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RESEARCH ARTICLE

Isocratic Reverse-Phase High-Performance Liquid Chromatographic Assay for Simultaneous Estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, and Rilpivirine in Fixed-Dose Combination Tablets

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Abstract: An isocratic reverse-phase high-performance liquid chromatographic (RP-HPLC) method was validated for the concurrent quantitative determination of emtricitabine, tenofovir disoproxil fumarate, and rilpivirine in triple-combination pharmaceutical dosage forms. Chromatographic separation was achieved on a Hypersil BDS C18 column (250 times 4.6 mm ID, 5 µm particle size) utilizing a mobile phase consisting of potassium dihydrogen phosphate buffer (adjusted to pH 4.0 with orthophosphoric acid) and acetonitrile (35:65 v/v) at a flow rate of 1.0 mL/min with ultraviolet detection at 280 nm. Chromatographic elution yielded sharp, symmetrical peaks with mean retention times of 2.692 min, 4.402 min, and 5.725 min for emtricitabine, tenofovir disoproxil fumarate, and rilpivirine, respectively, within a total run time of 10 min. Beer-Lambert linearity was confirmed over concentration ranges of 50-300 µg/mL for emtricitabine, 75-450 µg/mL for tenofovir disoproxil fumarate, and 6.25-37.5 µg/mL for rilpivirine, each exhibiting linear regression correlation coefficients (R^2) exceeding 0.999. Method precision demonstrated relative standard deviations below 1.0% across repeatability, intra-day, and inter-day evaluations. Standard addition recovery studies yielded mean percentages of 99.79-100.72% for emtricitabine, 99.47-100.03% for tenofovir disoproxil fumarate, and 99.58-99.98% for rilpivirine. Limits of detection were 0.0128 µg/mL, 0.380 µg/mL, and 0.0016 µg/mL, while limits of quantitation were 0.039 µg/mL, 1.154 µg/mL, and 0.0049 µg/mL, respectively. Deliberate modifications in operational parameters confirmed analytical robustness without altering critical chromatographic attributes. The established protocol provides reliable throughput and reproducibility suitable for routine pharmaceutical quality control.

Keywords: RP-HPLC; Emtricitabine; Tenofovir disoproxil fumarate; Rilpivirine; Method validation.

1. Introduction

Fixed-dose combination (FDC) antiretroviral therapy is a major therapeutic milestone in human immunodeficiency virus type 1 (HIV-1) management. Combined formulations enhance patient compliance, reduce pill burden, and decrease the probability of viral resistance emergence [1]. The combination of emtricitabine (FTC), tenofovir disoproxil fumarate (TDF), and rilpivirine hydrochloride (RPV) forms a potent complete single-tablet regimen widely prescribed for treatment-naive adult patients infected with HIV-1 [2].

Emtricitabine (4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one) is a synthetic nucleoside analogue of cytidine (Figure 1a) [3]. Intracellularly, emtricitabine undergoes sequential phosphorylation by cellular enzymes to form emtricitabine 5'-triphosphate. This active metabolite competitively inhibits HIV-1 reverse transcriptase, incorporating into nascent viral DNA and forcing premature DNA chain termination [4]. Emtricitabine also exhibits measurable inhibitory action against chronic hepatitis B virus infection [5].

Tenofovir disoproxil fumarate is an acyclic nucleoside phosphonate prodrug of tenofovir, chemically named 9-[(R)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate (Figure 1b) [6]. Following oral absorption, TDF undergoes rapid diester hydrolysis to tenofovir, which is subsequently phosphorylated to tenofovir diphosphate by cellular kinases. Tenofovir diphosphate acts as an adenosine 5'-monophosphate analogue, competing with natural deoxyadenosine 5'-triphosphate for incorporation into viral DNA, resulting in viral transcript termination [7].

Rilpivirine, identified as 4-[[4-[[4-[(E)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]pyrimidin-2-yl]amino]benzonitrile (Figure 1c), belongs to the diarylpyrimidine class of non-nucleoside reverse transcriptase inhibitors (NNRTIs) [8]. Rilpivirine binds directly to a

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non-essential hydrophobic pocket adjacent to the active catalytic site of HIV-1 reverse transcriptase. This binding induces a conformational change in the enzyme active center, suppressing catalytic conversion of viral RNA to complementary DNA [9]. The chemical flexibility of the diarylpyrimidine scaffold allows adaptation to mutations in the binding pocket, conferring higher genetic barriers to resistance compared to earlier NNRTI compounds [10].

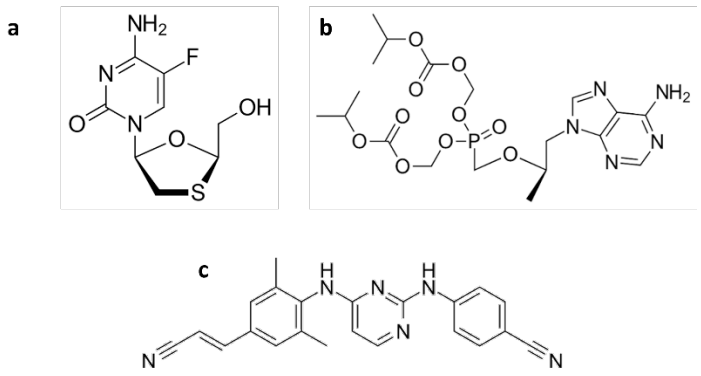

Figure 1. Structure of a. Emtricitabine b. Tenofovir disoproxil fumarate and c. Rilpivirine

Quality evaluation of multi-component pharmaceutical preparations requires validated analytical protocols capable of separating and quantifying distinct active ingredients simultaneously without mutual interference or degradation product overlap [11]. Survey of existing literature indicates various analytical strategies for individual active ingredients or dual-drug combinations, including spectrophotometric assays [12, 13], high-performance thin-layer chromatography (HPTLC) [14], liquid chromatography coupled with mass spectrometry (LC-MS/MS) for plasma matrixes [15–18], and high-performance liquid chromatography (HPLC) in biological fluids [19, 20].

Existing high-performance liquid chromatography procedures for simultaneous estimation of FTC, TDF, and RPV often utilize complex gradient elution profiles, long run times, or costly buffer systems [21, 22]. The objective of this study was to design and validate a simple, rapid, precise, and cost-effective isocratic reverse-phase high-performance liquid chromatographic method for the simultaneous quantification of emtricitabine, tenofovir disoproxil fumarate, and rilpivirine in solid oral dosage forms according to ICH Q2(R2) criteria [23].

2. Materials and Methods

2.1. Reagents and Reference Standards

Pharmaceutical reference standards of emtricitabine (99.8% purity), tenofovir disoproxil fumarate (99.6% purity), and rilpivirine (99.7% purity) were received as gift samples from Zydus Lifesciences Ltd. (Ankleshwar, India). Commercial combination tablets (Complera, containing 200 mg emtricitabine, 300 mg tenofovir disoproxil fumarate, and 25 mg rilpivirine) were procured commercially. HPLC grade acetonitrile and methanol were obtained from Rankem Chemicals. Potassium dihydrogen phosphate, orthophosphoric acid, and triethylamine of analytical reagent grade were obtained from Merck Life Science Pvt. Ltd. High-purity water was generated through a Millipore Milli-Q water purification system.

2.2. Instrumentation and Software

Chromatographic separations were carried out on a Shimadzu LC-2010 liquid chromatograph equipped with a quaternary pump, an auto-sampler with a fixed 10 µL loop, a column oven compartment, and a dual-wavelength ultraviolet/visible detector. System operations, data collection, and peak integration were managed using LC Solution software (Shimadzu Corporation, Kyoto, Japan). Precision analytical balances (Mettler Toledo) and a digital pH meter (pH 510, Eutech Instruments) were utilized for reagent preparations. Ultrasonic liquid degassing was conducted using a micro-processor controlled ultrasonic bath.

2.3. Chromatographic Conditions

Chromatographic separation was performed on a Hypersil BDS C18 stationary phase (250 mm times 4.6 mm ID, 5 µm particle size). The mobile phase comprised potassium dihydrogen phosphate buffer (adjusted to pH 4.0 using 10% v/v orthophosphoric acid) and acetonitrile in a 35:65 v/v ratio. Elution was executed in isocratic mode at a constant flow rate of 1.0 mL/min. The column

enclosure temperature was maintained at 30°C. The injection volume was fixed at 10 µL, and effluent detection was performed at a wavelength of 280 nm. The total chromatographic run time was established at 10.0 min.

2.4. Preparation of Solutions

2.4.1. Mobile Phase Preparation

Potassium dihydrogen phosphate (1.36 g) was dissolved in 1000 mL of purified water. The pH of the phosphate solution was adjusted to 4.0 ± 0.05 by dropwise addition of dilute orthophosphoric acid. The buffer solution was filtered through a 0.45 µm nylon membrane filter under vacuum. The final mobile phase was prepared by blending 350 mL of the aqueous phosphate buffer with 650 mL of HPLC grade acetonitrile (35:65 v/v). The mixture was degassed via ultrasonic agitation for 15 min prior to chromatographic use.

2.4.2. Diluent and Primary Standard Stock Solutions

The diluent consisted of acetonitrile and purified water mixed in a 50:50 v/v ratio. Primary stock standard solution was prepared by accurately weighing 20 mg of emtricitabine, 30 mg of tenofovir disoproxil fumarate, and 2.5 mg of rilpivirine standards into a 10 mL volumetric flask. Approximately 7 mL of diluent was added, and the contents were sonicated for 30 min to complete dissolution. The flask was allowed to cool to ambient temperature and diluted to volume with diluent, producing standard stock concentrations of 2000 µg/mL FTC, 3000 µg/mL TDF, and 250 µg/mL RPV. Working standard solutions were prepared by serial dilution of stock solutions using diluent.

2.4.3. Sample Solution Preparation from Dosage Forms

Twenty intact Complera tablets were weighed individually to calculate the average mass per tablet and ground into a uniform powder using a clean mortar and pestle. A portion of powdered tablet mass equivalent to 200 mg FTC, 300 mg TDF, and 25 mg RPV was transferred into a 100 mL volumetric flask containing 70 mL of diluent. The suspension was sonicated for 30 min with continuous agitation to ensure complete analyte extraction from tablet excipients. The flask was filled to volume with diluent and filtered through 0.45 µm PTFE syringe filters. Further volumetric dilutions were made with diluent to yield target working analytical concentrations of 200 µg/mL FTC, 300 µg/mL TDF, and 25 µg/mL RPV.

2.5. Method Validation

Validation was conducted in agreement with International Council for Harmonisation (ICH) Q2(R2) requirements for analytical procedure validation [23].

2.5.1. System Suitability and Specificity

System suitability parameters were evaluated by making six replicate injections of working standard solution containing FTC (200 µg/mL), TDF (300 µg/mL), and RPV (25 µg/mL). Chromatographic parameters, including peak retention time (t_R), theoretical plate count (N), peak tailing factor (T), and inter-peak chromatographic resolution (R_s), were recorded. Specificity was evaluated by comparing chromatograms obtained from diluent blanks, tablet excipient mixtures, standard reference solutions, and formulated tablet preparations to verify the absence of interfering peaks at the retention times of active analytes.

2.5.2. Linearity and Calibration Curves

Linearity was established by analyzing multi-level calibration standards prepared across six concentration levels ranging from 25% to 150% of the nominal assay concentration. The concentration ranges were 50-300 µg/mL for FTC (50, 100, 150, 200, 250, 300 µg/mL), 75-450 µg/mL for TDF (75, 150, 225, 300, 375, 450 µg/mL), and 6.25-37.5 µg/mL for RPV (6.25, 12.5, 18.75, 25, 31.25, 37.5 µg/mL). Calibration curves were constructed by plotting peak responses against corresponding analyte concentrations. Least-squares linear regression analysis was applied to evaluate slope, intercept, and correlation coefficient (R^2).

2.5.3. Precision

Instrument repeatability was evaluated through six sequential injections of nominal target standard solutions. Method intra-day precision was assessed by analyzing six separate sample solutions prepared from commercial tablet powder on the same day. Inter-day intermediate precision was evaluated by analyzing six independent sample solutions prepared and analyzed on three successive days. The percent relative standard deviation (%RSD) was calculated for peak area and assay content.

2.5.4. Accuracy and Recovery

Method accuracy was determined through standard recovery experiments using the standard addition technique. Known amounts of FTC, TDF, and RPV pure reference standards corresponding to 50%, 100%, and 150% of target levels were spiked into pre-analyzed sample tablet matrixes. Spike samples at each level were prepared in triplicate (n=3) and analyzed. Percentage recoveries and overall relative standard deviations were calculated for each drug component.

2.5.5. Limits of Detection and Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using standard deviation of the response (sigma) and slope of the calibration curve (S) according to mathematical expressions:

$$\text{LOD} = \frac{3.3 \times \sigma}{S}$$

$$\text{LOQ} = \frac{10 \times \sigma}{S}$$

where sigma represents the standard deviation of the y-intercepts obtained from six linear regression equations, and S represents the mean slope of the calibration curves.

2.5.6. Robustness

Robustness was evaluated by introducing deliberate perturbations into nominal chromatographic conditions. Parameter adjustments included mobile phase flow rate (1.0 ± 0.1 mL/min), column compartment temperature (30 ± 5°C), and organic mobile phase ratio (acetonitrile: buffer 65:35 v/v ± 5% v/v). Standard solutions were injected under altered conditions, and system suitability metrics were recorded to measure parameter sensitivity.

3. Results and Discussion

3.1. Method Optimization

Method development was aimed at achieving baseline separation of FTC, TDF, and RPV with sharp peak shapes within a short analysis time. During preliminary trials, various stationary phases (C8 and C18 columns) and mobile phase systems (methanol-water and acetonitrile-buffer combinations across pH values from 3.0 to 6.0) were tested. Methanol-based mobile phases generated higher column backpressures and broader peak shapes for RPV. Switching to acetonitrile provided faster elution with reduced backpressure. Adjusting the aqueous phase to 10 mM potassium dihydrogen phosphate buffer at pH 4.0 suppressed peak tailing for the basic nitrogen atoms present in rilpivirine and emtricitabine. A Hypersil BDS C18 column (250 mm times 4.6 mm ID, 5 µm) maintained at 30°C with an isocratic mobile phase of phosphate buffer (pH 4.0) and acetonitrile (35:65 v/v) at 1.0 mL/min yielded sharp, symmetrical peaks.

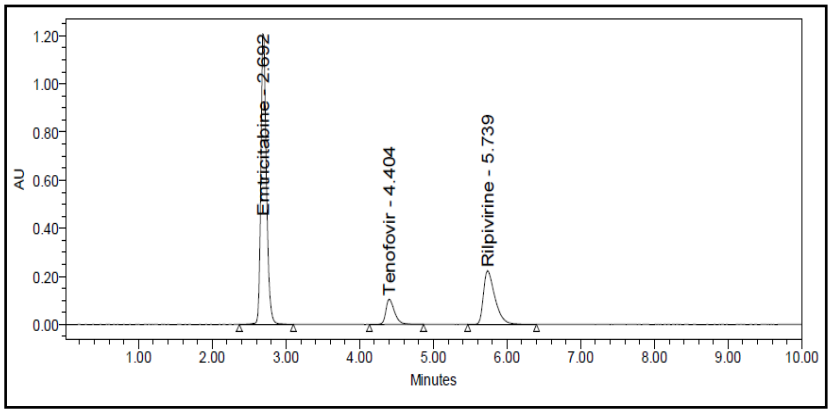

Figure 2. HPLC Chromatogram of Standard Solution Containing FTC (2.692 min), TDF (4.404 min), and RPV (5.739 min)

Ultraviolet spectrum indicated that a wavelength of 280 nm provided balanced absorbance sensitivity for all three active compounds, accommodating the lower dose concentration of rilpivirine (25 mg) relative to emtricitabine (200 mg) and tenofovir disoproxil fumarate (300 mg). Under these optimized conditions, retention times were 2.692 min for emtricitabine, 4.402 min for tenofovir disoproxil fumarate, and 5.725 min for rilpivirine, enabling complete analysis within 10.0 min (Figure 2).

3.2. Validation

3.2.1. System Suitability Parameters

System suitability was confirmed by six replicate injections of target standard mixture solutions. Theoretical plate counts exceeded 6,000 for FTC, 7,000 for TDF, and 6,500 for RPV. Tailing factors were below 1.6 for all three components, and inter-peak resolution values (R_s) were greater than 3.5, indicating adequate baseline separation. System suitability data are summarized in Table 1.

Table 1. System Suitability Parameters of the Chromatographic Method

Parameter	Emtricitabine	Tenofovir Disoproxil Fumarate	Rilpivirine	Acceptance Criteria
Retention Time (t_R, min)	2.692 ± 0.005	4.402 ± 0.048	5.725 ± 0.088	-
Theoretical Plates (N)	6032	7280	6569	> 2000
Tailing Factor (T)	1.27	1.29	1.54	le 2.0
Resolution (R_s)	-	5.82	4.31	> 2.0
Peak Area %RSD (n=6)	0.67%	0.65%	0.68%	≤ 2.0%

3.2.2. Linearity

Linearity was established over specified concentration ranges for FTC (50-300 µg/mL), TDF (75-450 µg/mL), and RPV (6.25-37.5 µg/mL). Linear regression analysis yielded high correlation coefficients ($R^2 \geq 0.9991$) for all three analytes, demonstrating proportional peak area responses across the concentration ranges evaluated. Detailed response values and regression parameters are detailed in Table 2.

Table 2. Linear Regression and Calibration Parameters

Drug Component	Concentration Range (µg/mL)	Mean Peak Area	Linear Regression Equation	Correlation Coefficient (R^2)
Emtricitabine	50	1,053,532	y = 20,708x + 23,812	0.9994
	100	2,006,616		
	150	3,192,503		
	200	4,272,452		
	250	5,191,551		
	300	6,231,250		
Tenofovir DF	75	168,954	y = 2,213.5x + 1,048	0.9991
	150	334,451		
	225	497,556		
	300	653,256		
	375	846,333		
	450	992,012		
Rilpivirine	6.25	515,704	y = 82,341x + 1,735	0.9996
	12.50	1,073,271		
	18.75	1,528,496		
	25.00	2,049,140		
	31.25	2,611,210		
	37.50	3,086,424		

3.2.3. Precision and Repeatability

Instrument precision (repeatability) evaluated by six replicate standard injections showed peak area % RSD values of 0.67% for FTC, 0.65% for TDF, and 0.68% for RPV. Intra-day assay precision yielded mean percentage recoveries of 99.92% (%RSD = 0.68%) for FTC, 99.49% (%RSD = 0.65%) for TDF, and 99.90% (%RSD = 0.69%) for RPV. Inter-day evaluation across three

consecutive days confirmed consistent analytical performance, with overall % RSD values remaining below 0.85%. Combined precision measurements are listed in Table 3 and 4.

Table 3. Intra-Day Precision Assay Results (% Recovery) for Emtricitabine, Tenofovir DF, and Rilpivirine

Sample Preparation	Emtricitabine (FTC)	Tenofovir Disoproxil Fumarate (TDF)	Rilpivirine (RPV)
Sample 1	99.66	98.93	100.32
Sample 2	99.47	99.67	99.68
Sample 3	99.01	99.17	100.47
Sample 4	100.66	100.62	100.67
Sample 5	100.00	98.88	99.03
Sample 6	100.71	99.66	99.23
Mean	99.92	99.49	99.90
Std. Dev.	0.67	0.65	0.68
%RSD	0.68%	0.65%	0.69%

Table 4. Inter-Day Precision Assay Results (% Recovery) for Emtricitabine, Tenofovir DF, and Rilpivirine

Sample Preparation	Emtricitabine (FTC)	Tenofovir Disoproxil Fumarate (TDF)	Rilpivirine (RPV)
Sample 1	101.27	98.92	100.14
Sample 2	100.75	101.03	101.92
Sample 3	100.22	101.04	101.43
Sample 4	100.44	100.27	101.63
Sample 5	99.03	100.22	101.38
Sample 6	99.52	100.22	100.54
Mean	100.20	100.28	101.17
Std. Dev.	0.81	0.77	0.68
%RSD	0.82%	0.77%	0.68%

3.2.4. Method Accuracy

Accuracy was verified through recovery studies at three spiked concentration levels (50%, 100%, and 150%). Mean recoveries ranged between 99.79% and 100.72% for emtricitabine, 99.47% and 100.03% for tenofovir disoproxil fumarate, and 99.58% and 99.98% for rilpivirine, with standard deviation values remaining below 0.80%. These high recovery percentages confirm method accuracy and demonstrate that common tablet excipients do not interfere with analyte recovery. Detailed recovery assessments are consolidated in Table 5.

Table 5. Recovery Results for Emtricitabine, Tenofovir DF, and Rilpivirine

Drug	Spike Level (%)	Target Concentration (µg/mL)	Standard Spiked (µg/mL)	Total Recovered (µg/mL)*	Mean Recovery (%)	Mean %RSD (n=3)
FTC	50%	100	200	299.79	99.79%	0.463%
	100%	200	200	401.44	100.72%	
	150%	300	200	500.98	100.33%	
TDF	50%	150	300	450.04	100.03%	0.769%
	100%	300	300	603.06	100.03%	
	150%	450	300	747.63	99.47%	
RPV	50%	12.5	25	37.49	99.92%	0.261%
	100%	25.0	25	49.89	99.58%	
	150%	37.5	25	62.49	99.98%	

** Average of three determinations at each spike concentration level.*

3.2.5. Limits of Detection and Quantitation

Calculated LOD values were 0.0128 µg/mL for FTC, 0.380 µg/mL for TDF, and 0.0016 µg/mL for RPV. Calculated LOQ values were 0.0390 µg/mL for FTC, 1.1540 µg/mL for TDF, and 0.0049 µg/mL for RPV. These low quantification thresholds demonstrate high method sensitivity, enabling accurate detection of trace degradation products or low active ingredient concentrations.

3.2.6. Robustness

Deliberate variations in flow rate (± 0.1 mL/min), column temperature (± 5°C), and mobile phase organic composition (± 5% v/v) produced minimal shifts in system suitability criteria. Theoretical plate counts remained above 6,000, and tailing factors stayed below 1.6 across all modified conditions, confirming method robustness. Operational robustness parameters are presented in Table 6.

Table 6. Method Robustness Testing Under Modified Operational Parameters

Parameter	Modification	FTC Peak Area*	FTC t_R (min)	TDF Peak Area*	TDF t_R (min)	RPV Peak Area*	RPV t_R (min)
Optimized Base	1.0 mL/min, 30°C, 35:65 v/v	4,936,742	2.689	648,585	4.507	2,051,395	5.918
Flow Rate	0.9 mL/min	3,699,782	2.985	735,412	4.878	849,093	6.344
	1.1 mL/min	4,315,222	2.447	577,164	3.980	1,821,382	5.158
Temperature	25°C	4,994,809	2.691	659,027	4.351	2,070,379	5.504
	35°C	4,994,030	2.686	659,340	4.305	2,070,379	5.504
Mobile Phase	40:60 v/v	2,266,221	2.691	370,312	4.307	939,732	5.555
	30:70 v/v	3,350,456	2.700	665,550	4.483	1,362,362	5.851

** Mean of three observations.*

3.3. Assay of Commercial Formulation

The validated RP-HPLC method was applied for the quantitative determination of emtricitabine, tenofovir disoproxil fumarate, and rilpivirine in commercial fixed-dose combination tablets (Complera). Mean assay yields were 99.92 ± 0.68% for FTC, 99.49 ± 0.65% for TDF, and 99.90 ± 0.69% for RPV relative to labeled claims. Chromatograms obtained from commercial tablet preparations showed clear analyte separation without secondary peaks or interference from formulation excipients such as microcrystalline cellulose, lactose monohydrate, povidone, magnesium stearate, or film-coating agents.

4. Conclusion

An isocratic reverse-phase high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of emtricitabine, tenofovir disoproxil fumarate, and rilpivirine in solid fixed-dose combination dosage forms. Isocratic separation on a Hypersil BDS C18 column using potassium dihydrogen phosphate buffer (pH 4.0) and acetonitrile (35:65 v/v) resolved all three analytes within a 10 min run time. Validation according to ICH Q2(R2) guidelines confirmed linearity ($R^2 > 0.999$), high precision (%RSD < 1.0%), accuracy (99.47-100.72% recovery), and operational robustness. The simple sample preparation procedure and short elution time support the suitability of this method for routine quality control assay of active pharmaceutical ingredients and solid dosage preparations.

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