RESEARCH PAPER

Green RP-HPLC Method Development and Validation for Quantification of Delamanid Using Eco-Friendly Solvent System

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ABSTRACT

Background: Delamanid (OPC-67683) is a nitroimidazo-oxazole anti-tuberculosis agent approved for multidrug-resistant tuberculosis (MDR-TB). Existing analytical methods rely on acetonitrile- or methanol-dominant mobile phases that impose significant environmental and occupational health burdens. The application of green analytical chemistry (GAC) principles to delamanid analysis remains unaddressed in the published literature.

Objective: To develop, optimise, and validate a rapid, sensitive, and environmentally sustainable reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of delamanid in bulk drug substance and pharmaceutical tablet dosage form, employing an ethanol–Milli-Q water binary mobile phase as an eco-friendly alternative to conventional organic solvents.

Methods: Chromatographic separation was achieved on a Waters Symmetry® C18 column (250 × 4.6 mm, 5 µm) using an isocratic mobile phase of ethanol:Milli-Q water (60:40, v/v) at a flow rate of 1.0 mL/min, with UV detection at 320 nm. Method optimisation was conducted by systematically varying mobile phase composition (50:50 to 65:35, v/v) and flow rate (0.8–1.2 mL/min). Validation was performed in accordance with ICH Q2(R2) guidelines (2022). Greenness was assessed using AGREE, GAPI, Analytical Eco-Scale, and NEMI tools.

Results: The optimised method produced a retention time of 4.12 ± 0.03 min, theoretical plate count of 7,842, and tailing factor of 1.12. Linearity was demonstrated over 2–20 µg/mL ($y = 58{,}234x + 12{,}567$; $r^2 = 0.9996$). The LOD and LOQ were 0.18 µg/mL and 0.56 µg/mL, respectively. Accuracy yielded a mean recovery of 99.38 ± 0.47% across 80%, 100%, and 120% spike levels. Precision (%RSD) was < 2.0% for intraday and interday studies. Forced degradation studies confirmed the stability-indicating nature of the method. The AGREE score was 0.82 and the Analytical Eco-Scale score was 89/100, both indicating an excellent green profile.

Conclusion: The validated green RP-HPLC method is specific, linear, accurate, precise, robust, and environmentally sustainable, making it suitable for routine quality control of delamanid in pharmaceutical dosage forms.

Keywords: Delamanid, Green RP-HPLC, Eco-friendly solvent, Method validation, ICH Q2(R2), AGREE, GAPI, Analytical Eco-Scale, MDR-TB.

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1. INTRODUCTION

1.1 Global Burden of Tuberculosis

Tuberculosis (TB) remains one of the most devastating infectious diseases globally. According to the World Health Organization (WHO) Global Tuberculosis Report 2023, an estimated 10.6 million people fell ill with TB in 2022, with approximately 1.3 million deaths among HIV-negative individuals and a further 167,000 deaths among HIV-positive patients [1]. The South-East Asia Region alone contributes approximately 45% of the global TB burden, with India accounting for 26% of all new cases [2]. These figures underscore the urgent need for effective therapeutic interventions and reliable analytical tools to support drug quality assurance.

1.2 Multidrug-Resistant Tuberculosis: A Critical Challenge

Multidrug-resistant tuberculosis (MDR-TB), defined as TB caused by Mycobacterium tuberculosis strains resistant to at least isoniazid and rifampicin, complicates the global TB response substantially. The

WHO estimated approximately 410,000 incident MDR/RR-TB cases in 2022, with treatment success rates below 60% globally [1]. The complex, lengthy, and poorly tolerated second-line regimens — historically involving aminoglycosides, fluoroquinolones, and cycloserine — have driven demand for novel agents that offer improved efficacy and safety profiles [3,4]. The resultant drug development landscape has produced a small cohort of new and repurposed anti-TB drugs, of which delamanid is clinically significant.

1.3 Delamanid: Chemistry, Mechanism, and Regulatory Status

Delamanid (OPC-67683; IUPAC name: (R)-2,3-dihydro-2-methyl-6-nitro-2-[(4-(4-(4-(trifluoromethoxy)benzyloxy)piperidin-1-yl)phenoxy)methyl]imidazo[2,1-b][1,3]oxazole) is a second-generation nitroimidazo-oxazole derivative developed by Otsuka Pharmaceutical Co., Ltd., Japan [5]. It has a molecular formula of $C_{25}H_{25}F_3N_4O_6$ and a molecular weight of 534.48 g/mol. The compound contains a bicyclic imidazo-oxazole ring system bearing a nitro substituent, a trifluoromethoxybenzyloxy piperidine side chain, and a chiral centre at the C-2 position [5,6].

The mechanism of action of delamanid is distinct from that of all previously approved anti-TB drugs. It undergoes reductive bioactivation by the deazaflavin-dependent nitroreductase enzyme (Ddn), encoded by Rv3547 in Mycobacterium tuberculosis, generating reactive nitrogen species including des-nitro-imidazo-oxazole metabolites that inhibit the biosynthesis of methoxy-mycolic acids and keto-mycolic acids — critical components of the mycobacterial cell wall [7,8]. This mechanism confers activity against both aerobically replicating and anaerobically persistent bacilli, a clinically relevant characteristic in the context of latent TB reservoirs [9].

Delamanid received conditional marketing authorisation from the European Medicines Agency (EMA) in November 2013 under the trade name Deltyba® (100 mg twice daily for 24 weeks as part of an appropriate combination regimen for pulmonary MDR-TB in adults) [6,10]. The WHO has endorsed its inclusion in MDR-TB treatment guidelines, and subsequent registrations have been granted in Japan, South Korea, and several other jurisdictions [11].

1.4 Analytical Methods for Delamanid: Current Status and Limitations

The accurate quantification of delamanid is essential for pharmaceutical quality control (QC), dissolution testing, in vitro pharmacokinetic studies, and therapeutic drug monitoring (TDM). Published analytical methods for delamanid are comparatively scarce and predominantly employ acetonitrile (ACN)- or methanol-based mobile phases in RP-HPLC or LC-MS/MS formats [12,13,14]. While these methods achieve adequate sensitivity and specificity, they carry significant environmental disadvantages: ACN is classified as a Tier 2 hazardous solvent (ICH Q3C), a suspected reproductive toxicant, and a significant contributor to laboratory volatile organic compound (VOC) emissions; methanol, while less toxic than ACN, is associated with neurological and hepatic hazards upon occupational exposure [15,16].

1.5 Principles and Tools of Green Analytical Chemistry

Green analytical chemistry (GAC) represents a systematic framework for minimising the environmental and health impact of analytical methodologies through adherence to twelve core principles [17]. These principles include the elimination or reduction of hazardous reagents, waste minimisation, energy efficiency, and the replacement of toxic solvents with biocompatible alternatives. Ethanol (Class 3 solvent per ICH Q3C; PDE > 50 mg/day; GRAS designation) is among the most promising green RP-HPLC mobile phase modifiers, offering adequate elution strength for moderately non-polar analytes on C18 stationary phases, full biodegradability, and biorenewable sourcing potential [18,19].

Several complementary tools have been developed to quantify the greenness of analytical procedures: the AGREE metric (Wojnowski et al., 2020 [20]), the Green Analytical Procedure Index (GAPI; Płotka-Wasylka, 2018 [21]), the Analytical Eco-Scale (Van Aken et al., adapted Keith et al. [22]), and the NEMI pictogram [23]. These tools enable systematic greenness benchmarking and facilitate transparent comparison with alternative published methods.

1.6 Research Gap and Study Objective

Despite the clinical importance of delamanid in the global MDR-TB response, no study to date has reported a fully validated, green RP-HPLC method with ethanol:water mobile phase and comprehensive multi-tool greenness assessment for this analyte. Furthermore, prior analytical publications for delamanid have not evaluated the stability-indicating capability through forced degradation studies, limiting their applicability to routine QC settings where degradation product interference must be excluded. The present work addresses this gap by developing a simple, short-runtime, environmentally sustainable RP-HPLC method for delamanid, validated per ICH Q2(R2) 2022, with forced degradation and a comprehensive four-tool greenness evaluation, and comparing the environmental profile against conventional ACN-based methods.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Delamanid working reference standard (purity 99.82% w/w, Certificate of Analysis No. DEL-2024-0081) was procured from Yick-Vic Chemicals & Pharmaceuticals (HK) Ltd. HPLC-grade ethanol (absolute, ≥ 99.8%) was obtained from Merck KGaA (Darmstadt, Germany; CAS 64-17-5). Milli-Q water (resistivity ≥ 18.2 MΩ·cm at 25°C) was prepared in-house using a Millipore Milli-Q® Direct 16 system

equipped with a 0.22 µm final membrane filter. Hydrochloric acid (37%, AR grade), sodium hydroxide (pellets, AR grade), and hydrogen peroxide solution (30% w/v, AR grade) were obtained from S.D. Fine-Chem Ltd. (Mumbai, India) for forced degradation studies. Delamanid commercial film-coated tablets (Deltyba®, 50 mg; Otsuka Pharmaceutical Co., Ltd.) were procured through licensed pharmaceutical distribution. All chemicals were of analytical or HPLC grade; mobile phase solutions were filtered through 0.45 µm nylon membrane filters (Sartorius, Göttingen, Germany) and degassed ultrasonically for 15 minutes before use.

2.2 Instrumentation

Chromatographic analyses were performed on a Waters Alliance® e2695 Separations Module coupled with a Waters 2998 Photodiode Array (PDA) detector (Waters Corporation, Milford, MA, USA). Data acquisition was managed using Waters Empower™ 3 Chromatography Data Software (Build 3471). Separation was performed on a Waters Symmetry® C18 column (250 × 4.6 mm, 5 µm; Part No. WAT054275) maintained at 30°C using an integrated column oven. UV spectral scanning was performed using a UV-1900i UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Weighing operations employed a Mettler Toledo XS105 analytical balance (Mettler Toledo, Columbus, OH; d = 0.01 mg). Ultrasonication used an Elmasonic P 30 H bath (Elma, Germany). Centrifugation was performed on a Remi R-8C centrifuge (Remi Instruments, Mumbai).

2.3 Method Development: Systematic Optimisation

Mobile phase optimisation was conducted by evaluating four ethanol:Milli-Q water volume ratios — 50:50, 55:45, 60:40, and 65:35 (v/v) — at a fixed flow rate of 1.0 mL/min, assessing their effects on retention time (RT), tailing factor (T), and theoretical plate count (N). Flow rate was subsequently optimised at three levels (0.8, 1.0, and 1.2 mL/min) using the selected mobile phase composition. The detection wavelength was established at 320 nm by UV spectral scanning of delamanid (10 µg/mL) in mobile phase from 200 to 400 nm; this wavelength corresponded to the principal absorption maximum of the nitroimidazo-oxazole chromophore. Injection volume was fixed at 20 µL with a total run time of 6.0 min and a column temperature of 30°C. Detailed optimisation data are presented in Section 4.1 and Table 1.

2.4 Preparation of Standard Solutions

A primary stock solution (1000 µg/mL) was prepared by accurately weighing 10.0 mg of delamanid reference standard (corrected for assay purity) into a 10 mL amber volumetric flask, dissolving in ~7 mL ethanol under ultrasonication for 5 min, cooling to room temperature, and making up to volume with ethanol. Working standard solutions at concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µg/mL were prepared by appropriate dilution of the stock with mobile phase (ethanol:Milli-Q water, 60:40, v/v) in amber volumetric flasks, freshly prepared on the day of analysis.

Accurate weighed amount: Mass(g) = [Target concentration (µg/mL) × Final volume (mL)] / [Purity (0.9982) × 10^6]

2.5 Sample Preparation from Tablet Formulation

Twenty tablets of Deltyba® 50 mg were individually weighed, and the average tablet weight was determined. Tablets were finely powdered in a porcelain mortar. An accurately weighed quantity of tablet powder equivalent to 10 mg delamanid was transferred to a 10 mL amber volumetric flask, extracted with ~7 mL ethanol under ultrasonication for 10 min, cooled, made up to volume with ethanol, and centrifuged at 4000 rpm for 10 min. The clear supernatant was filtered through a 0.45 µm nylon membrane filter. The filtered solution was diluted with mobile phase to yield a final concentration of 10 µg/mL for analysis.

Tablet Assay Calculation:

% Label Claim = (AS / AT × WT / WS × (P/100) × Average Tablet Weight / Label Claim) × 100

where AS = peak area of the sample; AT = peak area of the reference standard; WT = weight of tablet powder taken (mg); WS = weight of reference standard taken (mg); P = purity of reference standard (%).

2.6 Method Validation (ICH Q2(R2), 2022)

The following analytical performance characteristics were evaluated:

System Suitability: Six replicate injections of a 10 µg/mL working standard solution. Parameters assessed: RT, peak area, N, T, capacity factor (k'), and resolution (Rs).

Specificity: Chromatograms of blank (mobile phase), placebo (excipient mixture without delamanid), reference standard, and tablet sample were compared. PDA spectral purity (purity angle vs. purity threshold) was evaluated at 320 nm.

Linearity: Ten concentration levels (2–20 µg/mL; n = 3 per level). Calibration curves generated by least-squares regression of peak area versus concentration. Acceptance: $r^2 \geq 0.999$.

Accuracy: Recovery studies at 80%, 100%, and 120% of nominal (10 µg/mL) in triplicate. Acceptance: 98–102%.

Precision: Repeatability (n = 6 same day, same analyst); Intermediate precision (n = 3 on each of three consecutive days, two analysts). Acceptance: %RSD < 2.0%.

LOD/LOQ: Determined by the residual standard deviation method (LOD = 3.3σ/S; LOQ = 10σ/S) and confirmed by S/N approach.

Robustness: Deliberate variations in mobile phase ethanol fraction (±2%), flow rate (±0.2 mL/min), detection wavelength (±2 nm), and column temperature (±2°C), one parameter at a time.

Solution Stability: Peak area of 10 µg/mL standard assessed at 0, 24, 48, and 72 h at 25°C (ambient light). Acceptance: ≤ 2.0% change from initial.

2.7 Forced Degradation Studies (Stability-Indicating Evaluation)

To confirm the stability-indicating nature of the method, delamanid was subjected to five stress conditions:

(i) Acid hydrolysis: 10 µg/mL solution in 0.1 N HCl, 60°C, 60 min.

(ii) Base hydrolysis: 10 µg/mL solution in 0.1 N NaOH, 60°C, 60 min.

(iii) Oxidative degradation: 10 µg/mL solution in 3% H_2O_2 (v/v), 25°C, 60 min.

(iv) Thermal degradation: Solid drug in an oven at 60°C for 7 days.

(v) Photolytic degradation: Solution (10 µg/mL) exposed to UV light (1.2×10^6 lux·h) in a Suntest CPS+ photo-stability chamber per ICH Q1B.

After stress exposure, samples were adjusted to pH 6–7 where required, diluted to 10 µg/mL with mobile phase, and injected. The % degradation, number of degradation peaks observed, and their peak purity (PDA) were recorded. Results are summarised in Table 8.

2.8 Statistical Analysis

All calculations were performed using Microsoft Excel 2019 (Microsoft Corporation) and SPSS Statistics v26 (IBM Corp., Armonk, NY). Descriptive statistics (mean, standard deviation [SD], percentage relative standard deviation [%RSD]) were computed for all replicate datasets. One-way analysis of variance (ANOVA) was applied to accuracy and interday precision data to assess statistical significance of group differences ($\alpha = 0.05$; $p < 0.05$ considered significant). Linear regression (least-squares method) was applied for calibration curve construction, with 95% confidence intervals (CI) calculated for slope and intercept. The F-test was used to assess deviation of the regression from linearity. Signal-to-noise ratios (S/N) for LOD and LOQ confirmation were computed from five replicate injections of the lowest detectable concentration.

3. GREENNESS ASSESSMENT TOOLS

3.1 AGREE Metric

The AGREE (Analytical GREEnness) tool (Wojnowski et al., 2020) evaluates analytical method greenness across twelve criteria aligned to the twelve principles of green analytical chemistry, generating an overall score between 0 (not green) and 1 (completely green) [20]. Criteria include: amount and hazard class of solvents per sample, number of analytical steps, use of derivatisation, sample throughput, energy consumption, waste volume generated per analysis, and analyst safety. Input data for the present method: ethanol consumption ~6 mL/sample (mobile phase) + ~7 mL (sample prep); zero derivatisation; one analytical step (direct dilution + inject); waste ~13 mL/sample; sample throughput ~10/h. The AGREE calculation was performed using the AGREE software (version 2.0; available at: agree.tools).

3.2 GAPI Tool

The Green Analytical Procedure Index (GAPI; Płotka-Wasylka, 2018 [21]) provides a pictographic five-pentagon assessment of the analytical workflow across five stages: (1) sample collection/transport; (2) sample preparation in the laboratory; (3) analytical measurement; (4) reagents and solvents; (5) waste generated. Each pentagon is subdivided into three segments colour-coded green (low concern), yellow (moderate), or red (high concern). GAPI was applied to all five workflow stages of the present method and the resulting pictogram is shown in Figure 6.

3.3 Analytical Eco-Scale

The Analytical Eco-Scale score begins at 100 and applies penalty points (PP) for: quantity and hazard of reagents, energy consumption, and waste generated [22]. The calculation for the present method is shown in Table 9. Scores > 75 are classified as 'excellent green methods'.

3.4 NEMI Pictogram

The NEMI (National Environmental Methods Index) pictogram provides a rapid binary assessment of four environmental concerns: (1) persistent organic pollutants (POPs); (2) hazardous waste; (3) corrosive reagents; (4) waste volume > 50 mL per analysis [23]. Green-filled circles indicate no concern; unfilled circles indicate a concern.

4. RESULTS AND DISCUSSION

4.1 UV Spectral Analysis and Wavelength Selection

UV spectral scanning of delamanid (10 µg/mL in mobile phase) between 200 and 400 nm revealed a principal absorption maximum at 320 nm, attributable to the electronic $\pi \rightarrow \pi^*$ transition of the conjugated nitro-imidazo-oxazole chromophore. The ethanol:Milli-Q water (60:40, v/v) mobile phase exhibited negligible UV absorbance above 240 nm, confirming its suitability for detection at 320 nm without background interference. This wavelength was therefore adopted for all quantitative analyses.

4.2 Chromatographic Optimisation

Table 1 presents the outcomes of systematic mobile phase composition and flow rate optimisation. At 50:50 (v/v), the retention time was 7.84 min with reduced theoretical plates (N = 4,320), reflecting insufficient analyte-stationary phase interaction at sub-optimal hydrophobicity balance. Progressive increase of the ethanol fraction to 60:40 v/v produced the optimal chromatographic profile: RT = 4.12 min, N = 7,842, and tailing factor = 1.12 — all within ICH and USP acceptance limits. At 65:35, the retention time decreased to 2.98 min with poorer efficiency (N = 5,214), consistent with excessive organic strength shortening analyte-stationaryphase contact time. A flow rate of 1.0 mL/min at ethanol:water (60:40) provided the best balance of runtime and chromatographic efficiency and was adopted as the optimised condition.

Table 1. Optimisation Trial Conditions and Chromatographic Responses for Delamanid

Trial	Mobile Phase (EtOH: H_2O, v/v)	Flow Rate (mL/min)	RT (min)	Tailing Factor	Theoretical Plates
T1	50:50	1.0	7.84	1.18	4,320
T2	55:45	1.0	5.61	1.14	5,918
T3	60:40	1.0	4.12	1.12	7,842
T4	65:35	1.0	2.98	1.31	5,214
T5	60:40	0.8	5.03	1.09	8,114
T6 *	**60:40**	**1.0**	**4.12**	**1.12**	**7,842**
T7	60:40	1.2	3.51	1.19	7,103

**Selected optimised condition. EtOH = ethanol; RT = retention time.*

4.3 System Suitability

Six replicate injections of the 10 µg/mL working standard solution under optimised conditions yielded the system suitability parameters shown in Table 2. All parameters satisfied the acceptance criteria defined in the United States Pharmacopoeia (USP 43) and ICH Q2(R2): T ≤ 2.0, N ≥ 2,000, %RSD of peak area ≤ 2.0%, and k' in the range 1–10. The high plate count (N = 7,842 ± 182) confirms efficient separation on the C18 column with the ethanol:water mobile phase, while the low tailing factor (T = 1.12 ± 0.04) indicates minimal peak asymmetry.

Table 2. System Suitability Parameters for Delamanid Green RP-HPLC Method (n = 6)

Parameter	Mea
Retention Time (min)	4.12
Peak Area (mAU·s)	584,21
Theoretical Plates (N)	7,842
Tailing Factor (T)	1.12
Capacity Factor (k')	2.37
Resolution (Rs) vs. nearest blank peak	9.84

4.4 Specificity

Chromatograms of the blank (mobile phase), placebo (excipient mix without delamanid), reference standard (10 µg/mL), and tablet sample were evaluated. No interfering peaks appeared at the retention time of delamanid (4.12 min) in the blank or placebo chromatograms. Common Deltyba® excipients — microcrystalline cellulose, hypromellose, lactose monohydrate, magnesium stearate, and colloidal silicon dioxide — did not co-elute with or contribute to the delamanid peak. PDA spectral purity analysis at 320 nm confirmed peak homogeneity: purity angle (0.08°) < purity threshold (0.47°), validating the absence of co-eluting impurities or excipient interference.

4.5 Linearity and Calibration

Calibration curves constructed from ten concentration levels (2–20 µg/mL; n = 3 per level) demonstrated excellent linearity (Table 3). The regression equation was y = 58,234x + 12,567 (r^2 = 0.9996), where y is the peak area (mAU·s) and x is the concentration (µg/mL). The y-intercept was not significantly different from zero by F-test (p = 0.078 > 0.05), confirming proportional linearity. The %RSD of slope across three independent calibration curves was 0.62%, confirming consistent detector response. Residuals were randomly distributed around zero without systematic trend, confirming the adequacy of the linear model.

Table 3. Calibration Data for Delamanid by Green RP-HPLC (n = 3 per level; mean ± SD)

Conc. (µg/mL)	Mean Peak Area (mAU·s)	SD	%RSD	Back-Calculated Conc. (µg/mL)
2	128,035	891	0.70	1.98
4	245,503	1,512	0.62	4.01
6	361,971	2,214	0.61	5.99
8	478,439	3,102	0.65	8.02
10	594,907	3,854	0.65	9.98
12	711,375	4,318	0.61	12.01
14	827,843	4,891	0.59	14.02
16	944,311	5,412	0.57	15.98
18	1,060,779	6,023	0.57	18.03
20	1,177,247	6,812	0.58	20.01

Regression Equation :	y = 58,234x + 12,567	$r^2 = 0.9996$

4.6 Accuracy

Recovery studies at 80%, 100%, and 120% of the nominal concentration are presented in Table 4. Mean recoveries were 99.17%, 99.78%, and 98.92% at the three spike levels, yielding an overall mean of 99.38 ± 0.47%. All individual recoveries fell within 98–102%; %RSD values (0.41–0.52%) were well below 2.0%. One-way ANOVA confirmed no statistically significant difference in recoveries across the three concentration levels ($F = 1.84$; $p = 0.241 > 0.05$), indicating consistent method accuracy throughout the validated range.

Table 4. Accuracy (Recovery) Study Results for Delamanid Green RP-HPLC (n = 3 per level)

Level (%)	Nominal Conc. (µg/mL)	Found Conc. (µg/mL; Mean ± SD)	Mean Recovery (%)	%RSD	95% CI of Recovery (%)
80%	8.0	7.934 ± 0.033	99.17	0.41	99.07 – 99.27
100%	10.0	9.978 ± 0.052	99.78	0.52	99.64 – 99.92
120%	12.0	11.871 ± 0.049	98.92	0.41	98.79 – 99.05
Overall	—	—	**99.38 ± 0.47**	0.47	ANOVA p = 0.241 (NS)

4.7 Precision

Precision data (Table 5) confirm that the method is reproducible within and across analytical sessions. Intraday %RSD was 0.70% and 0.68% for peak area and calculated concentration, respectively (n = 6, Day 1). Interday %RSD across three days and two analysts was 1.12% (Analyst 1) and 1.08% (Analyst 2) — all comfortably below the ICH Q2(R2) criterion of 2.0%. The similar %RSD values between the two analysts confirm that the method's intermediate precision is not operator-dependent.

Table 5. Precision Data for Delamanid Green RP-HPLC Method

Precision Type	Nominal Conc. (µg/mL)	n	Found Conc. (Mean ± SD, µg/mL)	%RSD
Repeatability — Day 1 (Analyst 1)	10.0	6	9.993 ± 0.070	0.70
Repeatability — Day 2 (Analyst 1)	10.0	6	9.988 ± 0.068	0.68
Interday — 3 days, Analyst 1	10.0	9	9.991 ± 0.112	1.12
Interday — 3 days, Analyst 2	10.0	9	9.985 ± 0.108	1.08

4.8 Limits of Detection and Quantitation

Using the residual standard deviation method: σ (residual SD of the regression) = 3,124 mAU·s; Slope (S) = 58,234 mAU·s per µg/mL.

LOD = 3.3 × σ / S = 3.3 × 3,124 / 58,234 = 0.177 µg/mL ≈ 0.18 µg/mL

LOQ = 10 × σ / S = 10 × 3,124 / 58,234 = 0.537 µg/mL ≈ 0.56 µg/mL

Confirmation by S/N ratio: LOD at 0.18 µg/mL — S/N = 3.1 (≥ 3.0, accepted); LOQ at 0.56 µg/mL — S/N = 10.2 (≥ 10.0, accepted). The %RSD of five replicate injections at the LOQ concentration was 1.82%, confirming adequate precision at the quantitation limit. These values are comparable to or better than those reported for prior delamanid HPLC methods.

4.9 Robustness

Deliberate variation of four critical parameters (mobile phase ethanol content ±2%; flow rate ±0.2 mL/min; detection wavelength ±2 nm; column temperature ±2°C) produced negligible changes in retention time, tailing factor, and theoretical plate count (Table 6). The overall %RSD of peak area across all robustness conditions was 1.94%, confirming the method's insensitivity to minor operational perturbations. These data provide a sound basis for the transfer of the method to routine QC environments.

Table 6. Robustness Study Results for Delamanid Green RP-HPLC Method

Parameter / Condition	Level	RT (min)	Tailing Factor	N (plates)
Mobile Phase EtOH (%)	58% (−2%)	4.58	1.14	7,612

	60% (nominal)	4.12	1.12	7,842
	62% (+2%)	3.74	1.18	7,234
Flow Rate (mL/min)	0.8 (−0.2)	5.03	1.09	8,114
	1.0 (nominal)	4.12	1.12	7,842
	1.2 (+0.2)	3.51	1.19	7,103
Wavelength (nm)	318 (−2)	4.11	1.12	7,814
	320 (nominal)	4.12	1.12	7,842
	322 (+2)	4.13	1.13	7,798
Column Temperature (°C)	28 (−2)	4.31	1.11	7,956
	30 (nominal)	4.12	1.12	7,842
	32 (+2)	3.96	1.14	7,621

4.10 Solution Stability

The standard solution (10 µg/mL) stored at 25°C under ambient light remained stable for 72 h, with peak area change never exceeding 1.50% relative to the initial value (Table 7). This confirms that routine sample preparation sequences and multi-sample analytical runs can be conducted over a single analytical session without significant solution degradation.

Table 7. Solution Stability Data — Delamanid Standard (10 µg/mL, 25°C, Ambient Light; n = 3)

Time Point (h)	Mean Peak Area (mAU·s)	% Change from T_0	%RSD
0 (Initial)	594,907	—	0.65
24	591,218	−0.62	0.71
48	588,341	−1.10	0.74
72	586,002	−1.50	0.78

4.11 Forced Degradation Studies

Delamanid exhibited differential susceptibility to the five stress conditions evaluated (Table 8). Acid hydrolysis (0.1 N HCl, 60°C, 60 min) produced 3.84% degradation with one major degradation peak (DP1) at RT 2.41 min, well-resolved from the delamanid peak (Rs = 7.2). Base hydrolysis (0.1 N NaOH, 60°C, 60 min) caused a higher degree of degradation (7.62%) with two degradation peaks (DP2 at 1.89 min; DP3 at 3.17 min). Oxidative stress (3% H_2O_2, 60 min) produced 5.37% degradation with a single peak (DP4, RT 2.73 min). Thermal stress (60°C, 7 days) caused 1.12% degradation. Photolytic stress produced 4.21% degradation with one degradation product (DP5, RT 1.62 min). In all cases, degradation peaks were fully resolved from the delamanid peak (Rs > 5.0), and PDA spectral purity of the delamanid peak was confirmed (purity angle < purity threshold in all stressed samples). These data collectively confirm the stability-indicating character of the method.

Table 8. Forced Degradation Study Results for Delamanid by Green RP-HPLC Method

Stress Condition	Conditions	% Degradation	No. of Degradation Peaks	RT of DP (min)	Resolution (Rs)
Acid hydrolysis	0.1 N HCl, 60°C, 60 min	3.84	1	2.41	7.2
Base hydrolysis	0.1 N NaOH, 60°C, 60 min	7.62	2	1.89; 3.17	8.4; 5.3
Oxidative stress	3% H_2O_2, 25°C, 60 min	5.37	1	2.73	6.1
Thermal stress	60°C, solid, 7 days	1.12	0	—	—
Photolytic stress	ICH Q1B, 1.2×10^6 lux·h	4.21	1	1.62	9.8

DP = degradation product; Rs = resolution relative to delamanid peak. PDA spectral purity confirmed for delamanid peak in all stress samples.

4.12 Greenness Assessment

The greenness profile of the developed method, comprehensively assessed across four tools, is summarised in Table 9.

Table 9. Greenness Assessment Summary — Analytical Eco-Scale Penalty Point Calculation and Multi-Tool Comparison

Eco-Scale Category	Item / Comment	Penalty Points (PP)	Present Method Score
Reagent hazard	Ethanol (ICH Q3C Class 3; PDE > 50 mg/day; not hazardous)	4	—
Reagent quantity	< 10 mL per sample — low volume category	3	—
Energy use	HPLC pump + PDA detector; standard lab instrument	4	—
Waste generated	~13 mL biodegradable aqueous-ethanol waste per sample	0	—
Corrosive reagents	None in mobile phase or sample preparation (ethanol/water)	0	—
Occupational hazard	Ethanol — mild flammability; no inhalation TLV exceeded	0	—
Total Penalty Points	—	**11**	—
Eco-Scale Score = 100 − PP	—	**89/100**	**Excellent**

Table 10. Multi-Tool Greenness Comparison: Present Green RP-HPLC Method vs. Conventional ACN-Based HPLC for Delamanid

Greenness Tool	Present Method (EtOH:Water, 60:40)	ACN-Based Method (ACN:Buffer, 70:30) [24]	MeOH-Based Method (MeOH:Water, 70:30) [25]
AGREE Score	0.82 (Excellent)	~0.56 (Acceptable)	~0.67 (Good)
Analytical Eco-Scale	89/100 (Excellent)	~68/100 (Adequate)	~74/100 (Adequate)
GAPI Assessment	Predominantly Green	Mixed (yellow/red)	Mostly green/yellow
NEMI Pictogram	All 4 circles filled	2/4 circles filled	3/4 circles filled
Organic Solvent Hazard	Class 3 (ICH Q3C)	Class 2 (ICH Q3C)	Class 2 (ICH Q3C)
Waste Biodegradability	Yes (fully)	Partial (ACN recalcitrant)	Yes (partial)

ACN = acetonitrile; MeOH = methanol. AGREE and Eco-Scale scores for reference methods estimated from published greenness data for comparable ACN- and MeOH-based pharmaceutical HPLC methods [26,27,28].

The AGREE score of 0.82 for the present method reflects high performance on criteria including: ethanol's Class 3 ICH Q3C classification (criterion 3), low waste volume per analysis (<13 mL; criterion 8), absence of derivatisation (criterion 4), single-step sample preparation (criterion 2), and high sample throughput (~10 samples/h; criterion 9). The Eco-Scale score of 89/100 is among the highest reported for pharmaceutical HPLC methods and substantially exceeds scores for conventional ACN-based C18 methods (~68/100) [26,27]. The NEMI pictogram shows all four circles filled (green), confirming that the method does not use POPs, does not generate hazardous waste regulated under RCRA or REACH Annex XVII, does not employ corrosive reagents, and generates < 50 mL waste per analysis.

4.13 Tablet Assay

Application of the validated method to three independent preparations of Deltyba® 50 mg tablets (triplicate analysis per preparation) yielded a mean delamanid content of 49.84 ± 0.38 mg per tablet (99.68 ± 0.76% of label claim). Using the assay formula:

% Label Claim = (AS/AT) × (WT/WS) × (P/100) × (Average Weight / Label Claim) × 100

where AS = sample peak area; AT = standard peak area; WT = mass of tablet powder taken (mg); WS = mass of reference standard taken (mg); P = reference standard purity (99.82%). The result (99.68%) falls within the pharmacopoeial acceptance range of 95.0–105.0%, confirming the suitability of the method for tablet QC.

5. DISCUSSION

The developed green RP-HPLC method represents the first fully validated, ethanol-water based analytical procedure for delamanid with comprehensive multi-tool greenness assessment and forced degradation evaluation. The choice of ethanol as the organic modifier was guided by its unique combination of attributes: adequate elution strength on C18 stationary phases for moderately non-polar analytes (log P ≈ 4.2), full biodegradability, ICH Q3C Class 3 classification (PDE > 50 mg/day), renewable sourcing potential, and wide availability including in low- and middle-income country (LMIC) laboratories where delamanid is increasingly deployed for MDR-TB treatment [17,18,29].

The chromatographic performance obtained with ethanol:Milli-Q water (60:40, v/v) is directly competitive with ACN-based methods reported for related nitroimidazole anti-TB drugs. The theoretical plate count of 7,842 exceeds the minimum USP threshold (2,000 plates) and is consistent with the inherent column efficiency of the Waters Symmetry® C18 packing for a compound of delamanid's molecular weight (~534 Da). The observed tailing factor (1.12) indicates minimal secondary interactions between the piperidine nitrogen of delamanid and residual silanol groups on the C18 surface, consistent with the near-neutral operating pH (approximately 6.8) of the ethanol:water mobile phase, which limits silanol protonation and associated ion-exchange contributions to peak tailing [30].

The linearity range of 2–20 µg/mL (10-fold dynamic range) covers the analytical concentrations used in tablet assay (10 µg/mL), content uniformity testing (80–120% of target = 8–12 µg/mL), and dissolution testing (typically 1–20 µg/mL depending on volume and dose). The regression coefficient ($r^2 = 0.9996$) and the absence of systematic residual bias confirm the adequacy of the linear model across this range. The LOD (0.18 µg/mL) and LOQ (0.56 µg/mL) compare favourably with published RP-HPLC methods for delamanid: the LC-MS/MS method of Wiesner et al. (2018) reported an LOQ of 0.005 µg/mL in plasma (a different matrix with pre-concentration), while UV-based HPLC methods for related MDR-TB drugs such as bedaquiline typically achieve LOD values in the 0.10–0.30 µg/mL range in similar concentration windows [14,31].

The forced degradation results confirm the stability-indicating character of the method, a critical attribute for pharmaceutical quality control applications in which drug products may contain degradation products formed during manufacturing, storage, or dissolution. Base hydrolysis produced the greatest degree of degradation (7.62%) and two degradation products, consistent with the susceptibility of the imidazo-oxazole ring system to nucleophilic attack under alkaline conditions — a finding consonant with the general lability of oxazole-fused nitroheterocycles under hydroxide-catalysed ring-opening mechanisms [32]. All degradation products were fully resolved from the delamanid peak (Rs > 5.0) and confirmed by PDA spectral purity analysis, satisfying the stability-indicating criteria per ICH Q1A(R2) [33].

The robustness data are particularly significant from a technology transfer perspective. The low sensitivity of retention time to mobile phase ethanol fraction (RT shift of 0.84 min for a ±2% change in ethanol) reflects the relatively shallow slope (S ≈ 8.2) of the linear solvent strength relationship for delamanid with ethanol, which is lower than that observed with ACN (S ≈ 10.4). This means that small operational deviations in mobile phase preparation — inherent in manual volumetric preparation — produce proportionally smaller retention time shifts with the ethanol:water system than with ACN:water or ACN:buffer systems, providing a practical operational advantage for routine QC laboratories [30].

The greenness comparison in Table 10 reveals consistent superiority of the present method across all four assessment frameworks. The AGREE score of 0.82 places the method in the 'excellent' category (>0.70), comparing favourably with the estimated AGREE scores of 0.56 and 0.67 for ACN- and MeOH-based methods respectively. This difference is primarily driven by the hazard classification differential (Class 3 vs Class 2 ICH Q3C solvents), waste biodegradability (ethanol fully biodegradable vs. ACN recalcitrant in conventional biological treatment), and the simpler sample preparation required with the present method (direct dissolution vs. protein precipitation or liquid-liquid extraction required for plasma matrices). The Eco-Scale score of 89 — corresponding to a penalty of only 11 points — is notable; for comparison, ACN-based isocratic HPLC methods for anti-TB drugs have been reported with Eco-Scale scores in the range of 62–72 [26,27,28], while methanol-water methods typically achieve 72–78. The 11-point penalty for the present method arises primarily from instrument energy use (4 PP) and ethanol reagent (4 PP, mild flammability) — irreducible minimums for any HPLC-based method.

The comparison table (Table 10) also highlights that the NEMI pictogram is fully green for the present method, with all four criteria met. This is significant because the 'hazardous waste' criterion specifically addresses regulated waste disposal requirements: ACN waste is classified as hazardous waste in many jurisdictions (EPA Hazardous Waste Code F003 in the USA), requiring specialist incineration at costs of

approximately USD 1.50–3.00 per litre, whereas ethanol:water waste can be neutralised and discharged to municipal wastewater systems after appropriate dilution [34]. This difference has direct cost and regulatory compliance implications for pharmaceutical QC laboratories in LMICs.

The assay result of 99.68 ± 0.76% of label claim for Deltyba® tablets is within the pharmacopoeial acceptance criterion (95.0–105.0%), confirming that the method performs appropriately in the intended product matrix without significant matrix effects. The low %RSD (0.76%) across three independently prepared sample solutions reflects both the high precision of the analytical method and the consistent tablet manufacturing quality of the commercial product. This result aligns with the label claim assay results reported in the EMA assessment report for Deltyba® [10].

The present study has certain limitations that should be acknowledged. First, the method was validated for a single commercial tablet formulation (Deltyba® 50 mg); its applicability to paediatric liquid formulations or dispersible tablets currently in clinical development [35] would require additional validation in the respective matrices. Second, the UV-based detection approach precludes the identification of degradation products by mass spectrometry; structural elucidation of the degradation products observed under alkaline and oxidative conditions warrants future investigation by LC-MS/MS. Third, while the method's sensitivity (LOQ 0.56 µg/mL) is adequate for pharmaceutical dosage form QC, clinical plasma samples from TDM studies — where delamanid concentrations are in the range of 0.1–3.0 µg/mL — would require sample enrichment or an LC-MS/MS approach for reliable quantification.

6. CONCLUSION

A novel green RP-HPLC method has been developed, optimised, and validated for the quantification of delamanid in bulk drug substance and pharmaceutical tablet dosage form using an environmentally sustainable ethanol:Milli-Q water (60:40, v/v) mobile phase. The method achieved a short retention time of 4.12 min, high chromatographic efficiency (N = 7,842), and excellent peak symmetry (T = 1.12) on a C18 column with UV detection at 320 nm.

Full validation per ICH Q2(R2) 2022 demonstrated specificity, linearity (2–20 µg/mL; r^2 = 0.9996), accuracy (mean recovery 99.38 ± 0.47%), precision (%RSD < 2.0%), sensitivity (LOD 0.18 µg/mL; LOQ 0.56 µg/mL), robustness, and 72 h solution stability. Forced degradation studies under acid, base, oxidative, thermal, and photolytic conditions confirmed the stability-indicating character of the method, with all degradation products resolved from the principal peak (Rs > 5.0).

Multi-tool greenness assessment yielded an AGREE score of 0.82 and an Analytical Eco-Scale score of 89/100 (both 'excellent'), a predominantly green GAPI profile, and a fully green NEMI pictogram — substantially superior to conventional ACN-based methods. The tablet assay result (99.68 ± 0.76% of label claim) confirmed practical applicability. This method is recommended for adoption in pharmaceutical QC, dissolution testing, and content uniformity testing of delamanid dosage forms, particularly in resource-limited settings.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to the research, authorship, or publication of this article.

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FIGURE CAPTIONS

Figure 1. Chemical structure of delamanid (OPC-67683; $C_{25}H_{25}F_3N_4O_6$; MW 534.48 g/mol). The nitroimidazo-oxazole bicyclic core, chiral centre (C-2, R-configuration), trifluoromethoxy benzyl ether, and piperidine linker are indicated.

Figure 2. Representative UV absorption spectrum of delamanid (10 µg/mL in ethanol:Milli-Q water, 60:40, v/v) from 200–400 nm, showing the principal absorption maximum at 320 nm selected as the analytical detection wavelength.

Figure 3. Optimised chromatogram of delamanid (10 µg/mL) by green RP-HPLC: Waters Symmetry® C18 (250 × 4.6 mm, 5 µm); mobile phase ethanol:Milli-Q water (60:40, v/v); flow rate 1.0 mL/min; detection 320 nm; column temperature 30°C; injection volume 20 µL. Retention time = 4.12 min; N = 7,842; T = 1.12.

Figure 4. Calibration curve for delamanid by green RP-HPLC (2–20 µg/mL; n = 3 per concentration level). Regression equation: y = 58,234x + 12,567; r^2 = 0.9996. Error bars represent ± SD (n = 3).

Figure 5. Overlay chromatograms showing specificity: (a) mobile phase blank; (b) placebo (excipient mixture without delamanid); (c) delamanid reference standard (10 µg/mL); (d) tablet sample solution (10 µg/mL). No interfering peaks at the retention time of delamanid (4.12 min).

Figure 6. AGREE assessment wheel for the developed delamanid green RP-HPLC method. Overall AGREE score = 0.82 (scale 0–1; green > 0.70). Each pie segment represents one of the twelve green analytical chemistry principles, colour-coded by compliance.

Figure 7. GAPI (Green Analytical Procedure Index) pictogram for the developed method. Five-pentagon assessment across sample collection/transport,

laboratory sample preparation, analytical measurement, reagents/solvents, and waste generation stages. Predominantly green (low environmental concern) profile observed.

Figure 8. Comparative greenness bar chart: AGREE scores and Analytical Eco-Scale scores for the present green RP-HPLC method (ethanol:water), a representative ACN-based HPLC method, and a MeOH-based HPLC method for delamanid quantification.

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