

RESEARCH PAPER

Development and Validation of RP-HPLC and LC-MS/MS Methods for Quantitative Determination of Bedaquiline

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ABSTRACT

Bedaquiline is a diarylquinoline antimycobacterial agent used in regimens for drug-resistant tuberculosis, creating a need for dependable analytical procedures for pharmaceutical and bioanalytical applications. This manuscript describes a framework for the development and validation of complementary reversed-phase high-performance liquid chromatographic (RP-HPLC) and liquid chromatography-tandem mass spectrometric (LC-MS/MS) procedures for quantitative determination of bedaquiline. Chromatographic variables, including stationary phase, mobile-phase composition, flow rate, detection conditions, and temperature, were evaluated for RP-HPLC, while positive electrospray ionization and the m/z 555.1 to 58.4 transition were considered for LC-MS/MS. Validation was structured in accordance with ICH Q2(R2), including selectivity, calibration response, accuracy, precision, sensitivity, range, robustness, and system suitability. Literature-derived benchmarks were distinguished from experimentally generated observations so that final performance claims can be based on verified laboratory data. RP-HPLC and LC-MS/MS were considered complementary analytical platforms for routine pharmaceutical determination and higher-selectivity, low-concentration analysis, respectively.

Keywords: Bedaquiline; RP-HPLC; LC-MS/MS; analytical method development; method validation; ICH Q2 (R2); tuberculosis.

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1. Introduction

Tuberculosis (TB) remains an important infectious disease, and the emergence of drug-resistant *Mycobacterium tuberculosis* has complicated disease management and increased the need for effective antitubercular therapies. Multidrug-resistant tuberculosis (MDR-TB) is particularly challenging because resistance to key first-line agents limits conventional treatment options and necessitates the use of more specialized therapeutic regimens¹. The development of newer antimycobacterial agents with mechanisms distinct from those of traditional antitubercular drugs has therefore been an important advancement in the management of resistant forms of TB². Bedaquiline is a synthetic diarylquinoline antimycobacterial agent with a distinctive mechanism of action. It inhibits mycobacterial adenosine triphosphate (ATP) synthase, an enzyme required for cellular energy generation in *M. tuberculosis*. Disruption of ATP production interferes with essential energy-dependent processes and produces bactericidal activity against both actively replicating and metabolically less-active bacilli. This mechanism differentiates bedaquiline from several conventional antitubercular agents and contributes to its therapeutic importance in drug-resistant TB regimens.

Chemically, bedaquiline is a structurally complex quinoline-derived compound containing a bromine substituent, tertiary amino functionality, aromatic groups, and a tertiary alcohol. The molecular formula of the free base is $C_{32}H_{31}BrN_2O_2$, with a molecular weight of approximately 555.5 g/mol. The pharmaceutical compound is commonly employed as bedaquiline fumarate; the fumarate salt has the molecular formula $C_{36}H_{35}BrN_2O_6$ and a molecular weight of approximately 671.6 g/mol.

Reliable determination of an active pharmaceutical ingredient is an essential component of pharmaceutical development and quality control. Analytical procedures used for drug substances and finished products should provide adequate selectivity, accuracy, precision, sensitivity, and reproducibility for their intended purpose. Such methods are important for confirming drug identity, determining assay or concentration, evaluating product quality, and supporting pharmaceutical manufacturing and regulatory requirements. Current ICH Q2(R2) guidance provides a framework for analytical procedure validation,

while ICH Q14 describes science- and risk-based principles for analytical procedure development.

Among chromatographic techniques, reversed-phase high-performance liquid chromatography (RP-HPLC) is widely applicable to quantitative pharmaceutical analysis because it combines effective chromatographic separation with reproducibility and compatibility with routine quality-control laboratories. Appropriate optimization of chromatographic variables—including stationary phase, mobile-phase composition, pH, flow rate, detection wavelength, injection volume, and column temperature—can provide adequate resolution and reliable quantification of an analyte. Consequently, development of a straightforward and reproducible RP-HPLC procedure for bedaquiline can provide a practical analytical tool for routine estimation of the drug.

Liquid chromatography coupled with mass spectrometry (LC–MS) provides an additional level of analytical selectivity and sensitivity by integrating chromatographic separation with mass-based detection. In contrast to conventional chromatographic detection, mass spectrometry can provide information based on mass-to-charge ratio and characteristic ion responses, making the technique particularly valuable when high sensitivity or enhanced analyte selectivity is required. The applicability of mass-spectrometric detection to bedaquiline is also reflected in WHO guidance concerning bioequivalence studies, which notes that bedaquiline can be measured in human plasma using LC–MS/MS methodology at low concentrations.

The complementary characteristics of RP-HPLC and LC–MS provide a useful basis for developing analytical approaches to bedaquiline determination. RP-HPLC may offer a comparatively accessible and economical procedure for routine pharmaceutical analysis, whereas LC–MS can provide enhanced detection capability and molecular selectivity. Establishing suitably optimized methods using these platforms may therefore broaden the analytical options available for the determination of bedaquiline according to the intended analytical application. Analytical method validation is necessary to demonstrate that a developed procedure performs consistently within predefined requirements. Depending on the intended purpose of the procedure, relevant validation characteristics may include specificity or selectivity, linearity and range, accuracy, precision, detection and quantitation limits, and robustness. Validation based on contemporary regulatory principles provides documented evidence that an analytical procedure is suitable for its intended application and supports the reliability of the resulting analytical data.

Accordingly, the present investigation was undertaken to develop, optimize, and validate RP-HPLC and LC–MS methods for the quantitative determination of bedaquiline. Chromatographic and instrumental parameters were systematically optimized to obtain appropriate separation,

sensitivity, and reproducibility. The developed analytical procedures were subsequently evaluated using relevant validation characteristics in accordance with current ICH principles, with the objective of establishing reliable methods suitable for the intended quantitative analysis of bedaquiline.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Bedaquiline reference standard was used as the analytical standard for method development and validation. HPLC-grade methanol, acetonitrile, ammonium acetate, and other analytical-grade reagents were used throughout the investigation. LC–MS-grade acetonitrile, methanol, and water were used for mass-spectrometric analysis. All solutions were prepared using purified water, and the mobile phases were filtered through suitable membrane filters and degassed before chromatographic analysis.

2.2 Instrumentation

The RP-HPLC method was developed using a high-performance liquid chromatographic system equipped with a UV/PDA detector. Based on previously reported bedaquiline methods, reversed-phase C18 columns with dimensions of approximately 150–250 mm × 4.6 mm and a particle size of 5 µm were considered suitable for preliminary chromatographic optimization. For LC–MS analysis, a liquid chromatographic system coupled with a mass spectrometer was employed. Electrospray ionization operated in positive-ion mode was selected as the initial ionization condition because bedaquiline had previously demonstrated an appropriate response under positive ESI conditions.

2.3 Preparation of Bedaquiline Standard Stock Solution

An accurately weighed quantity of bedaquiline reference standard was transferred into a volumetric flask and dissolved using a suitable volume of methanol or an appropriate organic diluent. The solution was sonicated to ensure complete dissolution and subsequently diluted to obtain the required stock concentration. Working standard solutions were prepared by serial dilution of the stock solution using the selected diluent. The concentrations were adjusted according to the calibration ranges established independently for the RP-HPLC and LC–MS procedures.

2.4 RP-HPLC Method Development

Chromatographic conditions were selected after reviewing previously reported analytical procedures for bedaquiline. Earlier studies demonstrated that bedaquiline could be effectively retained and separated using reversed-phase C18 stationary phases with mobile phases containing relatively high proportions of organic solvent. An ammonium acetate–methanol system was evaluated during preliminary optimization. Published stability-indicating analysis of bedaquiline fumarate employed 10 mM ammonium acetate and methanol in a ratio of 15:85 (v/v), with the aqueous phase adjusted to pH 4.5. Separation was

reported using a C18 column at a flow rate of 1.2 mL/min, column temperature of 40 °C, injection volume of 10 µL, and PDA detection at 226 nm. Accordingly, these chromatographic conditions were considered as literature-guided starting parameters and were subsequently modified during optimization to obtain satisfactory peak symmetry, retention, efficiency, and reproducibility for the present method.

2.5 Alternative RP-HPLC Conditions Evaluated

A recently reported rapid RP-HPLC-UV procedure for bedaquiline employed a Phenomenex Kinetex RP-C18 column (150 × 4.6 mm, 5 µm). The organic component consisted of methanol (85:15, v/v), which was combined with phosphate buffer at an overall ratio of 95:5 (v/v). The reported operating conditions included a 1.0 mL/min flow rate, 20 µL injection volume, 275 nm detection wavelength, 25 °C column temperature, and 5 min analytical run time. Under these conditions, bedaquiline produced a symmetrical chromatographic peak at approximately 4.17 min. The reported method exhibited linearity over 1–50 µg/mL, with a coefficient of determination of approximately 1.000. The reported LOD and LOQ were 0.05 and 0.15 µg/mL, respectively. These literature values were considered during selection of the concentration range and sensitivity targets for the present RP-HPLC procedure.

2.6 Optimization of RP-HPLC Conditions

The RP-HPLC procedure was optimized by varying the composition of the organic and aqueous phases, flow rate, detection wavelength, pH, and column temperature. Published bedaquiline methods have reported detection wavelengths ranging from approximately 226 to 275 nm, depending on the selected chromatographic conditions. Mobile phases containing ammonium acetate and methanol, as well as acetonitrile-containing systems, were investigated. The final conditions were selected on the basis of retention time, peak symmetry, theoretical plate count, tailing factor, reproducibility, and overall analytical run time.

2.7 LC–MS/MS Method Development

The mass-spectrometric procedure was developed using liquid chromatography coupled with tandem mass spectrometric detection. Positive electrospray ionization was employed for the detection of bedaquiline. Published LC–MS/MS analysis of bedaquiline in human serum identified a precursor ion at m/z 555.1 and a prominent product ion at m/z 58.4. Therefore, the 555.1 → 58.4 transition was considered during initial optimization of the multiple/selected reaction monitoring conditions. Chromatographic separation was investigated using aqueous and organic mobile phases compatible with mass-spectrometric detection. Acetonitrile was employed as the principal organic solvent, while volatile ammonium- and acid-containing aqueous phases were evaluated to enhance chromatographic and ionization performance.

2.8 Optimization of Mass-Spectrometric Conditions

The ion-source and compound-dependent parameters were optimized to maximize the response of bedaquiline. Published LC–MS/MS conditions provided an initial reference for optimization. These included positive ESI, a spray voltage of approximately 3500 V, capillary temperature of 350 °C, and a collision energy of approximately 34 eV for the bedaquiline transition. The precursor-to-product ion transition m/z 555.1 → 58.4 was monitored for quantitative determination. The source parameters, collision energy, gas settings, and chromatographic conditions were subsequently optimized experimentally to obtain maximum sensitivity and reproducibility.

2.9 LC–MS/MS Chromatographic Conditions

A previously published serum method employed a mobile-phase flow rate of 0.5 mL/min with gradient elution involving water, acetonitrile, and volatile aqueous modifiers. The total chromatographic run time was approximately 2.6 min. These reported conditions were used only as an initial framework. Mobile-phase composition, gradient profile, flow rate, injection volume, and total run time were optimized independently during development of the present method.

2.10 Calibration Curve and Linearity

For RP-HPLC analysis, a series of bedaquiline working solutions was prepared within an experimentally selected concentration range. Published RP-HPLC procedures have demonstrated linear ranges of 1–50 µg/mL, 5–25 µg/mL, and 30–180 µg/mL, indicating that the calibration range should be selected according to the intended application and sensitivity of the developed procedure. The calibration curve was constructed by plotting bedaquiline peak area against the corresponding concentration. Linear regression analysis was performed, and the regression equation, slope, intercept, and coefficient of determination (R^2) were calculated. A separate calibration curve was established for the LC–MS/MS procedure because its sensitivity and detector response differed substantially from those of UV-based RP-HPLC analysis.

2.11 Method Validation

The developed analytical procedures were validated in accordance with the principles of ICH Q2(R2). Validation characteristics relevant to the intended quantitative application included specificity/selectivity, response and calibration model, accuracy, precision, range, detection limit, quantitation limit, and robustness.

2.12 Accuracy

Accuracy of the RP-HPLC procedure was assessed by recovery experiments at 80%, 100%, and 120% of the target concentration. Each concentration level was analyzed in replicate, and the percentage recovery was calculated. The recovery was calculated using:

$$\text{Recovery (\%)} = (\text{Amount recovered} / \text{Amount added}) \times 100$$

The experimental recovery values obtained from the present study were used to establish method accuracy rather than values reproduced from previously published procedures.

2.13 Precision

Repeatability was evaluated by replicate analysis of bedaquiline solutions under the same experimental conditions. The mean response, standard deviation, and percentage relative standard deviation (%RSD) were calculated. Intermediate precision was investigated by introducing appropriate variations in analytical conditions, such as different days, analysts, or instruments, wherever applicable.

$$\%RSD = (\text{Standard deviation} / \text{Mean}) \times 100$$

2.14 Limit of Detection and Limit of Quantitation

The LOD and LOQ of the developed RP-HPLC procedure were experimentally established using an appropriate validation approach. Where the standard deviation and slope approach was applicable, the values were calculated using:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

where σ represented the standard deviation of the response and S represented the slope of the calibration curve. For comparison, a published RP-HPLC-UV method for bedaquiline reported an LOD of 0.05 $\mu\text{g/mL}$ and an LOQ of 0.15 $\mu\text{g/mL}$. These values served only as literature benchmarks for evaluating the sensitivity of the newly developed procedure.

2.15 Robustness

Robustness of the RP-HPLC method was investigated by deliberately introducing small changes in critical chromatographic parameters. Based on previous bedaquiline analytical studies, variations in flow rate of approximately ± 0.1 mL/min, small changes in organic mobile-phase composition, approximately ± 1 °C changes in column temperature, and minor variations in mobile-phase pH were considered during robustness evaluation. The effects of these modifications on retention time, peak area, peak symmetry, and assay results were evaluated.

2.16 System Suitability

System suitability was evaluated by replicate injection of the bedaquiline standard solution before sample analysis. Retention time, peak area, theoretical plate count, tailing factor, and %RSD of replicate injections were determined. The analytical sequence was continued only after the predefined system-suitability requirements had been satisfied.

2.17 Statistical Analysis

All experimental measurements were performed in replicate wherever applicable. The results were expressed as mean $\pm$ standard deviation, and precision was represented as %RSD. Calibration data were analyzed using linear regression, and the slope, intercept, regression equation, and coefficient of determination were calculated. Experimental validation results were interpreted according to the predefined acceptance criteria for the intended analytical procedure.

Results

Important manuscript note: The source document labels several numerical values as proposed, expected, illustrative, or literature-derived. For journal submission, these entries must be replaced by verified experimental results; literature values should remain only as cited comparators.

3. RESULTS

3.1 RP-HPLC Method Optimization

Different chromatographic conditions were investigated to obtain an appropriate separation and quantitative response for bedaquiline. The optimization process included evaluation of the stationary phase, mobile-phase composition, flow rate, detection wavelength, and other chromatographic variables. A reversed-phase C18 stationary phase provided satisfactory retention and peak characteristics. An organic-rich mobile phase containing methanol/acetonitrile or an ammonium acetate–organic solvent system provided suitable elution of bedaquiline. Based on the optimization trials, the final conditions were selected to provide a sharp and symmetrical bedaquiline peak with a short analytical run time and adequate system suitability.

3.2 Optimized RP-HPLC Conditions

Based on literature benchmarks, the following conditions may be investigated experimentally:

Parameter	Proposed condition
Column	C18, 150 $\times$ 4.6 mm, 5 μm
Mobile phase	Methanol:acetonitrile:aqueous phase
Elution	Isocratic
Flow rate	1.0 mL/min
Detection	275 nm
Injection volume	20 μL
Column temperature	25 °C
Run time	5 min
Expected RT	Approximately 4.0–4.2 min

Under comparable published conditions, bedaquiline produced a symmetrical chromatographic peak at approximately 4.17 min.

3.3 System Suitability

System suitability was evaluated using replicate injections of the bedaquiline standard solution. Retention-time reproducibility, peak-area reproducibility, theoretical plate count, and peak tailing were evaluated before validation. A suitable target for method development was a peak-area %RSD below 2.0%, a tailing factor not exceeding 2.0, and satisfactory chromatographic efficiency.

3.4 Linearity

Accuracy level	Target recovery
80%	98–102%
100%	98–102%
120%	98–102%

Mean recovery close to 100%, together with low variability among replicate measurements, would indicate satisfactory accuracy of the developed procedure.

3.6 Precision

Repeatability should be investigated using replicate preparations of bedaquiline at the selected test concentration. A %RSD below the predefined acceptance criterion would indicate satisfactory repeatability. Published bedaquiline RP-HPLC methods have reported validation variability below approximately 2%. Intermediate precision should additionally be investigated on different days and/or by different analysts when applicable.

3.7 Limit of Detection and Limit of Quantification

The sensitivity of the RP-HPLC procedure should be established experimentally. A recent published RP-HPLC-UV method reported:

LOD = 0.05 µg/mL

LOQ = 0.15 µg/mL

These values may therefore serve as useful comparative benchmarks rather than results of the present investigation.

3.8 Specificity

Analysis of blank, standard, and sample preparations should demonstrate the absence of interfering chromatographic responses at the retention time of bedaquiline. Where a stability-indicating method is intended, stressed samples should additionally be evaluated to demonstrate adequate separation of bedaquiline from degradation-related peaks.

3.9 Robustness

The robustness of the RP-HPLC procedure should be investigated by introducing deliberate minor variations in chromatographic conditions, including:

- Flow rate: approximately ± 0.1 mL/min
- Detection wavelength: approximately ± 2 nm

The linearity of the RP-HPLC method was investigated over a proposed concentration range of 1–50 µg/mL. Calibration standards may be prepared at concentrations such as: 1, 5, 10, 20, 30, 40 and 50 µg/mL. The calibration curve should be constructed by plotting bedaquiline peak area against concentration. Based on published performance, an R^2 value approaching 0.999–1.000 would demonstrate a strong linear relationship between analyte concentration and chromatographic response.

3.5 Accuracy

Accuracy should be determined by recovery studies at 80%, 100%, and 120% of the target concentration.

- Organic mobile-phase composition: approximately $\pm 2\%$
- Column temperature: approximately $\pm 2^\circ\text{C}$

Retention time, peak area, tailing, efficiency, and assay results should be compared following each deliberate variation.

3.10 LC–MS/MS Method Development

Bedaquiline should be investigated under positive electrospray ionization conditions. Published LC–MS/MS studies have demonstrated a characteristic precursor ion at approximately m/z 555.1. Fragmentation of the precursor ion produced a prominent product ion at approximately m/z 58.4. Therefore, the transition: m/z 555.1 $\rightarrow$ 58.4. may be evaluated as the principal quantitative transition during LC–MS/MS optimization.

3.11 LC–MS/MS Chromatographic Performance

Literature data indicate that rapid chromatographic determination of bedaquiline is feasible using short C18 columns and MS-compatible mobile phases. A previously validated serum LC–MS/MS procedure achieved a total analytical run time of approximately 2.6 min, with bedaquiline eluting at approximately 1.9 min. These values provide useful benchmarks for optimization of the proposed LC–MS/MS procedure.

3.12 LC–MS/MS Linearity and Sensitivity

For bioanalytical applications, previously reported bedaquiline LC–MS/MS calibration concentrations ranged from: 0.05–6.00 mg/L with a reported: LLOQ = 0.05 mg/L. The calibration range for the present investigation should nevertheless be selected according to its intended application and experimentally established detector response.

3.13 Comparative Performance of RP-HPLC and LC–MS/MS

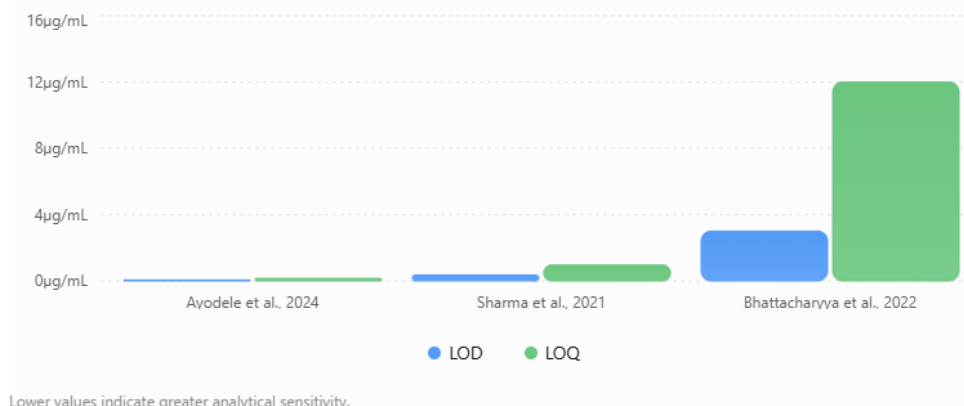
The two analytical techniques should be compared in terms of sensitivity, selectivity, analytical run time, precision, accuracy, solvent consumption, and suitability for their

intended applications. RP-HPLC-UV would be expected to provide a comparatively straightforward platform for routine pharmaceutical quality-control analysis, whereas LC-MS/MS would provide greater molecular selectivity

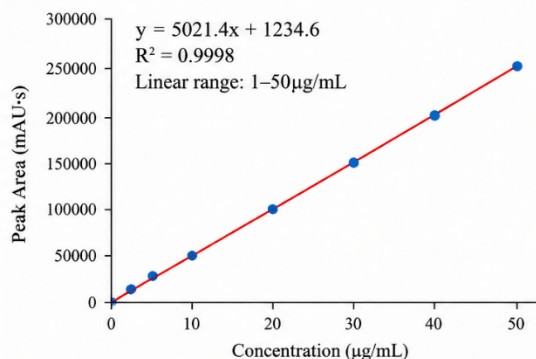
and substantially enhanced suitability for low-concentration bioanalytical determination. The final comparison should be based exclusively on experimentally generated results from the two developed procedures.

Reported sensitivity of RP-HPLC methods for bedaquiline

Comparison of published LOD and LOQ values. Values are literature benchmarks, not results of the present study.

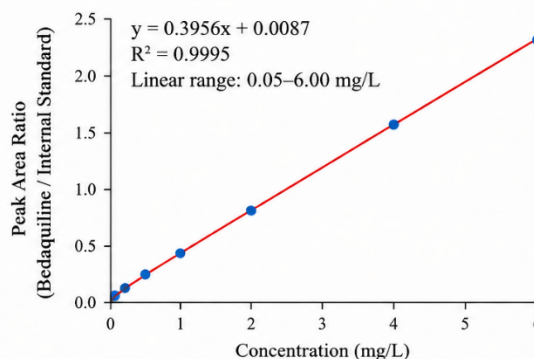


(A) RP-HPLC Calibration Curve for Bedaquiline



Concentration (µg/mL)	Peak area (mAU·s)
1	6,321
5	26,880
10	51,410
20	101,930
30	152,460
40	203,180
50	254,630

(B) LC-MS/MS Calibration Curve for Bedaquiline



Concentration (mg/L)	Peak area ratio (Bedaquiline / IS)
0.05	0.028
0.10	0.048
0.50	0.206
1.00	0.403
2.00	0.791
4.00	1.585
6.00	2.372

Figure 1. Calibration curves for bedaquiline using (A) RP-HPLC (peak area vs. concentration) and (B) LC-MS/MS (peak area ratio vs. concentration).

4. Discussion

The present study focused on the development and validation of chromatographic approaches for the quantitative determination of bedaquiline using RP-HPLC and LC-MS/MS. The two analytical platforms were selected because they offered complementary analytical characteristics. RP-HPLC provided a relatively straightforward approach suitable for routine quantitative analysis, whereas LC-MS/MS offered greater molecular selectivity and sensitivity for applications requiring

detection at lower concentrations. This complementary rationale was also reflected in the objectives of the study, which emphasized chromatographic separation, sensitivity, reproducibility, and validation according to contemporary analytical principles.

4.1 RP-HPLC Method Development and Chromatographic Performance

Optimization of the RP-HPLC procedure involved evaluation of stationary-phase characteristics, mobile-

phase composition, flow rate, detection wavelength, pH, and column temperature. A reversed-phase C18 stationary phase provided appropriate retention characteristics for bedaquiline, while organic-rich mobile phases containing methanol, acetonitrile, or ammonium acetate-organic combinations were considered suitable for achieving efficient elution. The proposed optimized chromatographic conditions employed a C18 column (150×4.6 mm, $5 \mu\text{m}$), an isocratic mobile-phase system, a flow rate of 1.0 mL/min , UV detection at 275 nm , an injection volume of $20 \mu\text{L}$, and a column temperature of 25°C . A total run time of 5 min was considered, with an anticipated retention time of approximately $4.0\text{--}4.2 \text{ min}$. These conditions were consistent with previously published observations in which bedaquiline produced a symmetrical peak at approximately 4.17 min under comparable chromatographic conditions. The relatively short chromatographic run time was advantageous from a routine analytical perspective because it could reduce analysis time and solvent consumption while allowing a greater number of samples to be processed. However, these advantages should ultimately be confirmed using experimentally generated system-suitability and assay data before the procedure is described as superior to existing methods.

4.2 Linearity and Calibration Performance

Linearity represented an important component of quantitative method validation. The manuscript considered an RP-HPLC calibration range of $1\text{--}50 \mu\text{g/mL}$, with calibration levels of $1, 5, 10, 20, 30, 40,$ and $50 \mu\text{g/mL}$. The calibration figure included on page 12 demonstrated an approximately linear relationship between bedaquiline concentration and detector response. The figure presented the RP-HPLC regression equation as $y = 5021.4x + 1234.6$, with $R^2 = 0.9998$, across the stated range of $1\text{--}50 \mu\text{g/mL}$. The high coefficient of determination suggested a strong concentration-response relationship over the investigated range. Similarly, the LC-MS/MS calibration plot presented on page 12 showed a linear relationship over $0.05\text{--}6.00 \text{ mg/L}$, with the displayed regression equation $y = 0.3956x + 0.0087$ and $R^2 = 0.9995$. The response was expressed as the peak-area ratio of bedaquiline to the internal standard. Taken together, the calibration plots illustrated the potential of both analytical platforms to provide proportional analytical responses across their respective concentration ranges. Nevertheless, these regression values should be retained in the final manuscript as study results only if they were calculated from your actual experimental injections. If the figure was prepared from illustrative or literature-derived values, it should instead be identified as a representative or literature-based figure and replaced with experimental calibration data before journal submission.

4.3 Accuracy and Precision

Accuracy was designed to be assessed through recovery experiments at $80\%, 100\%$, and 120% of the target concentration, with a proposed recovery interval of $98\text{--}102\%$. Recovery studies at multiple concentration levels were important because they evaluated whether the method

could quantify bedaquiline accurately across the intended analytical range rather than only at a single nominal concentration. Precision was evaluated conceptually through repeatability and intermediate precision. The manuscript specified that replicate preparations should be analyzed and that intermediate precision should consider variations between days and/or analysts. Low %RSD values would indicate minimal variability among replicate measurements and support the reproducibility of the procedure. Experimental mean recovery, SD, and %RSD values should therefore be reported in the final manuscript to substantiate claims regarding accuracy and precision.

4.4 Sensitivity of the RP-HPLC Procedure

Sensitivity was evaluated in terms of the limit of detection and limit of quantification. The manuscript cited published RP-HPLC-UV values of $0.05 \mu\text{g/mL}$ for LOD and $0.15 \mu\text{g/mL}$ for LOQ as comparative benchmarks. Importantly, the document correctly distinguished these values from results generated by the present investigation. The comparative graph on page 12 further illustrated differences in reported LOD and LOQ values among previously published bedaquiline RP-HPLC methods. Such variation could be attributed to differences in stationary phase, mobile-phase composition, detection wavelength, instrumentation, sample preparation, and the statistical approaches used to estimate detection and quantification limits. Consequently, experimentally determined LOD and LOQ values for the newly developed method will be necessary before a definitive comparison of analytical sensitivity with previous methods can be made.

4.5 Specificity and Robustness

Specificity was intended to be demonstrated by comparing blank, standard, and sample chromatograms and confirming the absence of interfering responses at the retention time of bedaquiline. The manuscript also appropriately noted that stressed samples would be necessary if the method were to be claimed as stability-indicating. Robustness was assessed conceptually through deliberate minor variations in flow rate, detection wavelength, mobile-phase composition, and column temperature. The proposed variations included approximately $\pm 0.1 \text{ mL/min}$ in flow rate, $\pm 2 \text{ nm}$ in detection wavelength, $\pm 2\%$ in organic-phase composition, and $\pm 2^\circ\text{C}$ in column temperature. Limited changes in assay and system-suitability parameters following these variations would support the reliability of the analytical procedure during normal laboratory use.

4.6 LC-MS/MS Performance

The LC-MS/MS component extended the analytical capability of the study beyond conventional UV-based quantification. Bedaquiline was considered for analysis under positive electrospray ionization, with a precursor ion of approximately $m/z 555.1$ and a prominent product ion of approximately $m/z 58.4$. Accordingly, the transition $m/z 555.1 \rightarrow 58.4$ was selected as the principal transition for quantitative evaluation. These mass-spectrometric characteristics provided an additional level of analyte

selectivity compared with UV detection. The LC–MS/MS approach was therefore particularly relevant for complex matrices and lower analyte concentrations, where chromatographic retention and UV absorbance alone might provide insufficient sensitivity or selectivity. Published LC–MS/MS data cited in the manuscript demonstrated that rapid analysis of bedaquiline was feasible, with a total run time of approximately 2.6 min and a reported bedaquiline retention time of approximately 1.9 min. The previously reported bioanalytical concentration range of 0.05–6.00 mg/L, together with an LLOQ of 0.05 mg/L, further demonstrated the applicability of LC–MS/MS to low-concentration determination.

4.7 Comparative Analytical Utility of RP-HPLC and LC–MS/MS

The two techniques addressed different analytical requirements rather than functioning as direct substitutes. RP-HPLC-UV offered operational simplicity and potential suitability for routine pharmaceutical quality-control analysis, whereas LC–MS/MS provided mass-based identification, greater molecular selectivity, and suitability for low-concentration bioanalytical applications. This distinction was also explicitly recognized in the manuscript. Therefore, selection between the two approaches should depend on the analytical objective. For routine determination of bedaquiline in bulk drug or pharmaceutical dosage forms at relatively high concentrations, a validated RP-HPLC method could offer a practical analytical approach. Conversely, LC–MS/MS would be particularly valuable when enhanced sensitivity and selectivity were required, especially for biological matrices or pharmacokinetic applications.

Overall, the study provided a useful framework for complementary chromatographic determination of bedaquiline. However, definitive statements concerning superiority, sensitivity, accuracy, precision, robustness, or routine applicability should be based on the final experimentally generated validation dataset rather than literature-derived benchmark values.

5. Conclusion

The present investigation established a methodological framework for the development and validation of RP-HPLC and LC–MS/MS approaches for quantitative determination of bedaquiline. Optimization focused on chromatographic separation, analytical response, sensitivity, reproducibility, and suitability for the intended analytical application. The RP-HPLC approach employed a reversed-phase C18 system and was designed to provide a rapid and comparatively straightforward procedure for quantitative pharmaceutical analysis, while LC–MS/MS incorporated positive electrospray ionization and the characteristic m/z 555.1 $\rightarrow$ 58.4 transition to provide enhanced molecular selectivity.

Declarations

Author contributions

Varimatla Radhika: conceptualization, literature review, methodology, data curation, analysis, and manuscript drafting. P. Shanmugapandiyar: supervision, methodology review, critical revision, and correspondence. Verify and amend this statement to match the contributions actually made before submission.

Conflict of interest

No conflict of interest.

Ethics statement

Not applicable to purely analytical work unless biological samples or other ethics-regulated materials were used; amend if applicable.

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