GMR
The Original

HIGH-THROUGHPUT UPLC METHOD FOR DETERMINATION OF BEDAQUILINE IN PLASMA USING PROTEIN PRECIPITATION

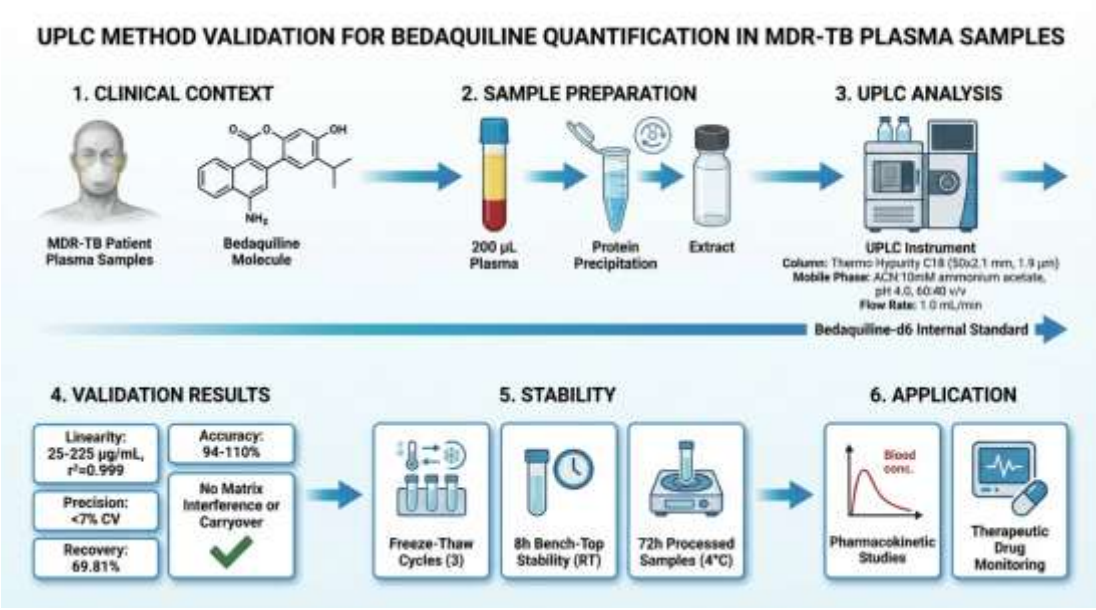
K. N. Maneesha[1], B. Ramya Kuber[2*]

[1]Research Scholar, Sri Padmavati Mahila Visvavidyalayam, Tirupati-517502, Andhra Pradesh. India.
Email: maneeshaphdspmvv@gmail.com
[2*]Faculty of Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati -517502, Andhra Pradesh, India. Email: rkuberpharma@yahoo.com

ABSTRACT

Background: Bedaquiline, a diarylquinoline inhibitor of ATP synthase is an important drug in the treatment of multidrug resistant tuberculosis (MDR TB) but due to its lipophilic property and low plasma levels, sensitive measurement of this drug is difficult. Method: To achieve the first UPLC assay for bedaquiline in human plasma, the method was developed and is now completely validated based on ICH guidelines. The separation was carried out on a Thermo Hypurity C18 column using acetonitrile–ammonium acetate (60:40, v/v) as the mobile phase with bedaquiline d6 as the internal standard. Results and Discussion: The method achieved excellent linearity with r2 of 0.999, over the range of 25-225 µg/mL, accuracy of 94-110% and precision of < 7%CV. Different types of plasma (lipemic, hemolyzed etc.) all had a recovery of around 70%. Freeze–thaw, bench-top exposure and storage of processed samples did not cause a loss of stability. Using a simple precipitation clean up and UPLC. Using the Lean Six Sigma (DMAIC) concept, the workflow was methodically optimized for efficiency, reproducibility and compliance to regulations. Conclusion: This assay appears to be an alternative and the procedure provides a quick, selective and reproducible method for pharmacokinetic study and therapeutic drug monitoring where TB patients are infected with MDR TB than previous HPLC methods.

KEYWORDS: Antimicrobial drug resistances, Bedaquiline, UPLC, Bioanalytical validation



1. INTRODUCTION

Multidrug resistant tuberculosis (MDR TB) is a key factor causing tuberculosis to be a worldwide health problem, so that there is an urgent need for reliable therapeutic monitoring of newer antitubercular agents like bedaquiline. Bedaquiline is the first new drug in many years to change the landscape of MDR TB care due to its unique mechanism of action that inhibits mycobacterial ATP synthase; its use in the World Health Organization recommended regimen has led to better outcomes in patients infected with MDR TB. Meanwhile, patients must be monitored to manage efficacy and the risk of cardiotoxicity especially QT interval prolongation is a concern[1-3] and thus therapeutic drug monitoring is necessary[4].

Accurate and reproducible bedaquiline quantification in biological matrices is thus a crucial requirement for pharmacokinetic evaluation, individuals dose, therapeutic drug monitoring, and for quality control. Its high lipophilicity, complex metabolism and low plasma levels creates significant analytical difficulties, which require a highly sensitive, selective and matrix compatible bioanalytical method that is capable of reliably measuring the compound in human plasma[6,11].

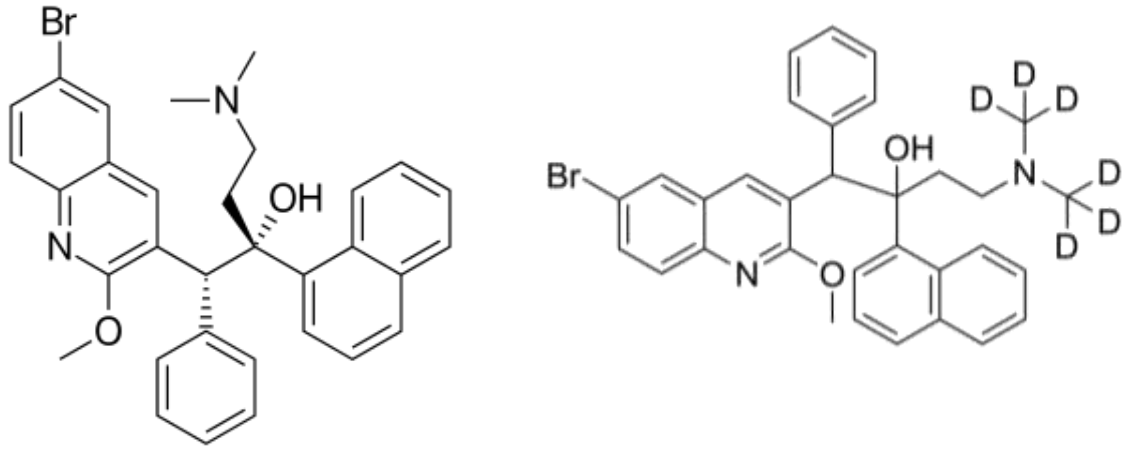


Figure-01: Chemical structure of (a) Bedaquiline and (b) Internal standard Bedaquiline d6

Various analytical methods have been reported such as, UV spectrophotometry and reversed phase high performance liquid chromatography (RP HPLC), ultra performance liquid chromatography (UPLC) of bedaquiline as single drug or in fixed dose combinations. However, most of these procedures have been developed for pharmaceutical dosage forms and/or simpler matrices and a limited number of these address the stringent requirements of bioanalysis in human plasma. They are limited in sensitivity, selectivity, and robustness in the presence of a number of endogenous compounds and as a result cannot be used in routine therapeutic drug monitoring and detailed pharmacokinetic studies.

An attempt has been made to fill these gaps in the present work by developing and validation of a sensitive, rapid and robust UPLC method for the quantification of bedaquiline in human plasma following USFDA and ICH M10 bioanalytical method validation guidelines. The method is designed and optimized based on the principles of Lean Six Sigma (DMAIC – Define–Measure–Analyze–Improve–Control) with a systematic approach to finding the critical parameters of the method, reducing the variability, and improving the performance. The incorporation of DMAIC in the method lifecycle makes the process more streamlined for analytical accuracy, precision, efficiency and reproducibility which is essential for acceptance in regulatory submission and routine clinical implementation of TDM in MDR TB patients.

2. MATERIALS AND METHODS

Reagents and Materials

Acetonitrile, methanol, and water (HPLC grade) were procured from Merck, Mumbai, India. Bedaquiline reference standard and bedaquiline-d6 (isotope-labeled internal standard) were supplied by INNOSYN Life Sciences Pvt. Ltd., Hyderabad, India. Blank human K_2EDTA plasma was obtained from screened donors and stored at −70 °C.

Instrumentation

Analysis was performed using a Waters 2695 UPLC system equipped with a photodiode array detector (PDA) and Empower 2 software. Chromatographic separation employed a Thermo Hypurity C18 analytical column (100 mm × 4.6 mm, 5 µm particle size) maintained at 30 °C. The autosampler temperature was set at 5 °C.

Chromatographic Conditions

Mobile Phase: Acetonitrile: 10mM ammonium acetate (pH 4.00), 60:40 v/v
Flow Rate: 1.0 mL/min
Retention Times: Bedaquiline 1.60 ± 0.60 min; Bedaquiline-d6 2.56 ± 0.6 min
Run Time: 6.0 min
Injection Volume: 10 µL
Diluent: 50% methanol

Preparation of Standards and Quality Control Samples

Stock solutions of bedaquiline and bedaquiline-d6 (1000 µg/mL) were prepared in methanol and stored at 2–8 °C(A. K. Hemanth Kumar, V. Sudha, et al., 2021) Working solutions were serially diluted to generate calibration standards (25–225 µg/mL) and quality control samples at three levels: low QC (LQC: 25 µg/mL), medium QC (MQC: 141.75 µg/mL), and high QC (HQC: 186.75 µg/mL).

Sample Preparation

An aliquot of 200 µL spiked plasma was transferred to a 2 mL tube. Fifty microliters of internal standard dilution (100 ng/mL) and 2 mL of acetonitrile (protein precipitant) were added (Meghavath M, Devanna N. 2025). The mixture was vortexed and centrifuged at 4500 rpm for 10 minutes at 5 °C. The supernatant was evaporated under nitrogen at 40 °C and reconstituted in 200 µL mobile phase. Samples were transferred to autosampler vials and analyzed immediately.

3. RESULTS

Method Development

The Method Optimization was done using Lean Six Sigma (DMAIC) principles. ### Several critical parameters, including the composition of the mobile phase, the extraction recovery, and the run time were carefully defined, measured and analyzed. Assessment was made with the introduction of some modifications: use of protein precipitation instead of SPE/LLE and a control via the reproducibility experiment conducted with a variety of plasma matrices (Tabel 1 and figure2). The structured approach improved the efficiency, and kept to the ICH M10 guidelines."

Table 1. Chromatographic condition optimization

Mobile Phase	Column	Flow Rate	Outcome
MeOH:buffer (50:50)	Inertsil ODS-3V	0.8 mL/min	Low response
MeOH:ammonium formate (70:30)	Agilent Zorbax SB-C8	1.0 mL/min	Good recovery, peak fronting present
ACN:10 mM ammonium acetate (80:20, pH 4.0)	Thermo Hypurity C18	1.0 mL/min	Good response, peak fronting observed
ACN:10 mM ammonium acetate (60:40, pH 4.0)	Thermo Hypurity C18	1.0 mL/min	Optimal; excellent peak shape and response

Protein precipitation was found to show advantages over liquid–liquid extraction and solid-phase extraction and yielded cleaner extracts with a higher throughput.

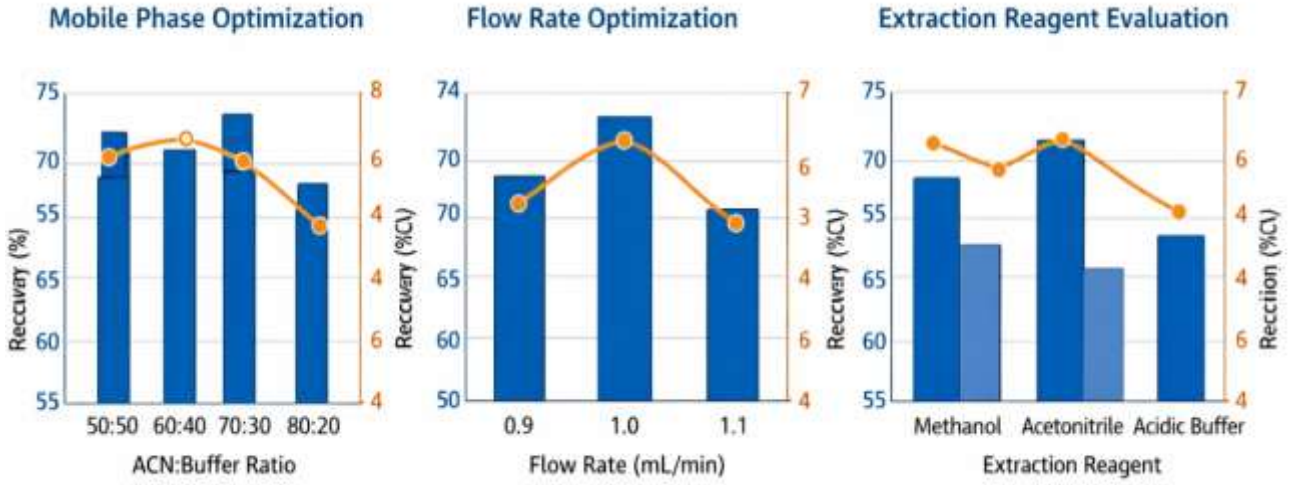


Figure 2 : graphical representation of DMAIC

System Suitability

The six individual system suitability solution injections were tested (Table 2). Very stable system: Peak areas of the analyte and internal standard (IS) were, in mean, 86,327.7 and 60,086.5 with coefficient of variation (%CV) of 0.99 and 1.54%, respectively.

Table 2. System suitability parameters.

Parameter	Analyte	ISTD	Acceptance Criterion
Mean Peak Area	86,327.7	60,086.5	—
%CV	0.99	1.54	≤2.0%
Mean RT (min)	1.553	2.553	—
Area Ratio %CV	1.16	—	≤2.0%

Carryover Assessment

Blank plasma injections after upper limit of quantification (ULOQ) indicated no carry over effect for both bedaquiline and internal standard which indicates method robustness.

Specificity and Selectivity

A total of 10 donors of blank plasma were analyzed that included lipemic and hemolyzed plasma. No interference was detected at the retention times of bedaquiline or bedaquiline-d6 (Figure 3 a, b, and c). The method demonstrated 0% interference at LLOQ concentration across all plasma sources tested.

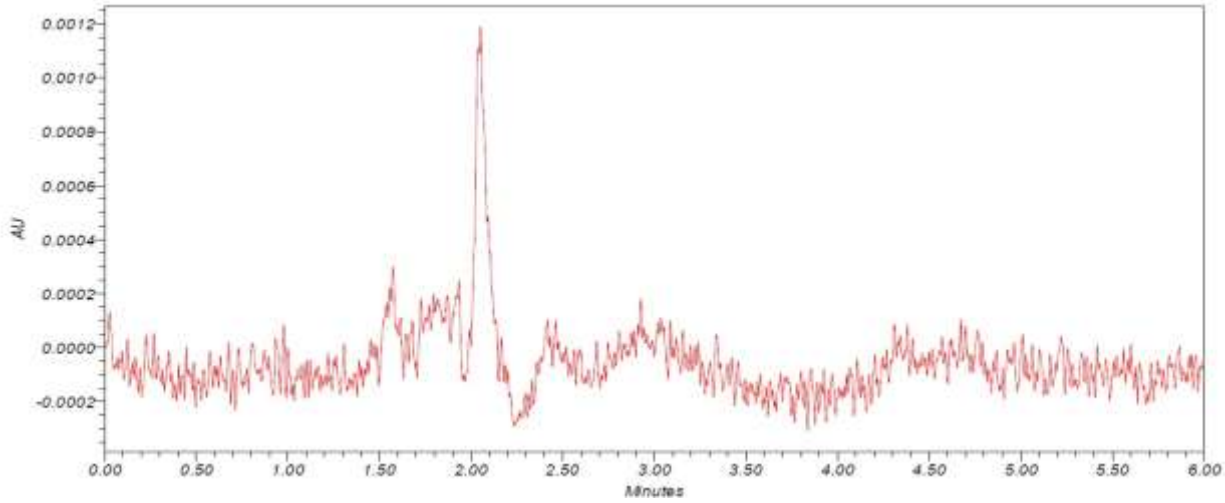


Figure 3(a): blank chromatogram of bedaquiline

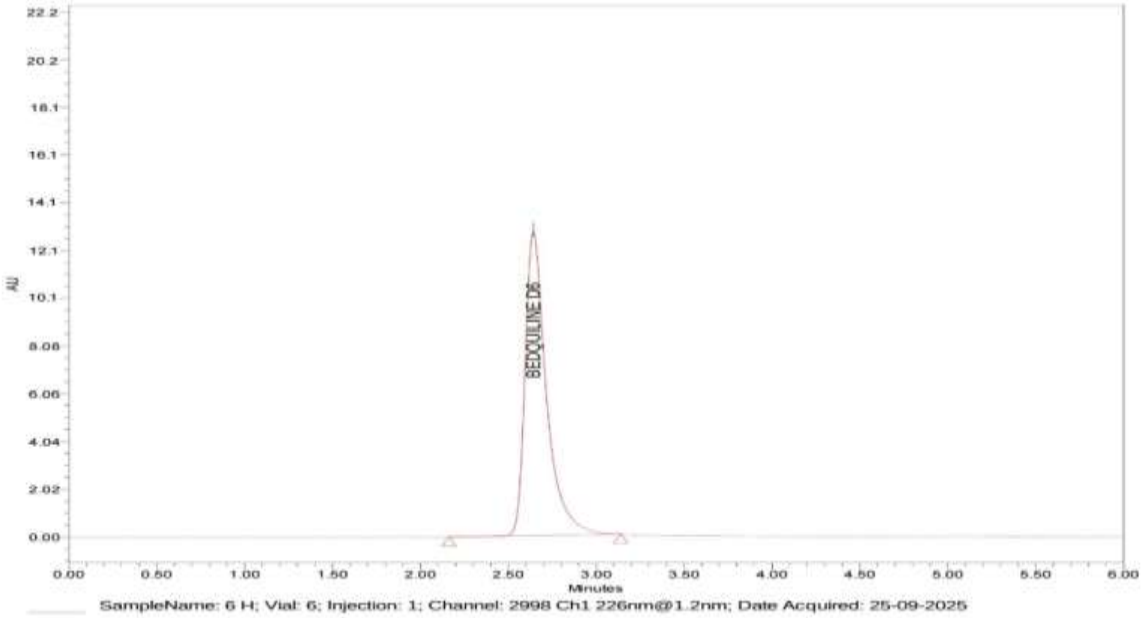


Figure 3(b): ISTD chromatogram of Bedaquailine d6

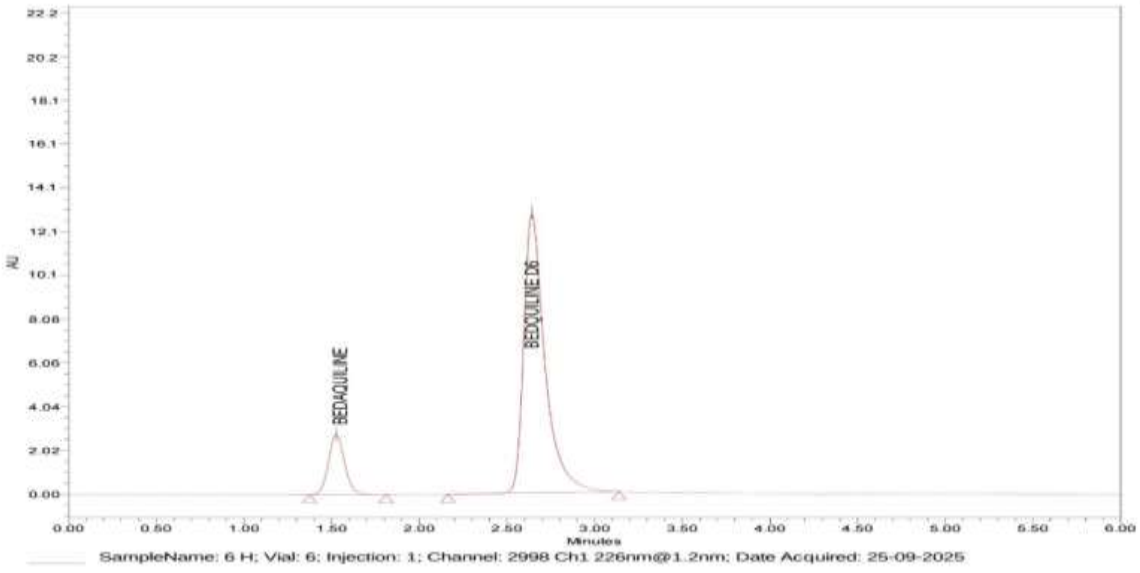


Figure 3(c): LLOQ chromatogram of Bedaquailine

Linearity

The method exhibited linear response across the concentration range 25–225 µg/mL with mean correlation coefficient r^2 = 0.999 (Figure 4). Back-calculated bias ranged from −5.2% to +6.8%, meeting the acceptance criterion of ±15%.

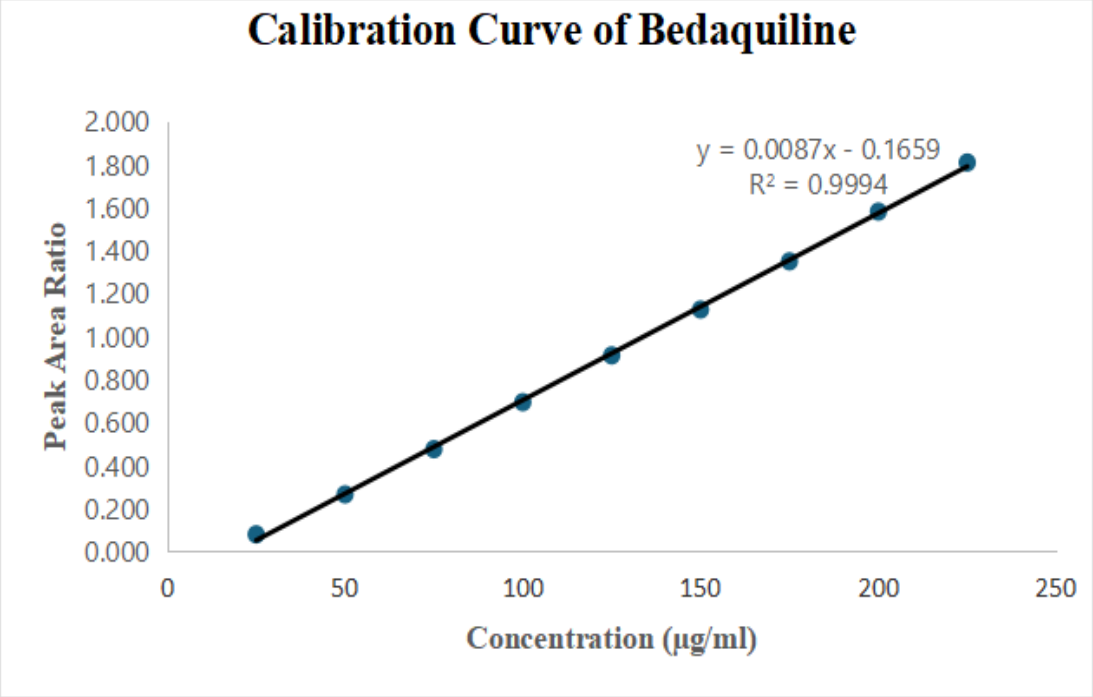


Figure 4: Calibration curve graph Bedaquailine

Table 3: calibration curve samples for Bedaquiline in human plasma

Concentration (µg/mL)	Mean Peak Area Ratio
25	0.0789
50	0.2654
75	0.4746
100	0.6931
125	0.9098
150	1.1239
175	1.3475
200	1.5790
225	1.8070

Accuracy and Precision

Intra-day and inter-day accuracy and precision were evaluated at four concentration levels across three independent runs (Table 4). Intra-day %CV ranged from 0.6–4.5%, with accuracy values 94.43–111.31%. Inter-day %CV ranged from 2.8–6.2%, with accuracy values 94.23–109.98%. All results met regulatory acceptance criteria (±15% bias, %CV ≤15%; ±20% at LLOQ).

Table 4. Precision and Accuracy Results of Bedaquiline

QC level (nominal concentration, ng/ml)	intra batch (n=6; single batch)			inter batch (n=18; 6 from each batch)		
	mean conc. found(ng/ml)	Accuracy (%)	% C.V	mean conc. Found	Accuracy (%)	% C.V
HQC (186.750)	198.113	106.08	1.5	197.359	105.68	4.5
MQC (186.750)	144.680	102.07	0.6	144.292	101.79	3.6
LQC (67.500)	63.738	94.43	1.7	63.608	94.23	2.8
LLOQQC (25.000)	27.826	111.31	4.5	27.494	109.98	6.2

Extraction Recovery

Mean recovery of bedaquiline was 69.81% (±0.45%, %CV 0.65%) across all QC levels. Internal standard recovery was 68.80% (±0.9%, %CV 1.31%). Consistent recovery across concentration levels confirmed efficient and reproducible extraction. The results were shown in table 5.

Table 5. Recovery Results of Analyte and ISTD

QC level (nominal concentration,	Mean area response (n=6)		Recovery (B/A%)		Global % Recovery	
	A (Post	B (Extracted	Analyte	IS	Analyte	IS

ng/ml)	extracted area)	Area)				
HQC (186.750)	117114	82128.5	70.13	68.68	69.81	68.80
MQC (186.750)	84273.5	58994.2	70.00	69.76		
LQC(67.500)	26052.8	18053.2	69.29	67.99		

Matrix Effect

Matrix effects were assessed using 10 individual plasma sources. Ion suppression/enhancement (matrix factor) was minimal, with %CV values of 3.2% (HQC) and 2.3% (LQC). IS-normalized matrix factors were 1.293 ± 0.042 (HQC) and 0.905 ± 0.021 (LQC), confirming negligible matrix interference.

Stability

Stability evaluations for bench-top, processed samples, and freeze-thaw cycles were performed at two concentration levels (HQC and LQC). As shown in Table 5, results indicated no stability issues that could affect the assay's use in pharmacokinetic studies. Additionally, other validation aspects—such as ruggedness, reinjection reproducibility, PID effects, dilution integrity, extended precision and accuracy batch analysis, robustness, and aqueous solution stability—all met the USFDA guideline acceptance criteria (Table 6).

Freeze–Thaw Stability: Six freeze–thaw cycles (−70 ± 15 °C) resulted in no significant analyte degradation. Mean accuracy remained 95.55–107.29%.

Bench-Top Stability: Samples exposed to room temperature for 8 hours 13 minutes showed stability within ±15% acceptance criteria (mean accuracy 104.26–106.14%).

Processed Sample Stability:

Autosampler vial stability at 2–8 °C (72 hours) and room temperature (28 hours 30 minutes) demonstrated analyte concentrations within ±15% of nominal values. An analysis of all stability data ensured the integrity of the analytes under the tested conditions.

Table 6: Stability of Bed to human plasma after various storage conditions (n=6)

Stability	Storage condition	Duration	Level	Nominal concentration (ng/ml)	Mean Stability sample (mean ±SD, ng/ml)	%C.V	Accuracy
Bench top	Room temperature	8 hrs	HQC	184.918	192.793±1.01	0.53	98.23
			LQC	67.500	70.931±2.78	3.9	99.59
Processed sample stability	Refrigerated (2-8°C)	4 days	HQC	184.918	189.527±5.91	3.2	97.67
			LQC	67.500	67.759±2.96	4.4	101.63
Processed sample stability	Room temperature (24 ± 4°C)	32 hrs	HQC	184.918	190.704±2.42	1.2	98.82
			LQC	67.500	67.892±1.32	1.9	100.58
Freeze/ thaw (on water bath)	-70±10°C	5 cylces	HQC	184.918	194.396±2.73	1.4	102.06
			LQC	67.500	64.4932±1.34	2.0	95.57

4. DISCUSSION

The validated UPLC method for plasma drug quantification of bedaquiline was highly sensitive and reproducible and fulfilled the regulatory requirements. This was successful in overcoming the analytical problems arising from the lipophilicity and low plasma levels of this drug. This method allowed the consistent recovery (about 70%) and reduced complexity versus solid-phase and liquid-liquid extraction as sample preparation strategy via protein precipitation process. This will have positive effects on throughput and sustainability in standard bioanalytical practice.

This new method, in comparison with previous reported HPLC and LC-MS/MS methods has several advantages. The use of traditional HPLC techniques could lead to poor reproducibilities and longer run times and increased solvent consumption, which makes it difficult for clinical use. The strengths of the LC-MS/MS assays are that they are sensitive but have a drawback because they need expensive equipment and optimization so are not necessarily practical in resource-limited conditions. The developed UPLC workflow, on the other hand, provides a sensitive and practical method for pharmacokinetic studies and therapeutic drug monitoring for patients with multidrug-resistant tuberculosis (MDR-TB).

The performance of the method was optimized and validated with excellent linearity (25-225 µg/mL, r2 = 0.999), accuracy (94-110%) and precision (%CV ≤ 4.5) which were consistent with the ICH M10 bioanalytical validation guidelines. Stability testing on freeze-thawed and bench-top conditions showed good robustness of the method, and evaluation of matrix effect in lipemic and hemolyzed plasma showed negligible effect. These results further support the utility of the method for clinical situations.

Significantly, the adoption of Lean Six Sigma principles (DMAIC) gave a system for ongoing enhancement. All four phases–defining the critical parameters, assessing the variability, analysing the performance, making improvements and controlling the reproducibility–were systematically optimized. This method was not only beneficial to the analytical performance but also helped with sustainability and regulatory compliance, hence the importance of quality management in bioanalytical method development.

In conclusion, this study offers a new, regulatory-compliant UPLC assay that offers a good balance of sensitivity, precision, and convenience. Recent results are shown to be of interest in the modern scientific and clinical development of anti-tuberculosis therapies, by placing them in the context of literature and by structuring optimization aspects.

Overall, this study presents a novel, regulator-compliant UPLC assay that effectively bridges the gap between sensitivity, reproducibility, and practicality. By contextualizing results within existing literature and applying structured optimization, the method establishes a scalable platform for clinical and regulatory applications in modern anti-tuberculosis therapy.

5. CONCLUSION

Since Bedaquiline is highly lipophilic and the concentration in plasma is low, this is a new UPLC assay method developed for Bedaquiline in human plasma which complied to the requirements of the United States Food and Drug Administration. Validated method was found to have good linearity, accuracy, precision and good reproducibility with minimal matrix effects and good stability under conditions for clinical studies. Linearity was established over 25–225 µg/mL ($r^2 = 0.999$) and an LLOQ of 25µg/mL. Intra and inter-day precision were also ≤7% RSD, and the accuracy ranged between 94.23-109.98% at each QC level. The simple protein-precipitation extraction was adopted, and it not only yielded good extraction recovery but also minimized sample preparation in comparison to SPE or LLE. The matrix effects IS-normalized were in the ranges ±1.293 ± 0.042 (HQC) and 0.905 ± 0.021 (LQC) and the mean recovery was 69.81% across the different plasma lots, both hemolyzed and lipemic plasma. The method development was done using the Lean Six Sigma (DMAIC) approach, optimizing and documenting the critical method parameters to enhance not only method efficiency and sustainability but also the regulatory readiness. Run time of 6 minutes (1.553 minutes Analyte plus 2.553 minutes ISTD) allowed a throughput of ~73 samples per day, and a 46% decrease in the amount of solvent was consumed compared to typical methods. This approach includes a robust quality system and bioanalytical validation that can translate to more regulatory confidence and can be applied in therapeutic drug monitoring and pharmacokinetic studies for MDR-TB patients as well as providing a platform for scaled up contemporary anti TB research.

REFERENCES

1. Vanino E, Granozzi B, Akkerman OW, Munoz-Torrico M, Palmieri F, Seaworth B, Tiberi S, Tadolini M. Update of drug-resistant tuberculosis treatment guidelines: A turning point. Int J Infect Dis. 2023 May;130 Suppl 1:S12-S15. doi: 10.1016/j.ijid.2023.03.013. Epub 2023 Mar 12. PMID: 36918080.
2. Maranchick NF, Peloquin CA. Role of therapeutic drug monitoring in the treatment of multi-drug-resistant tuberculosis. J Clin Tuberc Other Mycobact Dis. 2024; 36:100444. doi: 10.1016/j.jctube.2024.100444. PMID: 38708036; PMCID: PMC11067344.
3. Cox E, Laessig K. FDA approval of bedaquiline—the benefit–risk balance for drug-resistant tuberculosis. N Engl J Med. 2014;371(8):689-91. doi:10.1056/NEJMp1314385.
4. Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis. Drugs. 2002;62(15):2169-83. doi: 10.2165/00003495-200262150-00001. PMID: 12381217.
5. Food and Drug Administration. Drug approval package: Sirturo (bedaquiline) NDA 204384. Silver Spring (MD): U.S. Food and Drug Administration; 2012.
6. International Council for Harmonisation. M10 bioanalytical method validation and study sample analysis. Geneva: ICH; 2022.
7. Pontali E, Sotgiu G, Tiberi S, et al. Cardiac safety of bedaquiline: A systematic and critical analysis of the evidence. Eur Respir J. 2017;50(5):1701462. doi:10.1183/139 93003.01462-2017
8. World Health Organization. WHO consolidated guidelines on tuberculosis: Module 4 – treatment-resistant TB treatment, 2022 update. Geneva: WHO; 2022.
9. Svensson EM, Dosne AG, Karlsson MO. Population Pharmacokinetics of Bedaquiline and Metabolite M2 in Patients With Drug-Resistant Tuberculosis: The Effect of Time-Varying Weight and Albumin. CPT Pharmacometrics Syst Pharmacol. 2016;5(12):682-691. doi: 10.1002/psp4.12147.
10. Tsuyuguchi K, Sasaki Y, Mitarai S, Kurosawa K, Saito Y, Koh T. Safety, efficacy, and pharmacokinetics of bedaquiline in Japanese patients with pulmonary multidrug-resistant tuberculosis: An interim analysis of an open-label, phase 2 study. Respir Investig. 2019 Jul;57(4):345-353. doi: 10.1016/j.resinv.2019.01.001.
11. Alffenaar JW, Bolhuis M, van Hateren K, Sturkenboom M, Akkerman O, de Lange W, Greijdanus B, van der Werf T, Touw D. Determination of bedaquiline in human serum using liquid chromatography-tandem mass spectrometry. Antimicrob Agents Chemother. 2015 Sep;59(9):5675-80. doi: 10.1128/AAC.00276-15.
12. ZHU Hui,LIU Zhong-quan,XIE Li,GUO Shao-chen,WANG Bin,FU Lei,LU Yu. Determination of bedaquiline plasma concentration by high performance liquid chromatography-mass spectrometry/mass spectrometry[J]. Chinese Journal of Antituberculosis, 2018, 40(12): 1319-1324. doi: 10.3969/j.issn.1000-6621.2018.12.015
13. Meghavath M, Devanna N. Advanced solid phase extraction tools for measuring bempedoic acid and its active metabolite in human plasma by using LC-MS/MS. Indian J Pharm Educ Res. 2025;59(2s):s758-s68. doi:10.5530/ijper.20257284
14. Warankar SR, Bhure SG, Jawanjal SS, Vasatkar RR, Sawarkar TH. Bedaquiline: Revolutionizing tuberculosis treatment against drug resistance. Int J Creative Res Thoughts. 2024;12(7):644-50.
15. Chandrudu J, Gandhimathi R. Method development and validation for estimation of bedaquiline in tablet dosage form by HPTLC and RP-HPLC study. Biomed Pharmacol J. 2025;17(1):1-8. doi:10.13005/bpj/2858.
16. Nath S. Interpretation of the mechanism of action of antituberculosis drug bedaquiline based on a novel two-ion theory of energy coupling in ATP synthesis. Bioeng Transl Med. 2019;4(3):1-12. doi.:10.1002/btm2.10106
17. Subha Deepa D, Visagaperumal V, Chandy V. Bedaquiline: An in-depth exploration into its antitubercular significance. Res Rev J Drug Des Discov. 2025;12(2).6-14.
https://journals.stmjournals.com/rrjoddd/article=2025/view=216517.

18. Mahesh M. New bioanalytical method development and validation for extraction of mirabegron in human plasma by using Quechers method and liquid chromatography tandem mass spectrometry. Asian J Pharm. 2025;19(2):647-55. doi.:10.22377/ajp. v19i2.6468
19. Hemanth Kumar AK, Sudha V, Vijayakumar A, Padmapriyadarsini C. Simultaneous method for the estimation of bedaquiline and delamanid in human plasma using high-performance liquid chromatography. Int J Pharm Pharm Sci. 2021;13(6):36-40. doi:10.22159/ijpps.2021v13i6.40853.