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RESEARCH ARTICLE

Development and Validation of RP-HPLC for Determination Bedaquiline Fumarate in Nanostructured Lipid Carriers, Tablets, and Human Plasma Using Design of Experiment Method

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SUMMARY

A reversed-phase HPLC (RP-HPLC) technique was established to quantify Bedaquiline fumarate (BDQ), a hydrophobic drug for multidrug-resistant tuberculosis. Effective separation was achieved with a mobile phase of methanol and ammonium acetate buffer (pH 4.7) in a 85:15 (v/v) ratio, at a flow rate of 1.2 mL/min. The validated method exhibited excellent linearity in multiple matrices: tablet formulations (20-200 µg/mL; R^2=0.9993), nanostructured lipid carriers (NLCs) (40-300 µg/mL; R^2=0.9991), and human plasma (0.1-10 µg/mL; R^2=0.9975). Method sensitivity was confirmed with limits of detection (0.7483 µg/mL) and limit of quantification (2.2677 µg/mL). Comprehensive validation demonstrated satisfactory recovery rates (98-102%), precision, and robustness (relative standard deviation <2%), meeting all International Council for Harmonisation requirements. This study presents a systematically optimized RP-HPLC method for the quantification of BDQ. The main advantages over existing methods include a significantly shorter retention time (<3 minutes) achieved through DoE optimization and successful validation across diverse sample types (tablets, NLCs, and human plasma), and offering a reliable time-efficient solution for routine analysis.

Keywords: *Chromatography, design of experiments, validation method, lipid, tablet, plasma.*

Tasarım Deney Yöntemi Kullanılarak Nanoyapılı Lipid Taşıyıcılar, Tabletler ve İnsan Plazmasında Bedaquilin Fumarat Tayini İçin RP-HPLC Yönteminin Geliştirilmesi ve Validasyonu

ÖZ

Çoklu ilaca dirençli tüberküloz tedavisinde kullanılan hidrofobik bir ilaç olan bedaquiline fumaratın (BDQ) kantitatif analizi için ters fazlı HPLC (RP-HPLC) yöntemi geliştirilmiştir. Metanol ve amonyum asetat tamponunun (pH 4,7) 85:15 (v/v) oranında karıştırılmasıyla hazırlanan mobil faz ve 1,2 mL/dak akış hızı ile etkin bir ayırma sağlanmıştır. Validasyon çalışmaları, yöntemin farklı matrislerde mükemmel doğrusallık gösterdiğini ortaya koymuştur: tablet formülasyonları (20-200 µg/mL; R^2=0,9993), nanoyapılı lipid taşıyıcılar (40-300 µg/mL; R^2=0,9991) ve insan plazması (0,1-10 µg/mL; R^2=0,9975). Yöntemin hassasiyeti, tespit limiti (0,7483 µg/mL) ve kantifikasyon limiti (2,2677 µg/mL) ile doğrulanmıştır. Kapsamlı validasyon çalışmaları, tatmin edici geri kazanım oranları (%98-102), kesinlik ve sağlamlık (bağıl standart sapma <%2) ile Uluslararası Harmonizasyon Konseyi (ICH) gerekliliklerini karşıladığını göstermiştir. Bu çalışma, BDQ kantitatif analizi için sistematik olarak optimize edilmiş bir RP-HPLC yöntemi sunmaktadır. Mevcut yöntemlere kıyasla başlıca avantajları arasında Deneysel Tasarım (DoE) optimizasyonu ile elde edilen önemli ölçüde daha kısa tutulma süresi (<3 dakika) ve tabletler, NLY'ler ve insan plazması gibi çeşitli örnek tiplerinde başarıyla validasyonu yer almaktadır; bu da rutin analizler için güvenilir ve zaman açısından verimli bir çözüm sunmaktadır.

Anahtar Kelimeler: *Kromatografi, deneysel tasarım, validasyon yöntemi, lipid, tablet, plazma.*

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INTRODUCTION

In December 2022, the World Health Organization published therapeutic protocols for multidrug-resistant tuberculosis (MDR-TB), incorporating bedaquiline fumarate (BDQ) as a key therapeutic agent (World Health Organization, 2022). These updated recommendations introduce a more streamlined, patient-centred treatment paradigm with improved equity of access (Vanino et al., 2023). BDQ, commercially available as Sirturo® (Janssen-Cilag), is administered as 100 mg immediate-release uncoated tablets (Petersen et al., 2023). The drug faces significant bioavailability challenges (10-30%) due to its poor aqueous solubility, despite high permeability (Jaw-Tsai et al., 2023). These physicochemical properties classify BDQ as a Biopharmaceutics Classification System (BCS) Class II compound, underscoring the need for formulation strategies to improve its dissolution (Guglielmetti et al., 2020).

This study developed a reversed-phase HPLC (RP-HPLC) method for the analysis of BDQ in pharmaceutical formulations (tablets and lipid nanocarriers) and biological samples. The development of this methodology aimed to achieve a balance between analytical performance (efficiency, accuracy) and practical considerations, including cost and solvent consumption (Abd-AlGhafar et al., 2022). While several HPLC and LC-MS/MS methods for BDQ analysis have been reported, many exhibit prolonged retention times or lack a systematic approach for optimization. Previous analytical approaches for BDQ determination have predominantly employed gradient HPLC systems, typically exhibiting retention times greater than 5 minutes (Chandrudu & Gandhimathi, 2024). Isocratic methods reported in the literature demonstrate even more protracted elution profiles, with retention times extending to 7.5 minutes (Ayodele et al., 2024). Notably, mobile phases incorporating methanol as the organic modifier have been associated with particularly extended chromatographic run times, ranging from 10 to 30 minutes in specific analytical protocols (Pardhi et al., 2024). Consequently, the development of an optimized HPLC method employing an appropriate methanol-ammonium acetate buffer ratio represents a critical advancement, combining the economic benefits of methanol with the chromatographic efficiency afforded by ammonium acetate buffers.

The implementation of a multivariate strategy through Design of Experiments (DoE) methodology permits the simultaneous optimization of multiple critical parameters to enhance chromatographic performance (Ajayi et al., 2024). The selection of an appropriate experimental design for HPLC method development requires careful consideration of multiple factors, including study objectives, practical constraints, resource availability, and temporal considerations (Abdallah et al., 2023). Method validation followed a tiered approach, commencing with analysis of simple tablet formulations before progressing to more complex matrices such as nanostructured lipid carriers and human plasma specimens (Bezawada et al., 2024). This systematic validation strategy ensures methodological robustness across diverse sample types while permitting thorough evaluation of the technique's pharmaceutical applications. The developed analytical procedure incorporates several innovative aspects that collectively reduce hazardous solvent consumption, minimise chemical waste generation, and improve energy efficiency. The method underwent strict validation in accordance with ICH International Council for Harmonisation (ICH) guidelines (Ermer, 2025), including comprehensive statistical evaluation. This optimized HPLC methodology represents a significant advancement in BDQ analysis, addressing several limitations associated with existing techniques.

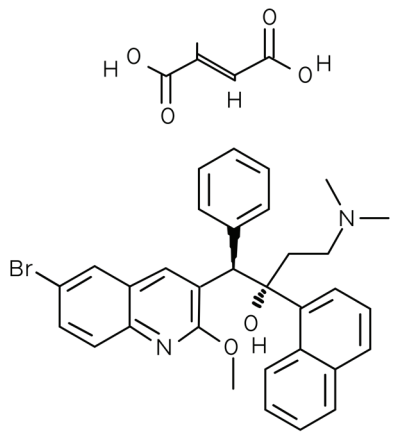


Figure 1. Chemical structure of Bedaquiline fumarate

MATERIALS AND METHODS

Materials

Bedaquiline fumarate was obtained commercially. All chemical reagents, including ammonium acetate, cetyl alcohol, oleic acid, Plantacare® 2000, and HPLC-grade methanol, were of analytical or pharmaceutical grade. Ultrapure water was produced using a laboratory water purification system. Marketed tablet formulation (Sirturo®) and fresh human blood samples were acquired for method validation.

Chromatographic apparatus and conditions

Chromatographic analysis was performed using an HPLC system equipped with a UV detector. Separation was achieved on a C18 column (250 × 4.6 mm, 5 µm) maintained at ambient temperature. The mobile phase, consisting of methanol and ammonium acetate buffer (pH 4.7) in a ratio of 85:15 (v/v), was delivered isocratically at a flow rate of 1.2 mL/min. Detection was carried out at 225 nm with an injection volume of 10 µL. Before use, the mobile phase was filtered through a 0.22 µm membrane filter.

Standard solution preparation

BDQ stock solutions (1000 µg/mL) were prepared by dissolving 10 mg of the compound in methanol within 10 mL volumetric flasks. Working standards were generated through serial methanolic dilutions of the stock solution, with thorough mixing ensured by vortex agitation. All solutions were filtered through 0.22 µm membrane filters before HPLC analysis to remove particulate matter. This dilution protocol produced the required concentration range for method validation while maintaining solution integrity.

Optimization and development of the RP-HPLC method

This study employed a full factorial design to optimize three critical parameters: flow rate (A), pH of buffer (B), and mobile phase ratio (C). As bedaquiline fumarate exhibits weak acidic properties, ammonium acetate buffer pH values (4.3, 4.5, 4.7) were selected to be slightly below the compound's pKa for enhanced reversed-phase retention (Pardhi et al., 2020). Methanol was chosen as the organic modifier due to its favourable low viscosity characteristics, which minimizes column backpressure (Abdu Hussen, 2022). Mobile phase compositions of buffer to methanol (15:85, 20:80, 25:75 v/v) were tested at flow rates (0.8, 1.0, 1.2 mL/min), with each parameter assessed at three coded levels (-1, 0, +1) for low, medium, and high values (Table 1.). A total of 27 experimental runs were executed to systematically assess the influence of these variables on two key chromatographic performance indicators: peak tailing factor (Y1), retention time (Y2), and theoretical plate count (Y3). The complete experimental matrix is detailed (Table 2.).

Table 1. Independent variables of the full factorial design method

Independent variables	Levels		
	-1	0	+1
Flow rate (mL/min)	0.8	1.0	1.2
Mobile phase ratio (buffer to methanol)	15:85	20:80	25:75
pH of buffer	4.3	4.5	4.7

Validation of analytical methods

Method validation followed ICH criteria, evaluating system performance, linear range, sensitivity (LOD/LOQ), precision, accuracy, and robustness (Chavan & Desai, 2022; Ermer, 2025).

Preparation of the mobile phase

The mobile phase comprised methanol-buffer mixtures ranging from 85:15 to 75:25 (v/v). A 10 mM ammonium acetate solution was prepared in 1000 mL HPLC-grade water, with pH precisely regulated to 4.3-4.7 using glacial acetic acid to achieve ideal separation conditions.

Table 2. 3^3 full factorial design optimization for Bedaquiline fumarate quantification

Run.	Independent variables			Dependent variables		
	Flow rate (mL/min) (A)	pH of Buffer (B)	Ratio of mobile phase buffer to methanol (%) (C)	Tailing Factor (Y_1)	Retention time (Y_2)	Theoretical plate (Y_3)
Run. 1	0.8	4.7	20:80	1.37	6.28	11916
Run. 2	0.8	4.7	25:75	1.30	12.83	12569
Run. 3	1.2	4.7	25:75	1.46	11.40	9851
Run. 4	1.2	4.3	25:75	1.97	9.32	8374
Run. 5	0.8	4.3	25:75	1.83	10.02	8670
Run. 6	1.2	4.5	20:80	1.51	4.63	9463
Run. 7	1.0	4.7	20:80	1.38	5.47	11120
Run. 8	0.8	4.3	20:80	1.37	3.66	11185
Run. 9	1.2	4.3	15:85	1.38	2.51	8838
Run. 10	1.0	4.5	25:75	1.53	10.68	9981
Run. 11	1.0	4.7	25:75	1.34	11.66	10752
Run. 12	1.2	4.5	25:75	1.62	11.41	9745
Run. 13	1.0	4.3	25:75	1.88	9.35	8608
Run. 14	0.8	4.3	15:85	1.32	3.21	9650
Run. 15	1.2	4.5	15:85	1.10	1.67	9084
Run. 16	0.8	4.5	25:75	1.52	9.50	9910
Run. 17	1.0	4.3	20:80	1.58	3.34	9207
Run. 18	0.8	4.5	20:80	1.48	5.57	10859
Run. 19	1.0	4.5	20:80	1.52	4.83	9521
Run. 20	1.2	4.3	20:80	1.58	3.41	8944
Run. 21	1.0	4.3	15:85	1.33	2.64	8964
Run. 22	0.8	4.7	15:85	1.09	3.46	10564
Run. 23	1.0	4.5	15:85	1.13	1.90	9715
Run. 24	1.0	4.7	15:85	1.08	2.77	10181
Run. 25	0.8	4.5	15:85	1.15	2.94	10777
Run. 26	1.2	4.7	20:80	1.38	5.39	11120
Run. 27	1.2	4.7	15:85	1.08	2.89	10417

System suitability

A 2.2 µg/mL BDQ solution was used to assess system suitability. Multiple injections verified compliance with acceptance criteria for peak area, tailing factor (TF), and theoretical plates (N). Mean values and percentage relative standard deviation (RSD) were calculated to assess method precision, ensure consistent chromatographic performance.

Linearity

The method showed strong linear responses within specified ranges: 20-200 µg/mL for BDQ tablets, 40-300 µg/mL for nanostructured lipid carriers (NLCs), and 0.1-10 µg/mL for human plasma. All standard curves achieved correlation coefficients exceeding 0.999, with satisfactory precision for all regression parameters.

Precision and accuracy

Method precision was investigated through both intra-day and inter-day studies (ICH Harmonised Guideline, 2022). Intra-day precision was evaluated by analyzing replicate calibration standard during a single session, whereas inter-day precision was assessed by conducting repeated tests over several days. Method accuracy was verified through recovery studies, in which a 100 µg/mL sample solution was spiked with standard solutions at 80%, 100%, and 120% of the original concentrations. Recovery percentages were then calculated to validate the method's trueness.

Selectivity

Method selectivity was rigorously evaluated by challenging the chromatographic system with potential interferents commonly present in the sample matrices. The standard addition method was employed for this purpose. A standard solution of BDQ was spiked into a placebo mixture mimicking the tablet excipients, blank nanostructured lipid carriers (without BDQ), and drug-free human plasma.

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

The limit of detection (LOD) and limit of quantification (LOQ) were derived from the calibration curve slope (SP) and the response standard deviation (r), following ICH criteria. LOD was defined as 3.3r/SP, whereas LOQ was calculated as 10r/SP. These values reflect the method's sensitivity and precision at low analyte concentrations.

Robustness

Method robustness was evaluated by intentionally modifying to chromatographic parameters, including mobile phase composition (83:17 to 87:13), flow rate (1.1-1.3 mL/min), and wavelength (224-226 nm). The impact on retention times and peak areas was analyzed using % RSD to verify consistency across altered conditions (Kohler et al., 2023).

Method application

Sample processing for BDQ in tablet dosage forms

The exact mass of Sirturo® 100 mg tablets was determined by precisely measuring the powdered tablet weight. BDQ tablet powder (100 mg) was dissolved in methanol within a volumetric flask for subsequent analysis.

Sample processing for BDQ in nanostructured lipid carriers

BDQ-loaded NLCs were prepared via ultrasonic emulsification (Hariyanti et al., 2023). The lipid phase (3.5% cetyl alcohol, 1.5% oleic acid). BDQ mixed into the lipid phase. The aqueous phase (4% PlantaCare® 2000, deionized water) was heated to 65°C. A heated microemulsion was prepared by homogenizing at 8000 rpm for 2 minutes, then probe-sonicated for 6 minutes (45:15 pulse cycle, 60% amplitude) to form NLCs. Following vortex mixing (2 min) and centrifugation (6000 rpm, 25 min) of 10 mL BDQ-NLC, 250 µL supernatant was diluted in methanol (2000 µL) and acetonitrile (3000 µL) before HPLC analysis.

Sample processing for BDQ in human plasma

Human plasma samples were obtained from Palang Merah Indonesia (PMI), Surabaya. 250 µL of human plasma was combined with BDQ standard solutions in separate centrifuge tubes. Following thorough mixing, protein precipitation was achieved

using 500 µL acetonitrile and 250 µL methanol, followed by 3 minutes of vortexing and 10 minutes of centrifugation at 15,000 rpm. The clear supernatant obtained was isolated and analyzed using the specified HPLC method.

Statistical analysis

A full factorial design was implemented in Minitab 21 (Minitab LLC, USA) to assess factor effects and interactions systematically.

RESULTS AND DISCUSSION

System suitability

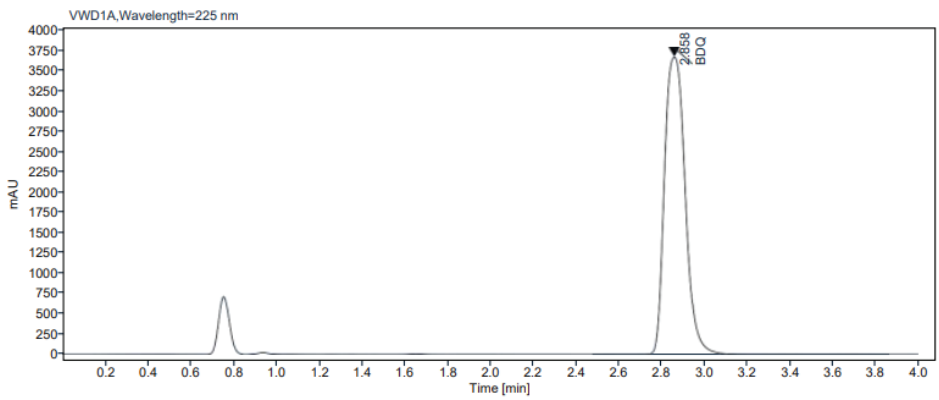
System suitability testing was conducted by determining %RSD for three critical chromatographic parameters: peak area reproducibility, tailing factor, and theoretical plate count (Isane et al., 2022). Analytical validation involved six replicate injections of a 2.2 µg/mL BDQ standard solution. The calculated %RSD values for both peak area and theoretical plate number demonstrated excellent precision, with all measurements yielding variations below the 2% threshold (Table 3.).

Table 3. System suitability test using 2.2 µg/mL

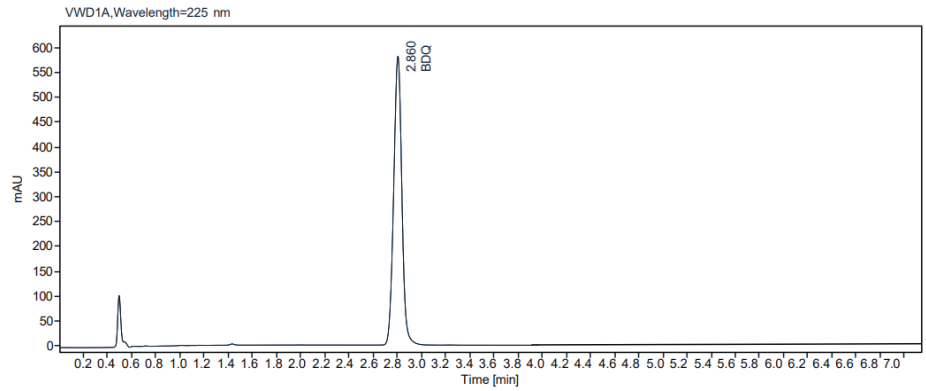
No.	Parameters	Results		
		Mean	SD	% RSD
1	Peak area	188.26	0.48	0.26
2	Number of theoretical plates	4536	29.04	0.64

Selectivity

The experimental protocol adhered strictly to the validation requirements outlined in ICH Q2(R1) guidelines (Rajmane & Shinde, 2023). Representative chromatograms (Figure 2.) illustrate the separation profiles for: (a) blank matrix, (b) BDQ standard solution (500 µg/mL), (c) tablet formulation extract, (d) nanostructured lipid carrier preparation, and (e) human plasma sample. The chromatographic system demonstrated consistent retention characteristics, with BDQ eluting at 2.858 ± 0.003 minutes across all sample matrices, confirming method robustness and also offering an environmental benefit through reduced solvent consumption per run due to the shorter analysis time compared to many existing methods.



(a)



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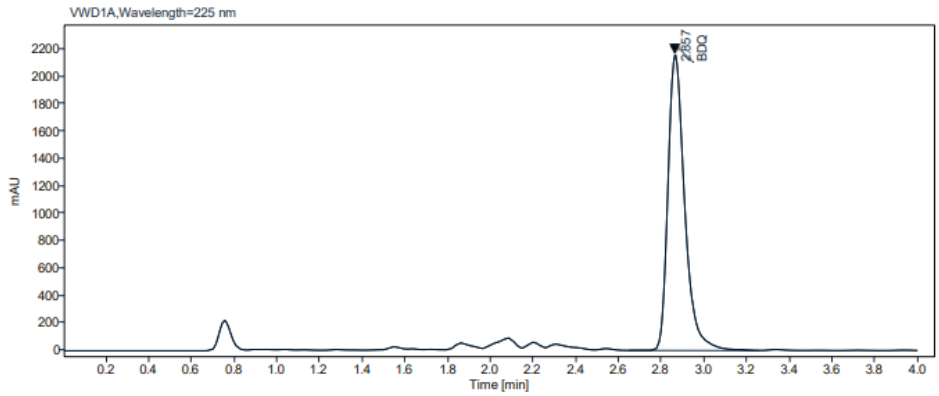

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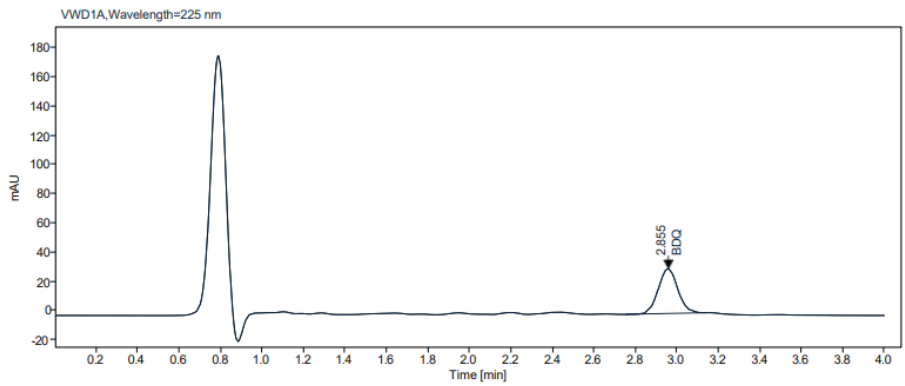


(d)

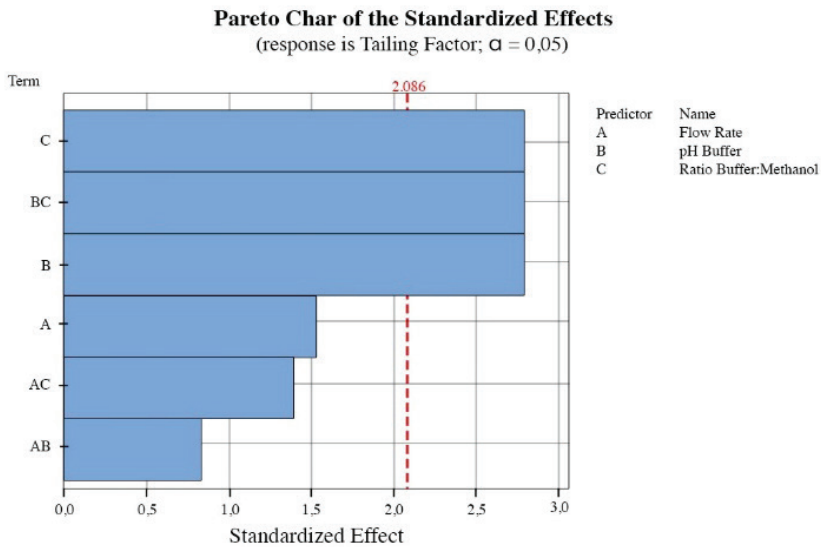
Figure 2. Chromatogram of BDQ in (a) standard at 500 µg/mL, (b) tablet dosage form, (c) nanostructured lipid carrier, (d) human plasma sample

Development and optimization of the RP-HPLC method

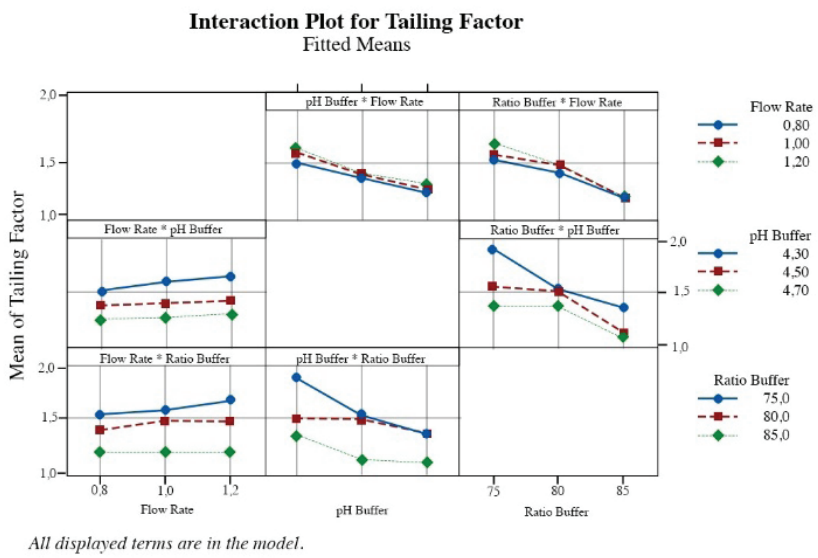
Twenty-seven chromatographic runs were performed, to evaluate peak area, tailing characteristics, and plate counts. Regarding the experimental design selection, a full factorial design (3^3) was deliberately chosen over more efficient response surface methodologies (RSM) such as Central Composite Design (CCD) or Box-Behnken Design (BBD). While CCD or BBD require fewer runs for a quadratic model, a full facto-

rial design provides the most reliable and unbiased estimates of all interaction effects without confounding (Montgomery, 2017). Minitab 21-generated Pareto analysis demonstrated statistically significant effects (p=0.011) of mobile phase composition (Factor C), buffer pH (Factor B), and their interaction (BC) on peak symmetry (Patil & Chalikwar, 2024). Flow rate variations proved inconsequential. Both linear and quadratic effects of pH and solvent ratio predominated in influencing tailing behaviour, either independently or synergistically. These results establish that mobile-phase pH and composition are pivotal parameters for chromatographic optimization. Visual representations of factor-response relationships are provided for standardized effects (Figure 4a.) and interaction plots (Figure 4b.), illustrating the dependence of chromatographic performance on these critical variables. The buffer pH (B), mobile phase ratio (C), and their interaction (BC) had statistically significant effects on chromatographic performance as their standardized effects exceeded the critical threshold, indicating that these parameters meaningfully influence separation outcomes.

a



b



All displayed terms are in the model.

Figure 3. Pareto chart illustrating standardised effects (a) and tailing factor interaction plot (b)

Minitab's response optimization tool established threshold values (minimum, target, maximum) for three critical responses (Table 4.), determining ideal operational parameters and corresponding desirability metrics (Sahu et al., 2018). The composite desirability index (D), scaled 0-1, quantified method robustness, with D=1 representing optimal performance as shown in (Table 4.) (Stojanović et al., 2021). The optimized chromatographic conditions comprised: 15:85 (v/v) buffer to methanol mobile phase, 1.2 mL/min flow rate, pH 4.7 buffer, and 30°C column temperature, demonstrating excellent robustness (D=1).

Table 4. Response optimization of a 3^3 full factorial design for RP-HPLC of BDQ

Parameters							Optimum condition pH = 4.7, methanol = 85%, flow rate 1.2 mL/min	
							Composite desirability (D) = 1	
Responses	Goal	Lower	Target	Upper	Weight	Importance	Predicted responses	Individual desirability (d)
TF	Minimize	1.08	1.0	1.97	1	1	1.08	1

The response optimization analysis established the ideal chromatographic conditions for BDQ analysis: ammonium acetate buffer (pH 4.7) and methanol in a 15:85 (v/v) ratio, delivered at a flow rate of 1.2 mL/min. This specific combination is grounded in fundamental reversed-phase chromatography principles to achieve an optimal balance of speed, efficiency, and peak integrity. The methanol-rich mobile phase (85%) is critical for the rapid elution of the highly hydrophobic BDQ (Nag et al., 2020). The high organic modifier content competitively reduces analyte interaction with the C18 stationary phase, thereby enabling the significantly shorter retention times (<3 minutes). This choice also enhances the method's greenness and cost-effectiveness compared to acetonitrile-based methods. The buffer pH of 4.7 was strategically selected based on BDQ's acid-base properties. Maintaining a pH slightly below its pKa ensures the molecule remains predominantly non-ionized, maximizing consistent hydrophobic interactions with the stationary phase (Dharuman et al., 2023). This controlled pH, synergized with the strong eluting power of 85% methanol, yields sharp, symmetrical peaks with minimal tailing. The flow rate of 1.2 mL/min represents an optimal compromise between analysis speed and chromatographic efficiency. While higher flow rates reduce retention time, they can compromise theoretical plate count, as evidenced by the negative coefficient for flow rate in the theoretical plate model (Y3). This selected rate provides fast analysis while maintaining high efficiency and acceptable backpressure.

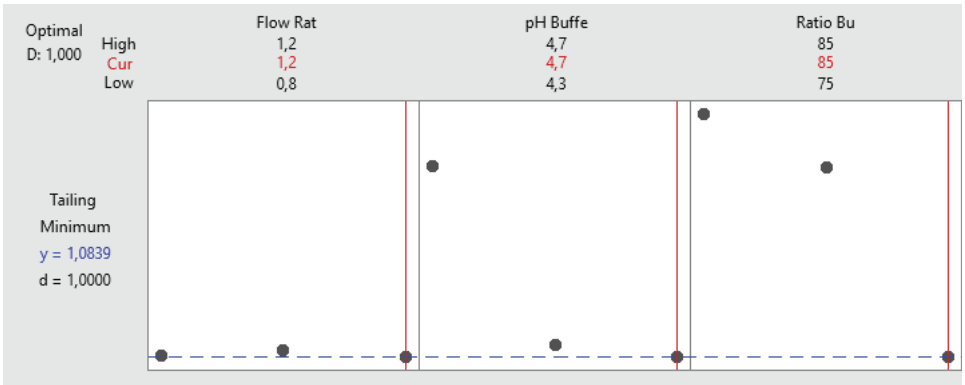


Figure 4. 3^3 Factorial design optimization plot for chromatographic parameter evaluation.

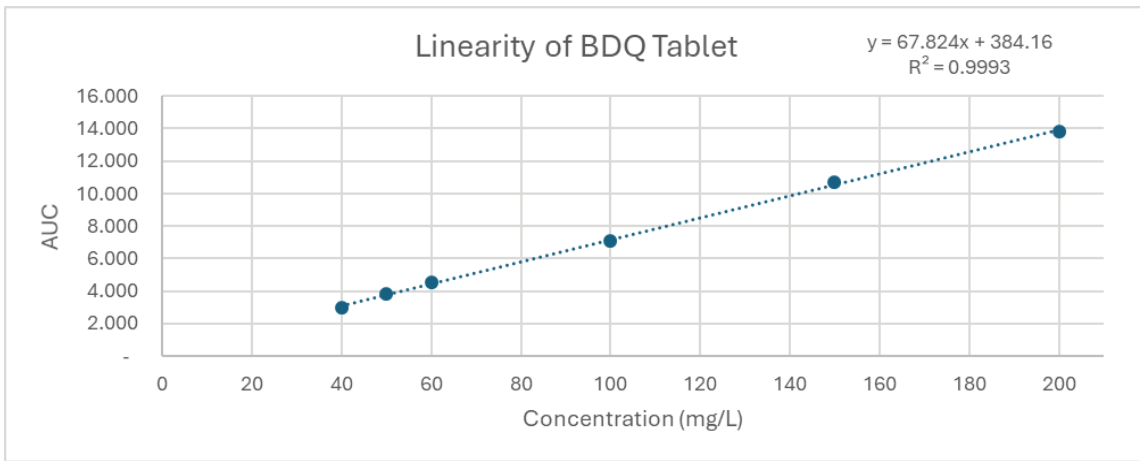
The model's adequacy was evaluated by analyzing pure error from central composite design points. Statistical assessment revealed significant model fit ($p<0.05$) for all response variables, confirming satisfactory predictive capability without evidence of lack of fit. Polynomial regression equations demonstrated quantitative relationships between independent and dependent variables, with positive regression coefficients indicating synergistic effects and negative values representing antagonistic interactions (Kumar et al., 2015). Coefficient magnitude directly correlated with parameter influence strength, with larger values denoting greater impact. The derived equations (Y1, Y2, and Y3) showed statistically significant correlations with all measured responses. The equations (1), (2), and (3) demonstrate how flow rate (A), buffer pH (B), and mobile phase ratio (C) affect chromatographic performance. A higher flow rate increases the tailing factor (Y1) but reduces the retention time (Y2). Alkaline pH decreases tailing while increasing theoretical plates (Y3). Mobile phase ratio shows minimal individual impact but interacts significantly with other parameters.

Tailing factor (Y1) = 27.13 + 5.17A – 5.60B – 0.309C – 0.508AB – 0.0337AC +0.0667BC	(1)
Retention Time (Y2) = -167 +25.6A + 49.5B +1.80C - 2.55 AB - 0.193AC – 0.536BC	(2)
Theoretical Plate (Y3) = -120.721 – 3.423A + 3.0516B + 1.382C – 653AB + 44AC – 318BC	(3)

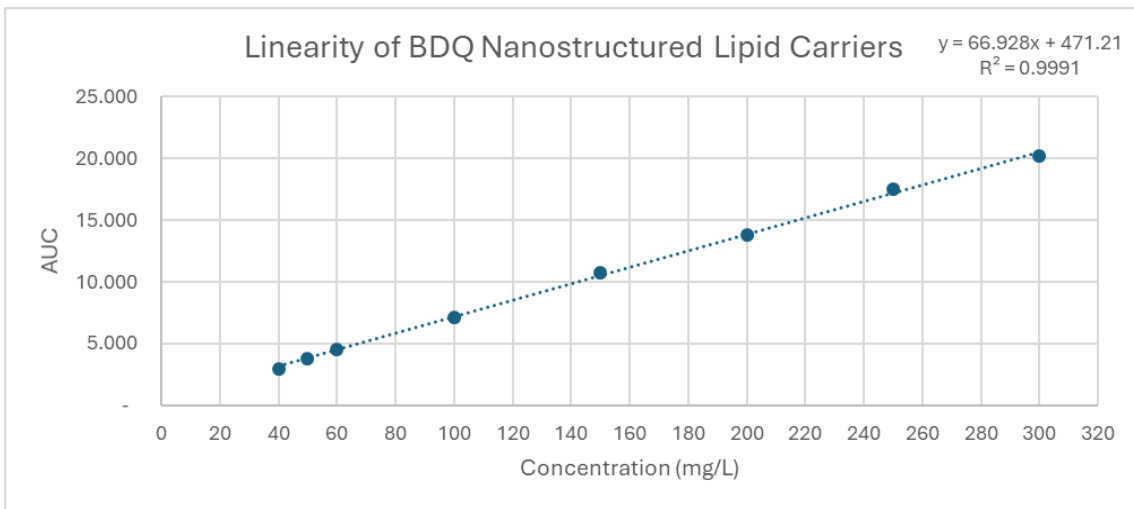
The model for the tailing factor (Y1) indicates that a higher flow rate (positive coefficient for A) adversely affects peak symmetry by increasing tailing. In contrast, an alkaline buffer pH (negative coefficient for B) is the most significant factor for improving peak shape by reducing tailing. While the mobile phase ratio (C) has a minimal standalone effect, its synergistic interaction with pH (positive BC coefficient) suggests that the beneficial effect of high pH is slightly enhanced at higher organic solvent concentrations. For retention time (Y2), buffer pH (B) has the profound positive effect, significantly increasing the analyte's residence time on the column. The flow rate (A) also shows a strong positive effect, which is counterintuitive but explained by its dominant interaction terms. This underscores that the system is best controlled by balancing these two parameters rather than adjusting them in isolation. The theoretical plate count (Y3), a measure of chromatographic efficiency, is primarily and favorably influenced by the buffer pH (B), as indicated by its significant positive coefficient. A higher flow rate (A) negatively impacts column efficiency. The most significant interaction is the strong antagonistic effect between flow rate and pH (negative AB coefficient), demonstrating that the combined increase of these two parameters drastically reduces the number of theoretical plates, thereby compromising separation efficiency. This highlights that achieving high efficiency requires a lower flow rate coupled with a higher pH.

Linearity

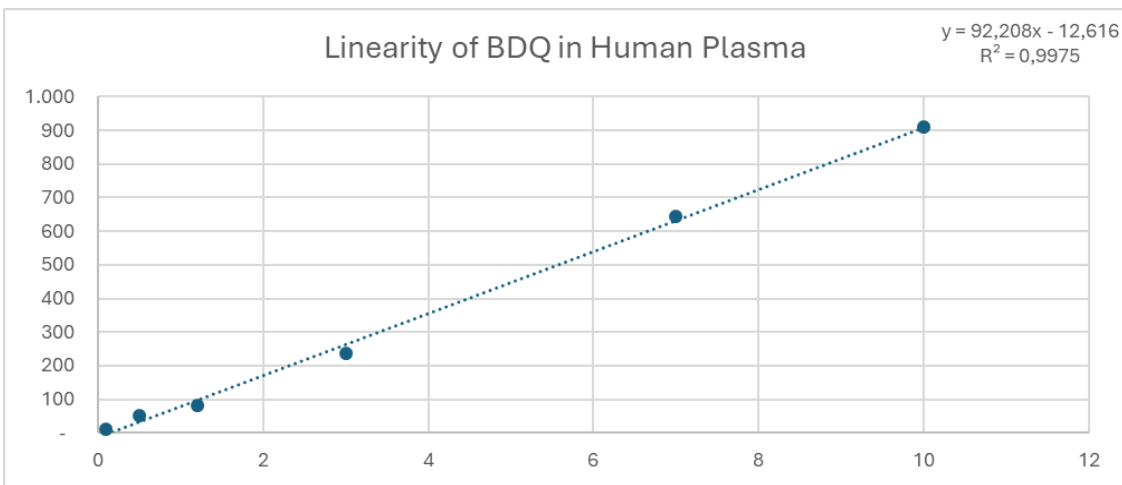
For tablet formulations Sirturo® (40-200 µg/mL), the linear regression equation $y = 67.8240x + 384.1611$ demonstrated excellent linearity (R^2=0.9993). Nanostructured lipid carrier analysis (40-300 µg/mL) showed comparable linearity (R^2=0.9991) with the equation $y = 66.9280x + 471.2101$. Plasma samples (0.1-10 µg/mL) showed satisfactory linearity (R^2 = 0.9975), described by $y = 92.2083x - 12.6161$. All calibration curves exhibited strong correlation coefficients exceeding 0.9975, confirming method validity across the studied concentration ranges.



(a)



(b)



(c)

Figure 5. Calibration Curve of BDQ in (a) tablet, (b) nanostructured lipid carrier, (c) human plasma sample

Precision

The analytical method's precision was rigorously assessed through intra-day (n=6) and inter-day variability studies, determined via peak area %RSD calculations (Çağlar et al., 2022). Accuracy validation examined BDQ in tablet formulations (70 µg/mL), nanostructured lipid carriers (240 µg/mL), and human plasma (2.5 µg/mL) through replicate analyzes. Both precision evaluations demonstrated exceptional reproducibility, with all %RSD values remaining below the 2% acceptance threshold (Table 5.), confirming method reliability for quantitative analysis across different matrices and time intervals.

Table 5. Intra-day and inter-day precision of BDQ in tablets, nanostructured lipid carriers, and plasma

Sample	Conc. (µg/mL)	Intra-day precision				Inter-day precision			
		Peak area	Mean area	SD	% RSD	Peak area	Mean area	SD	% RSD
BDQ in tablet	70	4,822.63	4,867.48	34.62	0.71	4,795.44	4,842.07	36.54	0.75
		4,891.44				4,837.76			
		4,843.36				4,861.11			
		4,922.59				4,895.24			
		4,884.07				4,866.09			
		4,840.80				4,796.77			
BDQ in nanostructured lipid carrier	240	16,264.32	16,295.29	36.83	0.23	16,508.97	16,427.55	193.78	1.18
		16,301.14				16,109.46			
		16,237.02				16,462.29			
		16,353.05				16,483.28			
		16,301.49				16,727.03			
		16,314.70				16,274.27			
BDQ in human plasma	2.5	188.50	188.26	0.48	0.26	188.11	188.28	0.24	0.13
		187.48				188.38			
		188.38				188.40			
		187.75				187.84			
		188.65				188,36			
		188.82				188.58			

Accuracy

Method accuracy was evaluated through standard addition at three concentrations (80%, 100%, 120%) across matrices: pure samples, nanostructured lipid carriers, and human plasma. Recovery rates demonstrated excellent accuracy, ranging from 98.46% to 101.11%, confirming the method's reliability for quantitative analysis.

Table 6. The percentage recovery of BDQ in tablet dosage form, nanostructured lipid carrier, and in human plasma

Sample	% Level	Conc. (µg/mL)	Found Conc. (µg/mL)	Mean	SD	% RSD	% Recovery
BDQ in tablet dosage form	80	80.01	80.02	80.42	0.43	0.54	100.52
			81.02				
			80.22				
	100	100.14	99.42	99.96	0.54	0.54	100.16
			100.52				
			100.56				
	120	120.03	119.99	119.33	0.49	0.41	99.44
			119.18				
			118.82				
BDQ in lipid-based nanocarriers	80	217.86	216.03	216.95	0.90	0.42	99.58
			218.18				
			216.65				
	100	236.67	235.97	236.02	0.39	0.17	99.90
			236.52				
			235.56				
	120	245.63	246.50	248.35	1.65	0.67	101.11
			248.04				
			250.51				
BDQ in human plasma	80	1.76	1.76	1.76	0.01	0.65	99.79
			1.76				
			1.74				
	100	2.20	2.17	2.17	0.01	0.15	98.46
			2.17				
			2.18				
	120	2.60	2.58	2.59	0.01	0.29	99.49
			2.59				
			2.60				

Limit of detection and limit of quantitation

Method sensitivity was established through signal-to-noise ratio calculations, with experimentally derived limits of 0.75 µg/mL for detection and 2.27 µg/mL for quantification, indicating robust bedaquiline measurement capability.

Robustness

The developed RP-HPLC method demonstrated consistent repeatability and specificity under optimized conditions. Robustness was evaluated through controlled variations in flow rate, temperature, and organic phase composition (Surapuraju & Juturu,

2022). Increasing temperature negligibly impacted retention time (%RSD < 2%) or BDQ peak area (recovery: 97.0–98.9%). Conversely, lower temperatures reduced retention time by 2.9% and slightly decreased peak stability (recovery: 93.2–96.7%). A 0.1 mL/min flow rate reduction delayed elution (9.66% difference) but maintained acceptable recovery (96.2–101.6%). These results confirm the method's reliability under minor operational modifications.

Table 7. Summary of BDQ validation parameter

Validation parameters	Tablet dosage forms	Nanostructured lipid carriers	Human plasma	Acceptance Criteria
Optimum wavelength (nm)	225	225	225	225
System suitability				
Peak area (%RSD)	0.71	0.23	0.26	
Retention time (%RSD)	0.13	0.13	0.25	<2%
Tailing factor (%RSD)	1.53	0.50	1.50	
Linearity				
Linearity range (µg/mL)	20-200	40-300	0.1-10	
Slope	67.8240	66.9280	92.2083	
Intercept	+384.1611	+471.2101	–12.6161	
Regression-coefficient (R^2)	0.9993	0.9991	0.9975	
Sensitivity				
LoD (µg/mL)	0.75			Sensitive
LoQ (µg/mL)	2.27			
Precision				
Intra-day (%RSD)	0.79	0.23	0.24	<2%
Inter-day (%RSD)	0.71	1.21	0.13	
Accuracy				
80% recovery (%)	100.52 ± 0.43	99.58 ± 0.90	99.79 ± 0.01	
100% recovery (%)	100.16 ± 0.54	99.90 ± 0.17	98.46 ± 0.15	98-102%
120% recovery (%)	99.44 ± 0.49	101.11 ± 1.65	99.49 ± 0.01	
Robustness				
Ratio of mobile phase (%RSD)	1.75			
Rate of flow (%RSD)	0.50			<2%
Detection wavelength (%RSD)	0.83			

Method application

Recovery assay in tablet dosage form

Content analysis of Sirturo® tablets demonstrated high BDQ purity (99.44–100.52%), with excellent reproducibility (%RSD 0.41–0.54). The developed method reliably quantified active pharmaceutical ingredient content, meeting pharmacopeial standards for drug formulation assessment (Sahu et al., 2018). This result is consistent with the findings of Chandrudu & Gandhimathi (2024), who reported a high BDQ purity of 100.78% in their analysis of Sirturo® tablets using an RP-HPLC method. The excellent reproducibility (%RSD 0.41–0.54) achieved in our study further corroborates the reliability of HPLC methods for the quality control of BDQ in pharmaceutical dosage forms.

Entrapment efficiency of BDQ nanostructured lipid carrier

The characterization of the NLCs has been streamlined to focus specifically on encapsulation efficiency and recovery data, as these parameters are most directly relevant to demonstrating the applicability and accuracy of the developed HPLC method for quantifying Bedaquiline in this complex formulation. The

developed NLCs demonstrated favourable physicochemical properties, with a monodisperse size distribution (157.88 ± 0.12 nm; PDI 0.254 ± 0.06) and excellent colloidal stability (zeta potential -45.9 ± 0.12 mV). High encapsulation efficiency (96.2 ± 0.24%) confirmed effective BDQ incorporation. Method validation through sextuplicate analyzes yielded consistent recovery rates (99.5 to 99.9%), verifying the reliability of the quantification approach (Table 6.).

Recovery assay in human plasma samples

LC-MS provides superior specificity and unambiguous compound identification through mass detection, unlike the non-specific UV detection in RP-HPLC. While advanced techniques such as LC-MS/MS are predominantly reported for BDQ analysis in biological samples (Gray et al., 2019), our validated RP-HPLC method offers a highly reliable, more accessible alternative. The excellent recovery rates (98.9-99.8%) confirm that this method provides the fundamental accuracy required for bioanalytical applications, such as therapeutic drug monitoring, without the need for sophisticated mass spectrometry instrumentation. This represents a critical first step in the bioanalytical method validation workflow, establishing the fundamental reliability for quantifying BDQ in plasma. However, it is acknowledged that analysing of plasma from patients undergoing BDQ treatment or from a formal pharmacokinetic study would have greater translational impact. Such applications would demonstrate the method's robustness in the presence of real-world metabolic interferences and its utility in therapeutic drug monitoring (TDM) or clinical pharmacokinetic research. Therefore, the application of this validated method to real patient samples is a clear and recommended direction for future work to fully leverage its potential in optimizing multi-drug-resistant tuberculosis therapy.

CONCLUSION

In conclusion, a rapid RP-HPLC method for BDQ was successfully optimized using a full-factorial DoE, achieving a key improvement analysis time under 3 minutes. The method was comprehensively validated across tablets, NLCs, and human plasma. The validation parameters confirmed the method's reliability, demonstrating excellent linearity (R^2 > 0.9975 across all matrices), high accuracy (mean recovery: 98.46-101.11%), exceptional precision (intra-day and inter-day %RSD < 2%), and satisfactory sensitivity (LOD: 0.75 µg/mL; LOQ: 2.27 µg/mL). Although the use of HPLC for BDQ analysis is established, a systematic DoE approach was developed to achieve rapid separation and validate it across a broad application scope. While the current study successfully demonstrates the method's suitability for quantifying BDQ in spiked human plasma, its application to pharmacokinetic studies or patient samples is a necessary next step to confirm its translational value in therapeutic drug monitoring and clinical research.

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AUTHOR CONTRIBUTION STATEMENT

Concept (AR, RM), Design (AR, RM), Supervision (RM, ST, NFK), Resources (AR, RM), Materials (AR, RM), Data Collection and/or Processing (AR), Analysis and/ or Interpretation (RM, ST, NFK), Literature Search (AR), Writing (AR), Critical Reviews (RM, ST, NFK).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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