetic acid & methanol as a diluent
Flow rate	1.0 mL/min
Detection (λ_{max})	272 nm
Detector	(SPD- M20A), PDA detector
Temperature	25ºC
Injection volume	20 µL
Retention time (t_R)	2.56 ± 0.01 min
Run time	10 min

Table 2: **Optimized Chromatographic conditions for EMCB -HPTLC**

HPTLC system parameters	**Conditions**
Stationary phase	Silica gel 60 F_{254} aluminum HPTLC plates (10 × 10 cm, 0.2 mm thickness) supplied byE-Merck prewashed with methanol.
Mobile phase	Toluene: Methanol: n-hexane (6:1:3 v/v/v)
Chamber saturation	10 min
Migration distance	80 mm
Activation of prewashed plate	15 min
Band width	6mm
Radiation source	Deuterium lamp
Scanning wavelength	287 nm
Injection volume	0.1 µL
Rf	0.48

Figure 2: UV absorption curve of Emtricitabine -



HPLC

Preparation of HPLC stock solution

EMCB (100 mg) was accurately weighed using an OHAUS Pioneer™ analytical balance (USA) and dissolved in water: acetonitrile (50:50, v/v), and the solution was sonicated for 5 minutes to ensure complete dissolution of the drug. The final volume was adjusted with the same diluent to obtain 1 mg/mL (1000 µg/mL) standard stock solution.

Preparation of HPLC working solution

Working standard solutions of EMCB in the concentration range of 10–60 µg/mL were prepared by suitable dilution of the stock solution. Aliquots of 0.1–0.6 mL were transferred into 10 mL volumetric flasks, diluted to volume with the selected diluent, and passed through a 0.45 µm membrane filter prior to chromatographic analysis.

Preparation of Marketed Tablet Formulation

Ten tablets, containing Emtricitabine (200 mg per tablet) were accurately weighed and finely powdered. An amount of the powder equivalent to 100 mg of EMCB was transferred to a 100 mL volumetric flask and dissolved in mobile phase to obtain a 1000 µg/mL solution. The resulting solution was filtered through a membrane filter, degassed, and injected into the chromatographic system for analysis. From that, take 0.3 mL solution and adjust the volume upto 10 mL with mobile phase to obtain concentration of 30 µg/mL solution.

System suitability testing

System suitability parameters were assessed using an EMCB standard solution of appropriate concentration of 20 µg/mL, prepared by appropriate dilution of the 1000 µg/mL stock solution. Six replicate injections (20 µL) were performed, and retention time, theoretical plates, tailing factor, and %RSD were evaluated. In accordance with ICH guidelines, acceptable system performance was confirmed by a %RSD below 2%, an asymmetry factor of less than 2, and a theoretical plate count exceeding 2000, indicating adequate column efficiency.

Validation of the RP-HPLC method

Method validation was performed using an Analytical Quality by Design (AQbD) strategy incorporating a central composite design (CCD), in accordance with the International Conference on Harmonisation ICH Q2(R2) guidelines[13-18].

Accuracy (% recovery)

Method accuracy was assessed by standard addition at 80%, 100%, and 120% of the nominal concentration (30 µg/mL), with analyses performed in triplicate. Accuracy was expressed as % recovery ± SD and %RSD, with %RSD maintained below 2%. Percentage recovery was calculated by comparing chromatographic peak areas of spiked samples with those of the standard. The method was considered acceptable when recovery values were within 98–102%.

Precision

Method precision was evaluated by both intra-day and inter-day precision at a concentration 10, 20 and 30 µg/mL. Intra-day precision was assessed within the same day by analysing each concentration in triplicate, whereas inter-day precision was evaluated over three consecutive days. Precision was expressed as %RSD of peak areas, with %RSD < 2% indicating acceptable precision.

Specificity

Method specificity was confirmed by comparing chromatograms of EMCB, placebo, and blank samples. No interfering or co-eluting peaks were observed at the retention time of EMCB, confirming selective analyte identification. Consistent retention time and peak purity across replicate injections further verified method specificity.

Linearity and range

Linearity of the analytical method was assessed across a concentration range of 10–60 µg/mL using working solutions of EMCB prepared in the mobile phase. Calibration plot was generated by plotting peak area versus concentration. The linear relationship was verified using the regression equation and correlation coefficient (R^2).

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated as per ICH Q2(R2) based on the standard deviation of the analytical response and slope of the calibration curve, applying factors of 3.3 (LOD) and 10 (LOQ). Method sensitivity was confirmed by acceptable signal-to-noise ratios (3:1 for LOD, 10:1 for LOQ) and %RSD ≤ 10% at low concentration levels.

Robustness Using the conventional method

Method robustness was evaluated by deliberate variation of chromatographic parameters, including column temperature (±5 °C), flow rate (±0.1 mL/min), mobile phase pH (±0.2), and injection volume (±5 µL). Studies were performed using triplicate injections of a 1 µg/mL solution following a one-factor-at-a-time (OFAT) approach. The method was considered robust as no significant changes in retention time, theoretical plates or tailing factor were observed under minor variations [12,19].

Robustness assessment employing the QbD approach for analytical method optimization

Robustness was evaluated using an Analytical Quality by Design (AQbD) framework. An Analytical Target Profile (ATP) was established to clearly define the objective of the analytical method, along with its expected performance characteristics and acceptance limits. Subsequently, the critical method attributes (CMAs) and critical quality attributes (CQAs) were systematically identified and a method operable design region (MODR) was established to determine factors significantly affecting method performance.

Risk assessment and factor screening were performed, followed by DoE-based optimization to enhance method robustness and reliability. High-risk variables influencing analytical performance were systematically identified and visualized using an Ishikawa (fishbone) diagram in Figure 3, ensuring controlled and consistent method performance throughout the analytical lifecycle [20,21,22].

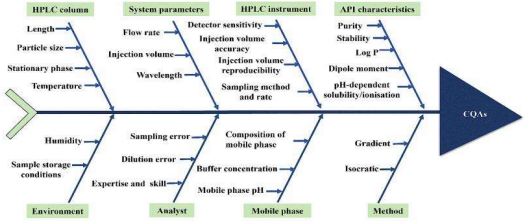


Figure 3: Ishikawa fishbone diagram showing causes and effects

Stress Degradation Studies[23-27]

Forced degradation studies were conducted using the optimized RP-HPLC method in accordance with ICH Q1A(R2) and ICH Q1B guidelines to assess the stability of EMCB under acidic, alkaline, oxidative, photolytic, and thermal conditions. Stress conditions were selected based on reported literature, and methanol was used as the solvent to ensure uniform exposure. Minor adjustments in mobile phase composition and flow rate ensured adequate separation of degradation products. Results are summarized in Table 8.

Acidic degradation: The stock solution (1000 µg/mL) was treated with 2 N HCl and heated at 60 °C for 30 min, The resulting solution was diluted with mobile phase to obtain 10 µg/mL and 10 µL of this solution was injected for chromatographic analysis.

Alkaline degradation: For base-induced degradation, stock solution was treated with 2 N NaOH, heated at 60 °C for 30 min, diluted to 10 µg/mL, and 10 µL aliquot was injected for analysis.

Oxidative degradation: Stock solution was treated with 3% H_2O_2 followed by heating at 60 °C for 30 min, diluted to 10 µg/mL, and 10 µL sample was injected.

Photolytic/thermal degradation: EMCB solution (10 µg/mL) was first exposed to UV radiation for 24 h followed by heating at 60 °C for 30 min, and analyzed by RP-HPLC.

Greenness assessment using AGREE and AGREEprep method

The environmental sustainability of the developed RP-HPLC method for EMCB was evaluated using AGREE and AGREEprep tools. AGREE assesses method greenness based on the 12 principles of green analytical chemistry, generating a 0–1 score displayed as a color-coded circular pictogram reflecting solvent use, reagent hazard, energy consumption, instrumentation, and waste generation. To complement this evaluation, AGREEprep was applied to specifically assess the sample preparation stage using ten criteria related to reagent toxicity, solvent volume, waste, and energy demand. The combined application of AGREE and AGREEprep provided a comprehensive, quantitative, and visual assessment of method greenness, supporting the development of an environmentally sustainable analytical method[28,29,30,31].

HPTLC- Spectral and Densitometric Evaluation

Preparation of Standard Stock Solution

EMCB (20 mg) was dissolved in methanol and sonicated for 10 min, and diluted to 10 mL to obtain a standard stock solution with a concentration of 2000 µg/mL (2000 ng/µL).

Preparation of Working Standard Solutions

Working standards for linearity study were prepared by serial dilution of the stock solution to produce concentrations ranging from 200–1200 ng/band, achieved by applying different volumes (0.1–0.6 µL) of the stock solution.

Application on HPTLC Plate

0.1 µL of each working standard solution was applied on the HPTLC plate using a CAMAG Linomat applicator as 6 mm bands, maintaining a 10 mm inter-band distance.

Analysis of Marketed Formulation:

Twenty tablets of the marketed formulation containing Emtricitabine (label claim: 200 mg per tablet) were accurately weighed and finely powdered. A quantity of tablet powder equivalent to 200 mg of Emtricitabine was transferred into a 100 mL volumetric flask. About 60 mL of methanol was added and the mixture was subjected to sonication for 15 minutes to facilitate complete extraction of the drug. The solution was cooled to room temperature and the volume was made up to 100 mL with methanol. The resulting solution was filtered through Whatman filter paper No. 41, to obtain a 2000 µg/mL sample stock solution.

Quantification

A volume of 0.4 µL of both standard and sample solutions (800 ng/band) was applied, and quantification was performed by comparing sample peak areas with standards applied at the same concentration.

Validation of HPTLC Analytical Method

The method was validated according to ICH Q2(R2) guidelines to ensure suitability, accuracy, precision, and reliability. Validation parameters included accuracy, precision, specificity, linearity, LOD, LOQ, range, robustness, and ruggedness.

Accuracy
Accuracy of the established analytical method was assessed by recovery studies with standard addition method, at 80%, 100%, and 120% levels.

Precision
Precision was assessed as system precision (repeat scanning of 800 ng/band) and method precision (repeatability) using multiple sample preparations, with acceptable %RSD values. Low %RSD values indicated acceptable precision of the method.

Specificity
It was confirmed by comparing chromatograms and overlay spectra of standard and marketed formulation, ensuring no interference.

Linearity and Range
Linearity of the developed method was evaluated over the concentration range of 200–1200 ng/band using EMCB working solutions prepared in the mobile phase. Calibration plot was generated by plotting peak area versus concentration, and method linearity was confirmed from the regression equation and correlation coefficient (R^2).

Limit of Detection (LOD and Limit of Quantitation (LOQ)
LOD and LOQ were calculated from the standard deviation of response and slope of the calibration curve in accordance with ICH guidelines (LOD = 3.3σ/S; LOQ = 10σ/S).

Robustness
Robustness was evaluated by making slight changes in chromatographic conditions such as mobile phase composition Toluene: methanol: n-hexane (6:1:3), Toluene: methanol: n-hexane (6:1.5:2.5). wavelength (289 and 285), saturation time (10 minutes, 15 minutes) activation of plates (15 and 10 minutes).

Ruggedness (intermediate precision)
Ruggedness was assessed by analysis performed by two analysts under identical conditions using 800 ng/band, demonstrating method consistency.

Forced Degradation Studies for Emtricitabine[9-12]
Forced degradation studies were performed to assess the stability of Emtricitabine under acidic, alkaline, oxidative, thermal, photolytic, and neutral conditions by determining the percentage of drug remaining after stress exposure.

Acidic and alkaline degradation: EMCB (20 mg) was subjected to acidic or alkaline stress by adding 3 mL of 0.1 N HCl or 0.1 N NaOH and allowing the mixture to stand at room temperature for 45 min. The solution was then made up to 10 mL with methanol and analyzed.

Oxidative degradation: A 20 mg sample of Emtricitabine (EMCB) was exposed to 3 mL of 3% hydrogen peroxide and allowed to stand at room temperature for 45 minutes. The resulting solution was then diluted with methanol and analyzed.

Thermal degradation: EMCB (20 mg) was exposed to dry heat at 40 °C for 30 min, the sample was then dissolved in methanol, diluted to a final volume of 10 mL, and subsequently analyzed.

Photolytic degradation: EMCB (20 mg) was exposed to UV light at 254 nm for 24 h, the sample was then dissolved in methanol, diluted to a final volume of 10 mL, and subsequently analyzed.

Neutral degradation: EMCB (20 mg) was treated with distilled water (3 mL) for 45 min at room temperature, diluted with methanol, and analyzed using the validated method.

RESULTS AND DISCUSSION

Method development of EMCB employing HPLC
In RP-HPLC method development, selection of the stationary phase and mobile phase was guided by the physicochemical properties of EMCB. Initial trials with 100% methanol, methanol–water, and acetonitrile–water mixtures resulted in rapid elution and poor resolution. Incorporation of an acetate buffer improved chromatographic performance, and the optimized mobile phase of acetate buffer: acetonitrile (70:30, v/v; pH 3.5) resulted in well resolved and symmetrical peaks. Separation was performed on a Hypersil C18 column with a retention time of approximately 2.5 min. All system suitability parameters met acceptance criteria (theoretical plates >2000, tailing factor <2). The method showed consistent retention for standard and marketed formulation (RT ≈ 2.56 min) in Figure 4 (A) and 4 (B) with 99.80% purity, confirming reliable and efficient chromatographic performance.

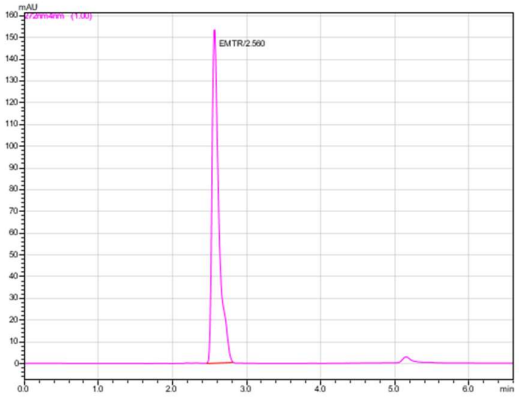


Figure 4 (A): Chromatogram of working standard containing Emtricitabine

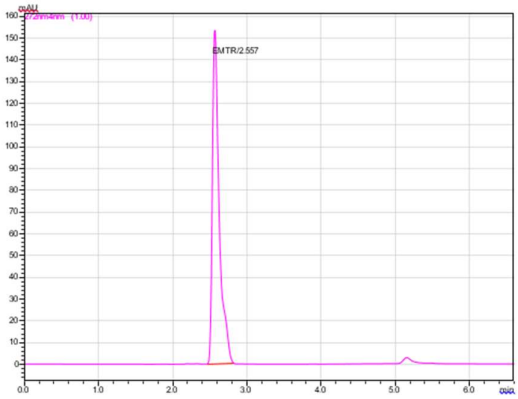


Figure 4 (B): Chromatogram of Emtricitabine in marketed formulation

System Suitability

System suitability was assessed by six replicate injections of the standard solution, evaluating retention time, theoretical plates, tailing factor, and %RSD. All parameters complied with ICH acceptance criteria, confirming consistent system performance (Table 3).

Table 3: A summary of the System suitability test parameters of EMCB

Parameter	HPLC assay
Concentration selected	20 µg/mL
Run time	10 min
Retention time	2.56 ± 0.01
Number of theoretical plates (N)	6015 ± 0.09
Tailing factor	1.232 ±0.08
Range	10-60 µg/mL
Linearity (regression equation)	Y=31697x + 28668
Correlation coefficient	R^2: 0.9989

Method Validation

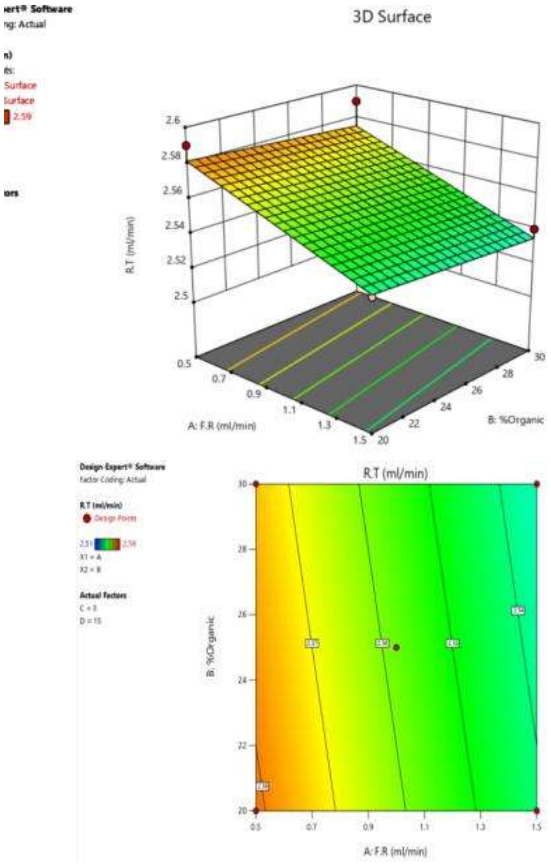
The method was validated in accordance with ICH guidelines, confirming its reliability, accuracy, and reproducibility. Linearity was established over 10–60 µg/mL (Table 4) with excellent correlation (R^2 = 0.9989) shown in Figure 9. Accuracy of the method was assessed at concentration levels of 80%, 100%, and 120% levels showed recoveries within 99.08–100.38%. Precision studies indicated minimal intra-day and inter-day %RSD (<2). The method showed good sensitivity with LOD and LOQ of 0.299 and 0.906 µg/mL, respectively. Robustness was confirmed by deliberate variations in flow rate, pH, and mobile phase composition using both conventional (Table 5) and AQbD (Box–Behnken design) approaches (Table 6). Validation results are shown in Table 5. Cause–effect relationships were evaluated using an Ishikawa (fishbone) diagram to systematically identify potential factors influencing method performance (Figure 3) [32,33]. Statistical analysis (ANOVA) (Table 7), response surface (Figure 5 (A–H)), contour plots (Figure 7), perturbation (Figure 6 (A-D)), and desirability plots confirmed significant models, defined design space Figure 8 and consistent performance under optimized conditions. The mathematical equations derived from the model are presented as follows:

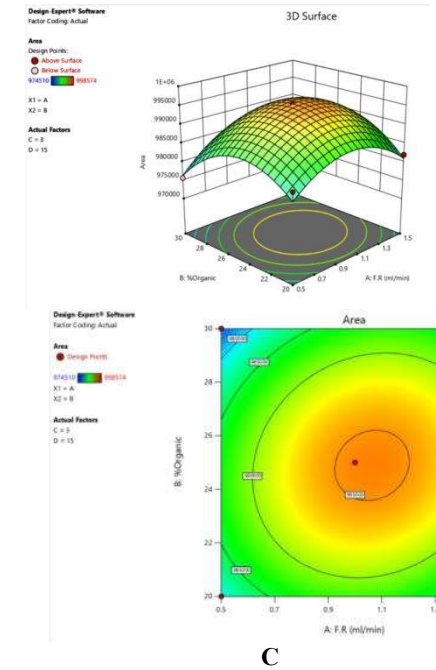
Retention Time Y_1 = +2.56 -0.0200A-0.0033B-0.0083C-0.0050D … (1)

Area Y_2 = +9.954+1822.00A-557.42B-2740.08C+1783.83D+1744.75AB-2000.75AC-1561.00AD+1351.75 BC-316.25BD-4368.75CD-7050.63A^2 -7963.75B^2 -3317.00C^2 -4738.87D^2 … (2)

Theoretical Plate Y_3 = +3194.00+23.33A+5.75B+36.75C-45.33D-3.50AB-54.00AC-16.00AD+14.25BC-3.50BD+41.00CD-55.96A^2+41.42B^2-6.33C^2-54.71D^2 … (3)

Tailing factor Y_4 = +1.68 + 0.1125 A+ 0.0017B+0.2042C+0.0550D- 0.0250AB-0.2050AC -0.3125AD-0.0975BC-0.0025BD-0.0600CD … (4)





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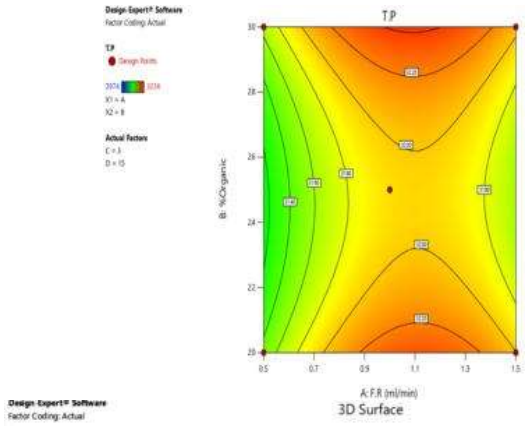

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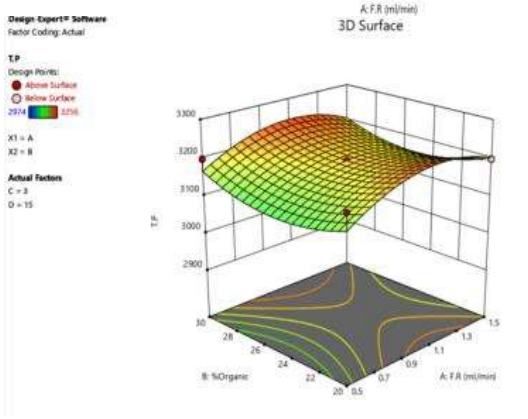

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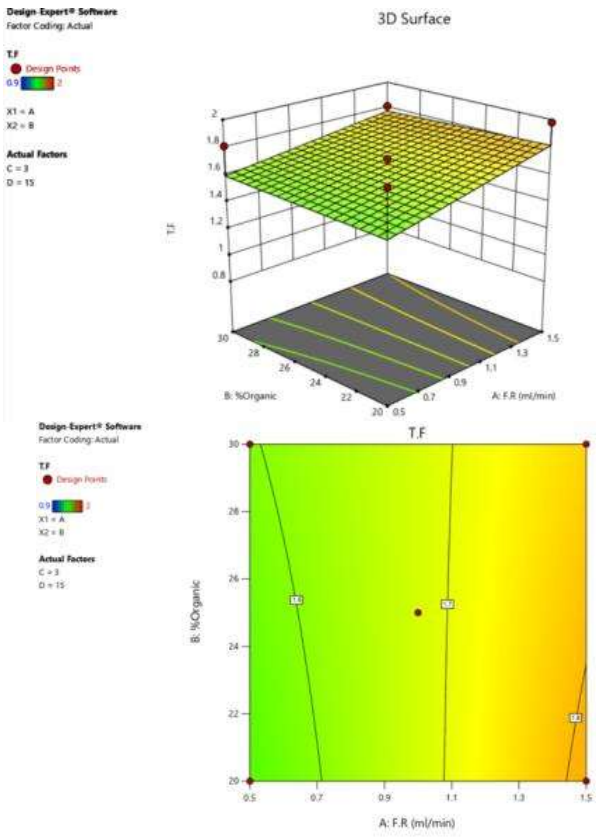

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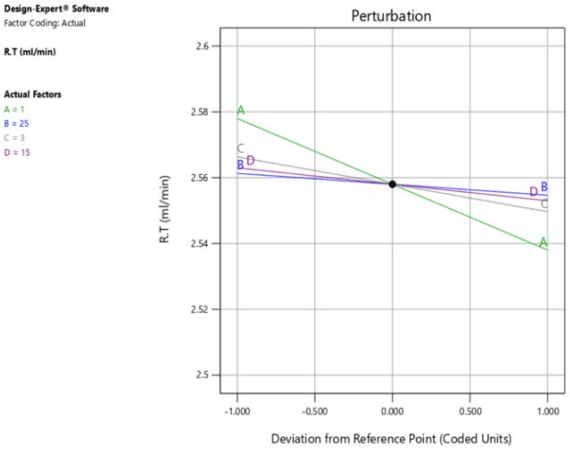
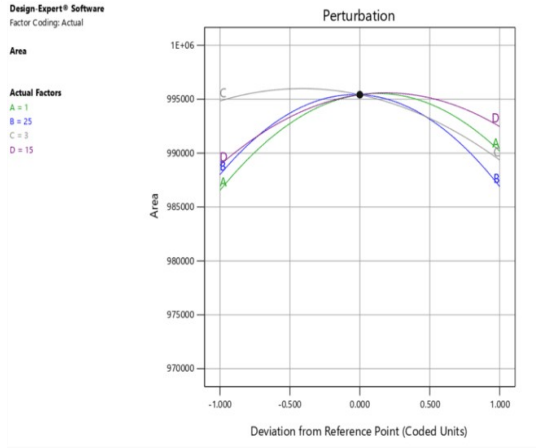
Figure 5: A 3D plot and control plot of responses R.T, Area, T.P, and T.F





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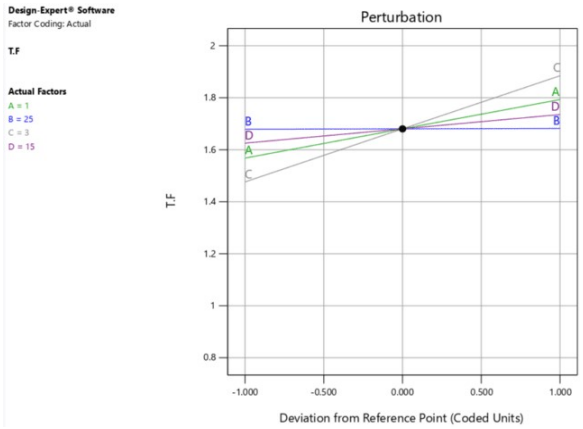

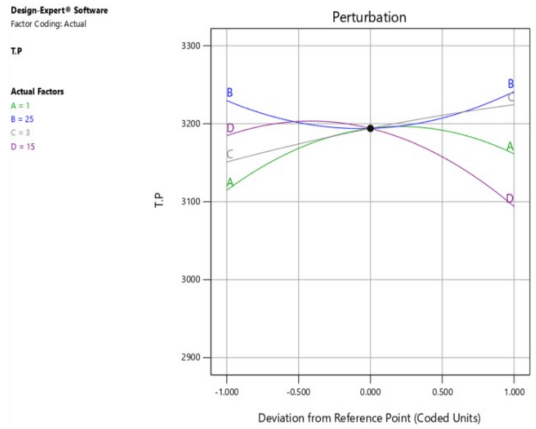

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Figure 6: Perturbation plots (A-D) showing the effect of factors on responses (RT, Area, TP, & TF)

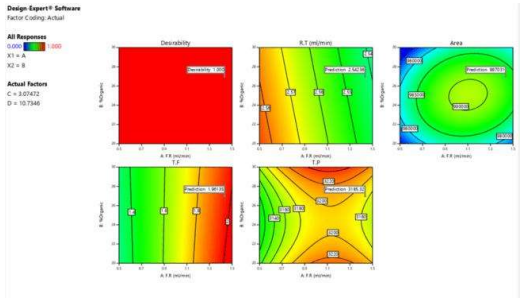

Figure 7: The desirability of contour plots

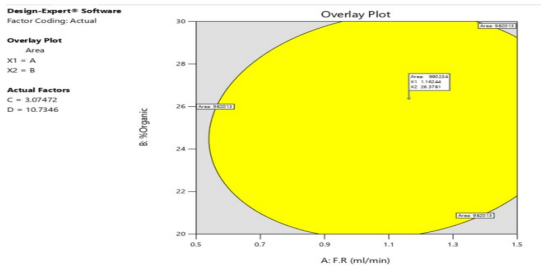

Figure 8: Overlay Plot

Table 4: Linearity of EMCB by RP-HPLC

Concentration (µg/mL)	Peak Area response (mV)
10	316918
20	692258
30	983217
40	1306102
50	1608346
60	1921450

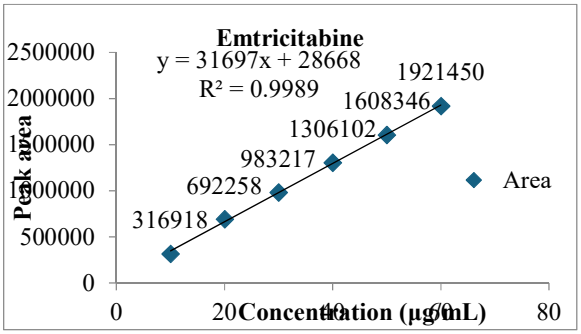

Figure 9: Calibration curve for EMCB showing linearity with area of peak (10-60 µg/ml)

Table 5: Validation results of emtricitabine -HPLC

Sr. No.	Parameter	Statistical analysis	
	Accuracy	80%	Mean % Recovery = 99.57, SD=0.54 %RSD=0.542
		100%	Mean % Recovery = 99.68, SD=0.39 %RSD=0.391
		120%	Mean % Recovery = 99.89, SD=0.48 %RSD=0.48
	Intraday Precision	**Concentration assayed (µg/mL) (n= 3)**	Mean ± SD, % RSD
		10	Mean=314261, SD=2442.71, %RSD=0.777
		20	Mean=694467, SD=2776.67, %RSD=0.400
		30	Mean=985963, SD=2505.89, %RSD=0.254

	Inter-day Precision	**Concentration assayed (µg/mL) (n= 3)**	Mean ± SD, % RSD
		10	Mean=312971, SD=3426.83, %RSD=1.095
		20	Mean=692277, SD=2727.55, %RSD=0.394
		30	Mean=987296, SD=3734.28, %RSD=0.378
	Linearity (10-60 µg/ml)	R^2 = 0.9989	
	LOD (µg/mL) & LOQ (µg/mL)	0.299 & 0.906	
	Robustness at Flow rate (mL/min) (±0.1)	0.9, 1.0, 1.1	Mean %RSD = 0.35
	Robustness at Composition of Mobile phase (v/v) (± 2%)	Acetate buffer: Acetonitrile pH 3.5 ± 0.05 in the ratio of 73:27 v/v	0.15%
		Acetate buffer: Acetonitrile pH 3.5 ± 0.05 in the ratio of 70:30 v/v	0.34%
		Acetate buffer: Acetonitrile pH 3.5 ± 0.05 in the ratio of 67:33 v/v	0.54%
	Robustness at pH of mobile phase (±0.2)	Acetate buffer: Acetonitrile (70:30 v/v) with pH 3.3 ± 0.05	0.92%
		Acetate buffer: Acetonitrile (70:30 v/v) with pH 3.5 ± 0.05	0.20%
		Acetate buffer: Acetonitrile (70:30 v/v) with pH 3.7 ± 0.05	0.52%

Table 6: Robustness employing AQbD approach (BBD)

	Factors			**Responses**				
Run	A: pH	B: % Organic	C: Flow rate	Injection volume	Retention Time	Area	Tailing Factor	Theoretical Plates
1	2.5	25	1	20	2.55	998574	1.79	3025
2	2.5	25	1.5	15	2.54	991540	1.72	3194
3	2.5	25	1	10	2.57	982145	1.42	3189
4	2.5	30	1	15	2.58	983211	1.49	3195
5	2.5	20	1	15	2.59	986541	1.08	3190
6	2.5	25	0.5	15	2.58	987541	0.9	2974
7	3	25	0.5	10	2.57	974510	1.08	3120
8	3	30	0.5	15	2.59	975681	1.81	3198
9	3	30	1	20	2.51	983237	1.59	3175
10	3	20	1	20	2.54	982541	1.68	3181
11	3	20	0.5	15	2.59	983217	1.88	3191
12	3	25	1.5	10	2.54	986281	1.92	3180
13	3	20	1	10	2.56	983280	1.89	3190
14	3	30	1.5	15	2.54	981456	1.81	3191
15	3	25	1	15	2.54	995420	1.72	3194
16	3	30	1	10	2.57	985241	1.81	3198
17	3	25	1.5	20	2.55	987542	1.59	2974
18	3	25	0.5	20	2.59	982015	2	2978
19	3	20	1.5	15	2.54	982013	1.98	3198

20	3.5	30	1	15	2.56	9823 54	1.81	3256
21	3.5	25	1	10	2.56	9852 64	1.71	3189
22	3.5	25	1	20	2.57	9842 18	1.84	3189
23	3.5	25	0.5	15	2.54	9842 81	1.85	3188
24	3.5	25	1.5	15	2.51	9802 77	1.85	3192
25	3.5	20	1	15	2.57	9802 77	1.79	3194

Table 7: Statistical parameters by ANOVA analysis for the responses

Parameters	SS	Df	MS	F-value	p-value	Model F-value	Model p-value	Prob > F
Response Y_1 (Retention Time)								
(A) pH	0.0008	1	0.0008	2.48	0.1313	4.50	<0.0500	The linear model is significant
(B) % Organic	0.0001	1	0.0001	0.3960	0.5363			
(C)Flow rate	0.0048	1	0.0048	14.26	0.0012			
(D) Injection volume	0.0003	1	0.0003	0.8911	0.3564			
Response Y_2 (Area)								
(A) pH	9.010	1	9.010	7.58	0.0203	3.19	<0.0500	The quadratic model is significant
(B) % Organic	3.729	1	3.729	0.3138	0.5877			
(C)Flow rate	3.984	1	3.984	3.35	0.0970			
(D) Injection volume	3.818	1	3.818	3.21	0.1033			
Response Y_3 (Tailing Factor)								
(A) pH	0.5002	1	0.5002	10.60	0.00057	2.76	<0.0500	The 2FI model is significant
(B) % Organic	0.0000	1	0.0000	0.00007	0.97992			
(C)Flow rate	0.1519	1	0.1519	3.22	0.09942			
(D) Injection volume	0.0363	1	0.0363	0.77 05	0.3949			
Response Y_4 (Theoretical Plates)								
(A) pH	16206.75	1	16206.75	5.73	0.03 77	3.06	<0.0500	The quadratic model is significant
(B) % Organic	396.75	1	396.75	0.1404	0.7157			
(C)Flow rate	6533.33	1	6533.33	2.31	0.1594			
(D) Injection volume	24661.33	1	24661.33	8.72	0.0144			

Forced degradation studies of EMCB by HPLC[34-36]

The forced degradation results indicates that Emtricitabine shows different stability profiles under multiple stress conditions. Under acidic stress (2 N HCl), the drug exhibited minimal degradation (2.58%), suggesting good stability in acidic environments. Alkaline conditions (2 N NaOH) caused moderate degradation (5.84%), indicating comparatively higher susceptibility than acid stress. The highest degradation was observed under oxidative conditions (3% H_2O_2), with 11.86% degradation, confirming that Emtricitabine is most sensitive to oxidative stress. In the case of photo-thermal stress (UV light with heat), the drug showed moderate degradation (3.82%), reflecting limited sensitivity to combined light and thermal exposure. Overall, the results demonstrate that Emtricitabine is relatively stable under acidic and photo-thermal conditions, moderately unstable in alkaline media, and most vulnerable to oxidative degradation. These results confirm the stability-indicating nature of the developed method as summarized in Table 8.

Table 8: Degradation products obtained for EMCB under stress conditions

Force Degradation	Percent recovery	Percent Degradation	RT of Drug and degraded products
Acidic degradation reflux at 60°C for 30 minutes with 2 N HCL	97.42±0.87%	2.57± 0.15%	2.24 and 1.937 min
Alkali degradation reflux at 60°C for 30 minutes with 2 N NaOH	94.15±1.74%	5.84± 0.54%	2.25 and 1.938 min
Oxidative degradation reflux at 60°C with 3 % H_2O_2 for 30 minutes	88.14±1.04%	11.85±1.02 %	2.241 and 0.389, 1.937 min
Photolytic degradation using a UV energy source with a minimum power of 200 watts for 30 minutes	96.18±1.24%	3.81%±1.22%	2.23 and 1.934 min

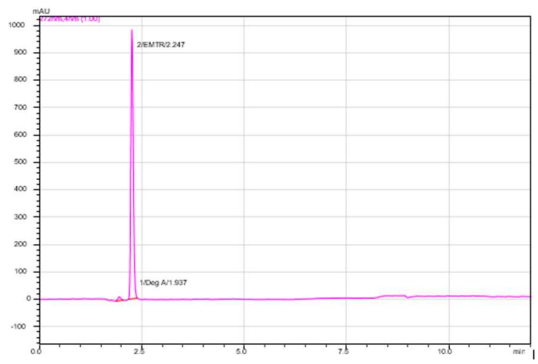

Figure 9(A): Chromatogram of Stability studies for Emtricitabine in Acid

Figure 9 (B): Chromatogram of Stability studies for Emtricitabine in Alkali

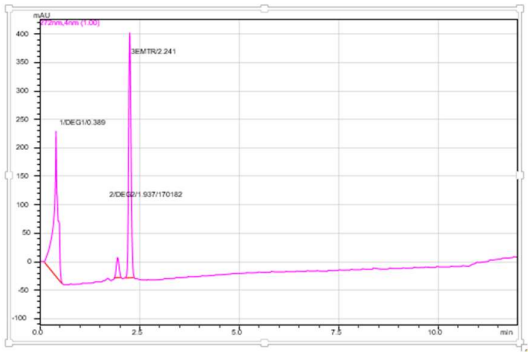

Figure 9 (C): Chromatogram of Stability studies for Emtricitabine in Oxidation

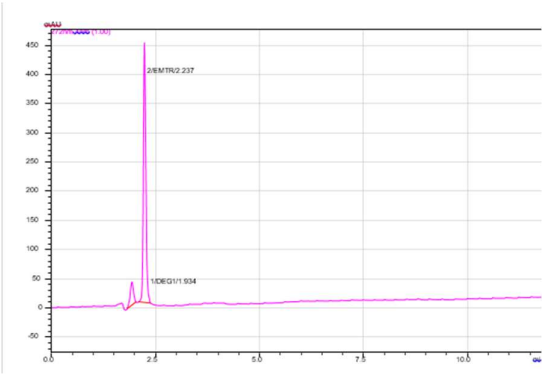

Figure 9 (D): Chromatogram of Stability studies for Emtricitabine in Photolytic condition

Greenness assessment using AGREE and AGREEprep method

The method showed that the developed method possesses good environmental sustainability. AGREEprep yielded a greenness score of 0.63 (Figure 10A), indicating acceptable performance, with scope for improvement in waste reduction, sample throughput, and post-sample preparation practices. AGREE evaluation provided a comparable score of 0.64 (Figure 10B), demonstrating strong adherence to the 12 principles of green analytical chemistry, particularly in reduced sample consumption, automation, and minimal derivatization. Minor limitations related to energy consumption and reagent toxicity were identified. Overall, the combined AGREE and AGREEprep assessment confirms the method's notable greenness while highlighting opportunities for further environmental optimization [29-31].

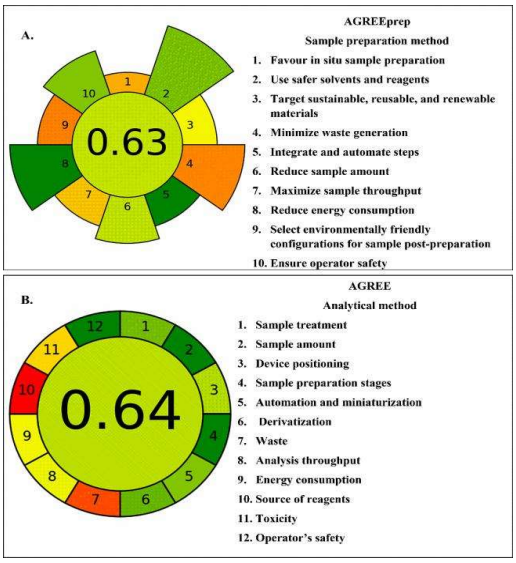

Figure 10 A and 10 B: AGREEprep assessment yielded a greenness score of 0.63, and 0.64, reflecting strong compliance with the 12 principles of green analytical chemistry

System Suitability Studies for Emtricitabine (HPTLC) [45-47]

System suitability was evaluated by repeated densitometric scanning of Emtricitabine (400 ng/band) under optimized HPTLC conditions. Consistency of Rf values, peak area repeatability, and %RSD were assessed. The results are summarized in Table 9.

Table 9: System Suitability Parameters for Emtricitabine (HPTLC)

Parameter	Observation
Rf value (mean ± SD)	0.48 ± 0.006
Rf range	0.47 – 0.49
Peak area (mean, AU)	5755.18
Standard deviation (SD)	5.12
%RSD of peak area	0.09
Peak shape	Sharp and symmetric
Interference at drug Rf	Not observed
System precision acceptance limit	%RSD < 2%
Compliance	Complies with ICH Q2(R2) criteria

Spectral and Densitometric Evaluation

The UV spectrum of Emtricitabine showed a sharp λmax at ~287 nm with good absorbance and spectral purity, confirming suitability for densitometric detection (Figure 11). Overlay spectra of the standard and marketed formulation exhibited complete overlap, indicating specificity and no excipient interference (Figure 12). Three-dimensional spectra further confirmed peak purity and spectral homogeneity (Figure 13). For HPTLC analysis, working standards of 200–1200 ng/band were prepared by applying 0.1–0.6 µL of stock solution, with overlay spectra shown in Figure 14 representative densitogram of the working standard (800 ng/spot) is shown in Figure 15. The method was successfully applied to the marketed formulation, producing a well-resolved peak matching the standard (Figure 16).

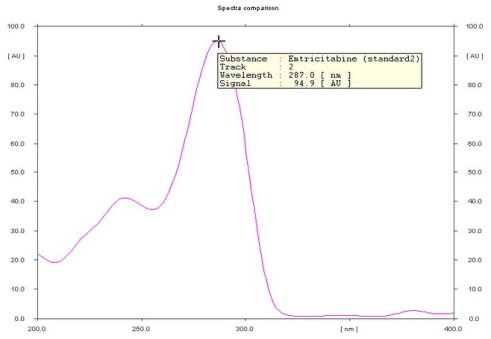

Figure 11: UV spectra for standard for Emtricitabine

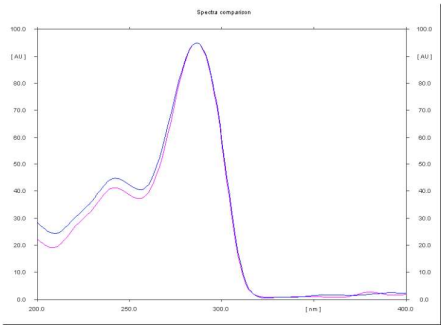

Figure 12: Overlay spectra for standard and marketed formulation of Emtricitabine

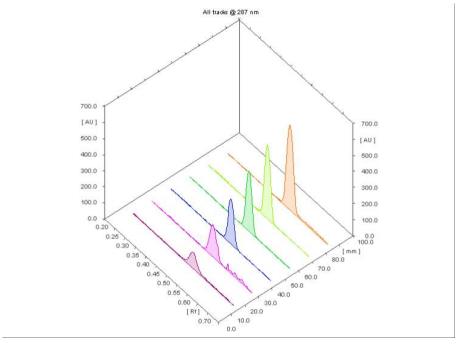

Figure 13: 3D Spectrum for standard drug

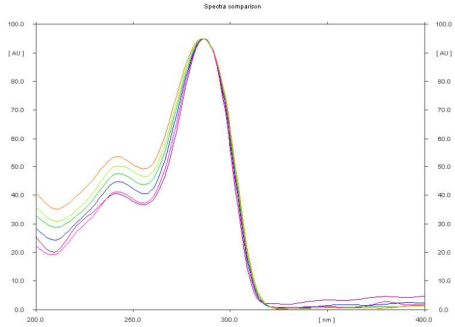

Figure 14: Overlay spectra of Working standard solutions (0.2–1.2 µg/mL/200 to 1200 ng/µL)

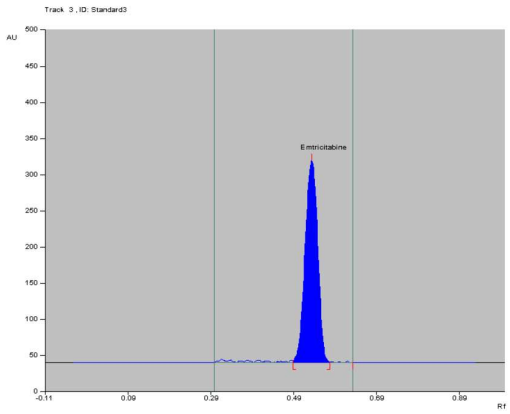

Figure 15: Densitogram of working standard of Emtricitabine (0.8 µg/800ng/spot)

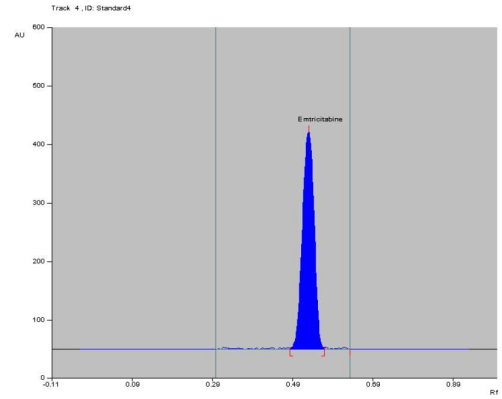

Figure 16: Densitogram of Marketed formulation of Emtricitabine

Validation of Analytical Method[47-53]

Accuracy was confirmed by recovery studies at 80%, 100%, and 120% levels, showing recoveries of 99.93%, 100.97%, and 99.90%, respectively, demonstrating excellent accuracy across the range System and method precision, evaluated at 800 ng/band, showed %RSD < 2%, confirming good repeatability and reproducibility Specificity was established by the absence of interfering peaks at the drug Rf. Linearity was observed over 200–1200 ng/band (Table 10) with a proportional response between concentration and peak area (Figure 17), complying with ICH Q2(R2). The LOD and LOQ were 1.65 and 5.01 ng/band, respectively, and the method was linear within the range of 200–1200 ng/band (0.2–1.2 µg/band). Robustness studies involving minor variations in chromatographic conditions confirmed method reliability. Intermediate precision (ruggedness), assessed using different analysts under identical conditions, demonstrated consistent results, confirming the ruggedness of the method. Overall results of validation are shown in Table 11.

Table 10: Linearity of Emtricitabine

Amount (ng)	Area (AU)
200	3009.01
400	5753.94
600	7122.2
800	9078.64
1000	11245.15
1200	14096.98

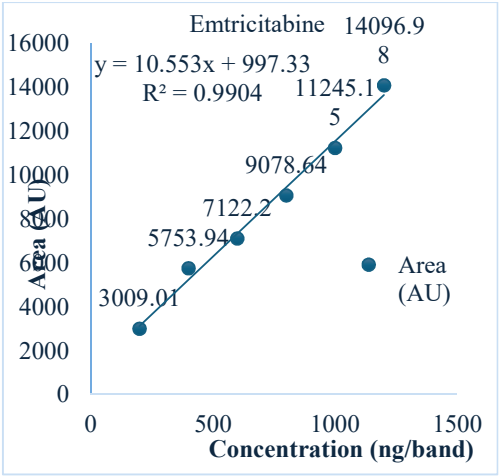

Figure 17: Calibration curve for Emtricitabine

Table 11: Validation results of emtricitabine - HPTLC

Sr. No.	Parameter	Statistical analysis	
	Accuracy	80%	Mean % Recovery = 99.93, SD=0.07, %RSD=0.07
		100%	Mean % Recovery = 100.97, SD=0.22, %RSD=0.22
		120%	Mean % Recovery = 99.90, SD=0.09, %RSD=0.09
	System precision	Mean peak area= 5755.18 ± SD = 5.12, % RSD = 0.09	
	method precision	Mean % Amount Found = 100.30 ± SD = 0.58, % RSD = 0.58	
	Intraday Precision	**Concentration assayed (µg/mL) (n= 3)**	Mean ± SD, % RSD
		200	Mean Area (AU)=3031.53, SD=1.19, %RSD=0.03

		400	Mean Area (AU)= 5759.23, SD=5.50, %RSD=0.09
		600	Mean Area (AU)= 7154.27, SD=28.34, %RSD=0.39
	Inter-day Precision	**Concentration assayed (µg/mL) (n= 3)**	Mean ± SD, % RSD
		200	Mean Area (AU)= 3089.37, SD=10.60, %RSD=0.34
		400	Mean Area (AU)= 5849.1, SD= 52.82, %RSD=0.9
		600	Mean Area (AU)= 7284.2, SD=36.95, %RSD=0.5
	Linearity (200-1200 ng/band)	R^2 = 0.9904	
	LOD (µg/mL) & LOQ (µg/mL)	*1.65* ng/band and 5.01 ng/band	

		Deliberate Variation Applied	**Rf**	**Mean Peak Area**	**± SD**	**%RSD**
	Robustness at different Composition of Mobile phase (v/v/v)	Toluene: methanol: n-hexane (6:1:3)	0.47	3029.73	1.418	0.04
		Toluene: methanol: n-hexane (6:1.5:2.5)	0.49	3029.8	2.09	0.06
	Robustness at different Wavelength	289	0.56	3034	5.5	0.18
		285	0.58	3183	37.05	1.16
	Robustness at different Saturation time	10 minutes	0.53	3030.13	3.91	0.12
		15 minutes	0.49	3029.46	4.46	0.14
	Robustness at different time of Activation of plates	15 minutes	0.50	3027.1	7.77	0.25
		10 minutes	0.48	3029.13	5.12	0.16
	Ruggedness	Analyst I	% Amount found= 100.30		0.58	0.58
		Analyst II	% Amount found= 100.22		0.57	0.57

Results of Forced Degradation Studies of Emtricitabine

Forced degradation studies demonstrated that Emtricitabine exhibits distinct stability behavior under different stress conditions. The drug showed maximum degradation under oxidative stress, with about 30.6% degradation, indicating high susceptibility to oxidation. Thermal stress caused moderate degradation (22.0%), suggesting sensitivity to elevated temperature. Under acidic conditions, Emtricitabine underwent moderate degradation (14.4%), whereas alkaline, photolytic, and neutral conditions resulted in less degradation (9.5%, 10.8%, and 6.5%, respectively), reflecting relatively better stability in these environments. Overall, the degradation behavior (Figure 18 A to 18 F) confirms that Emtricitabine is most vulnerable to oxidative stress, moderately sensitive to heat and acid, and comparatively stable under alkaline, photolytic, and neutral conditions (Table 12).

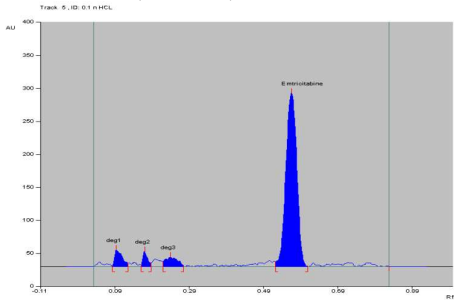

Figure 18 (A): Densitogram of Stability studies for Emtricitabine in Acid

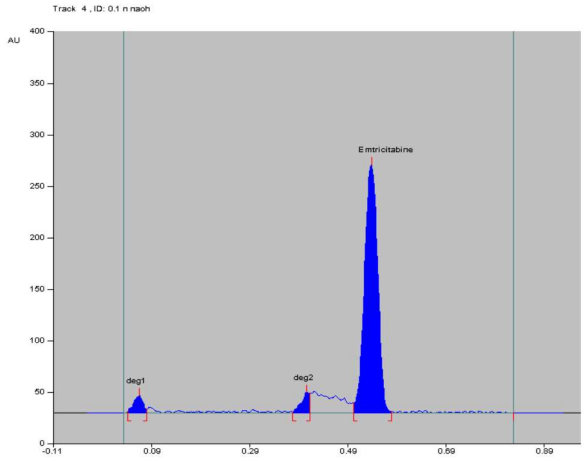

Figure 18 (B): Densitogram of Stability studies for Emtricitabine in alkali

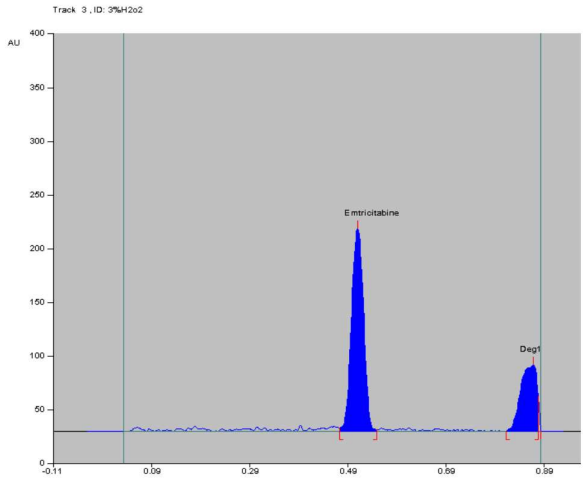

Figure 18 (C): Densitogram of Stability studies for Emtricitabine in oxidative condition

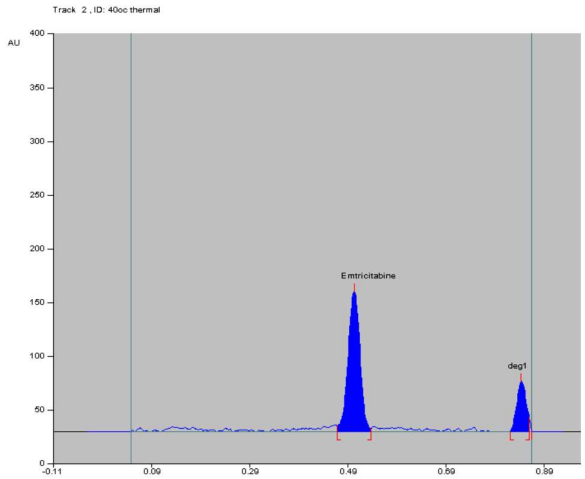

Figure 18 (D): Densitogram of Stability studies for Emtricitabine in Thermal condition

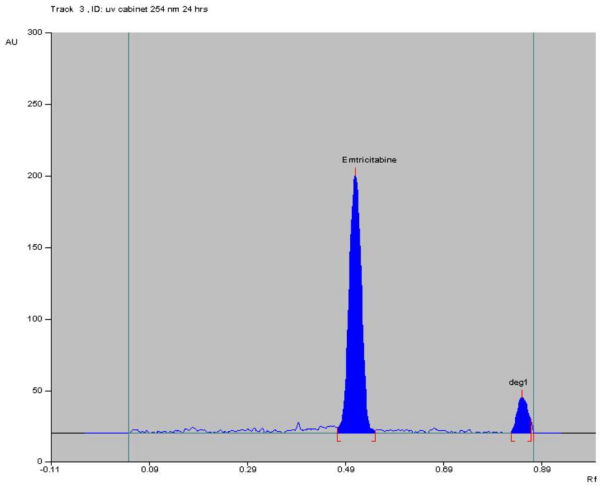

Figure 18 (E): Densitogram of Stability studies for Emtricitabine in photolytic condition

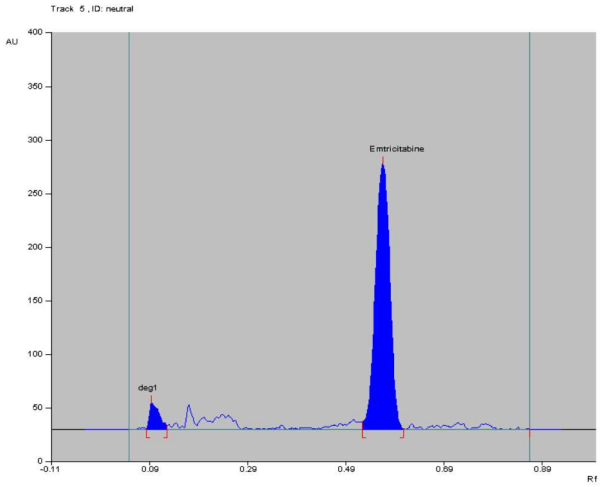

Figure 18 (F): Densitogram of Stability studies for Emtricitabine in Neutral condition

Table 12: Results of force degradation studies for Emtricitabine

Sr. no	Degradation Condition	Reagent / Condition	Time	Temperature	% Drug Undegraded	% Drug Degraded	Conclusion
1	Acid	HCl (0.1 N)	45 min	Room temperature	85.62	14.37	Moderate degradation
2	Alkali	NaOH (0.1 N)	45 min	Room temperature	90.45	9.54	Less degradation
3	Oxidation	3% H_2O_2	45	Room temp	69.41	30.59	Maximum

			min	eratu re			degr adat ion
4	Ther mal	Dry hea t	1 0 m in	40°C	77.9 7	22. 03	Mo dera te degr adat ion
5	Phot olyti c	UV ligh t	2 4 hr s	254 nm (UV cabi net)	89.1 9	10. 81	Les s degr adat ion
6	Neut ral	Wa ter/ Met han ol	4 5 m in	Roo m temp eratu re	93.5 5	6.4 5	Les s degr adat ion

Discussion

The developed AQbD-based RP-HPLC and HPTLC methods provided robust, reliable, and stability-indicating analysis of emtricitabine. Emtricitabine was selected due to its therapeutic importance in HIV treatment and the need for accurate quality control and stability assessment. A C18 column with acetate buffer: acetonitrile with glacial acetic acid (70:30, v/v; pH 3.5) in RP-HPLC and toluene: methanol: n-hexane (6:1:3, v/v/v) in HPTLC produced sharp, symmetrical peaks with excellent resolution, reproducibility, and acceptable retention/Rf values. The optimized pH minimized ionization and peak tailing, improving chromatographic performance. System suitability and validation results confirmed the methods were accurate, precise, robust, and compliant with ICH guidelines. Forced degradation studies demonstrated that emtricitabine undergoes degradation under various stress conditions, confirming the stability-indicating capability of both methods. Furthermore, AQbD using Box–Behnken Design enabled systematic optimization of critical parameters, resulting in a robust analytical method with improved reliability. Overall, the optimized RP-HPLC method is suitable for routine quality control and stability studies, while the HPTLC method offers a rapid and economical alternative for routine pharmaceutical analysis.

FINANCIAL ASSISTANCE

The authors declare no financial support.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTIONS

Varsha P. Patil designed the study, performed method development, validation, and degradation studies, analyzed data, and drafted the manuscript. Ravi Ajudia supervised the work, contributed to study design and analytical strategy, reviewed data, and revised and approved the manuscript. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication.

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