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A sensitive, rapid and cost-effective RP-HPLC-UV method for detection and quantification of bedaquiline in physiological fluid (pH 7.4)

Simisola Ayodele,[a] Armorel D. van Eyk,[b] Pradeep Kumar[a] and Yahya E. Choonara *[a]

Bedaquiline, a highly lipophilic molecule, is used in the treatment regimen of multi-drug resistant tuberculosis. A rare complication of pulmonary tuberculosis is tuberculous pericarditis. *Ex vivo* studies utilising animal pericardium can be used to investigate whether this drug is capable of diffusing across pericardial tissue into simulated pericardial fluid (pH 7.4) to indicate efficacy. For detection of bedaquiline in physiological fluid, a rapid, cost-effective and sensitive method is essential. The aim of this study was thus to develop and validate a simple and sensitive RP-HPLC-UV method for the detection and quantification of bedaquiline, encapsulated in a nanosystem, at pH 7.4 after permeation across excised pericardium. A HPLC Phenomenex Kinetex RPC18 column (150 × 4.6 mm, 5 μm) was utilized for analysis. The mobile phase consisted of 95 : 5 v/v (A : B), where (A) methanol : acetonitrile (85 : 15 v/v) : (B) triethylamine (1% v/v) : 0.15 mM KH_2PO_4 buffer (pH 7.4). Running conditions included the following: injection volume 20 μl, flow rate 1.0 ml min^{-1}, detection wavelength 275 nm, 25 °C and running time of 5 min. Bedaquiline eluted as a single symmetrical peak at a retention time of 4.17 min. The method was found to be linear within the range of 1–50 μg ml^{-1} ($R^2 = 1$). The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.05 μg ml^{-1} and 0.15 μg ml^{-1}, respectively (signal-to-noise ratio method). All validation parameters were found to be within acceptable limits (RSD < 2%). The method was fast, reliable, accurate, reproducible, and transient for the detection of bedaquiline in simulated physiological fluid (pH 7.4). This method can thus be applied to easily detect bedaquiline in body fluids (pH 7.4) *i.e.* blood and pericardial fluid without the accuracy being impacted by ionisation factors of the molecule.

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Introduction

Bedaquiline is chemically identified as (1*R*,2*S*)-1-(6-bromo-2-methoxy-3-quinolyl)-4-dimethylamino-2-(1-naphthyl)-1-phenylbutan-2-ol as shown in Fig. 1 below.

It is a diarylquinoline with tuberculocidal activity approved for the treatment of multi-drug-resistant tuberculosis (MDR-TB).[1] Bedaquiline works by inhibiting the proton pump of *M. tuberculosis* ATP synthase. It has a MIC of 0.03 mg l^{-1} against *M. tuberculosis* H37Rv and 0.12 mg l^{-1} against a range of *M. tuberculosis* clinical isolates.[2] It also forms two major metabolites through a series *N*-demethylation by cytochrome p450 isoenzyme 3A4 namely, *N*-monodesmethyl-bedaquiline (M2) and *N*-didesmethyl-bedaquiline (M3), with an *in vitro* potency of about 5- and 187-fold less than that of bedaquiline, respectively.[3] Bedaquiline has also been found to be slightly more potent against dormant *M.tb* strains as compared to rifampicin and isoniazid. It has a half-life of ~5.5 months.[4] TB pericarditis (TBP) is a rare complication of pulmonary tuberculosis caused by lymphatic spread of the TB bacilli from lymph nodes.[5] The extent of bedaquiline penetration across the pericardium into the pericardial fluid is not well known, resulting in uncertainty whether this drug reaches high enough levels in the fluid to effectively eradicate the bacilli. Bedaquiline is

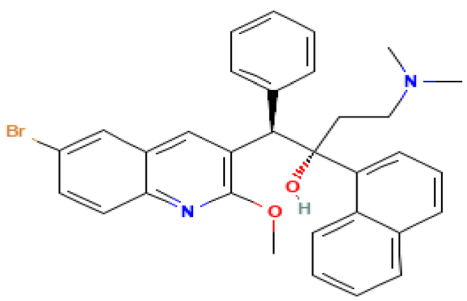


Fig. 1 Chemical structure of bedaquiline (https://pubchem.ncbi.nlm.nih.gov/).

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a lipophilic drug with poor solubility in aqueous fluid but high protein binding (99%), very low MIC and long half-life.[6] It is a positively charged molecule with pK_a values of 1.57 (imine); 8.91 (amine); and 13.61 (hydroxyl).[7] Pericardial permeation seems to favour drugs with low protein binding, thus incorporating drugs with a higher protein binding into nanosystems may result in improved drug penetration and bioavailability in pericardial fluid to effectively treat pericarditis and prevent constrictive pericarditis.[8]

Ex vivo studies of bedaquiline diffusion characteristics across porcine pericardium may serve as an affective model providing greater information on efficacy. Existing methods have utilized acidic buffers at pH 4.5 and a PDA detector to detect bedaquiline is official dissolution media,[9] because bedaquiline is an orally administered drug, such methods are suitable for experiments that involve the oral administration of the drug due to the pH of gastric media. Other methods have employed the use of a gradient method using HPLC connected to a mass spectrometer for the quantification of bedaquiline.[10] In addition, some isocratic methods yielded a longer retention time (R_t) of 5.4 min, whilst using a high ratio of acetonitrile.[11] Furthermore, use of methanol as an organic component of the mobile phase in the detection of bedaquiline, have shown to result in much longer retention times of more than 10 min, and to up 30 min in some HPLC detection methods.[12–14] A HPLC method was thus required that would incorporate a suitable ratio of methanol and acetonitrile that would ensure a fast, reliable and cost-effective method for the detection of bedaquiline, due to methanol's cost-advantage and acetonitrile's elution strength.

Studies that explore the incorporation of bedaquiline into nanoparticle injectables require more sensitive and pH congruent methods for detection in plasma and body fluids with a general pH of 7.4. Due to its pK_a of 8.91 (amine), bedaquiline is expected to act as a weak base and thus be slightly ionised in plasma and body fluids. To prevent further dilution of samples with acid after collection, a method with a pH that allows direct analyses of body fluids without the need to change pH of samples containing bedaquiline to fit existing methods, is required. This will maintain the charge of bedaquiline after sample collection and during elution to prevent tailing and splitting of peaks.[15]

The aim of this study was therefore to develop a sensitive, rapid and cost-effective RP-HPLC method with UV detection to quantify bedaquiline, incorporated into nanoparticles, for the quantification in release and *ex vivo* diffusion studies.

The method was validated based on the recommendations of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; ICH guidelines Q2(R1) ("ICH Guideline Q2(R1): Validation of Analytical Procedures: Text and Methodology", 2005).[16]

Materials and methods

Materials

The materials employed in this study were of the highest purity grade and were purchased from Sigma-Aldrich (Pty) Ltd (St. Louis, Missouri, United States). This included the following: bedaquiline (CAS 843663-66-1, 95% purity), methanol (CAS 67-56-1, 99.9% purity HPLC grade), acetonitrile (CAS 75-05-8, 99.9% HPLC grade), potassium dihydrogen orthophosphate (CAS 7778-77-0, 99.0% purity), triethylamine (CAS 121-44-8, 99.5% purity), orthophosphoric acid (CAS 7664-38-2, 85% purity).

Preparation of standards and phosphate buffer for mobile phase

Preparation of phosphate buffer. Phosphate buffer (0.15 mM) was prepared by weighing 20 mg of potassium dihydrogen orthophosphate (KH_2PO_4) and dissolving it in 1000 ml Milli-Q® water. Triethylamine (10 ml) was added to this mixture and the pH of the subsequent solution was adjusted to 7.4 with 10% (v/v) orthophosphoric acid. All samples and buffers were filtered (0.45 μm) before HPLC analysis.

Preparation of standard solutions. The mobile phase was prepared by mixing 5 ml of phosphate buffer prepared above, with 80.8 ml of methanol and 14.2 ml of acetonitrile. Bedaquiline (10 mg) was dissolved in 10 ml of mobile phase and further diluted down using a dilution factor of 0.1 (10%) to form 100 μg ml^{-1} of stock solution.

Running conditions. Previously, various methods have been developed and validated for the detection of bedaquiline, with most using a high organic phase ratios due to the lipophilic nature of bedaquiline. Preliminary studies using (A) methanol : acetonitrile (85 : 15) : (B) phosphate buffer in a ratio of 90 : 10 (A : B) were conducted and no peaks were obtained. Therefore, phosphate buffer containing triethylamine and adjusted with orthophosphoric acid to physiological pericardial pH of 7.4, was prepared and utilized instead of water. The lambda max was determined by dissolving bedaquiline in the proposed mobile phase and obtaining its wavelength on a UV/Vis spectrometer. Running conditions using ratios of (A) methanol : acetonitrile (85 : 15) : (B) phosphate buffer at 90 : 10, 85 : 15 and 95 : 5 (A : B) in a run time of 5 min were tested and the chosen condition was based on system suitability, characteristics of the peak, and retention time. The chromatographic apparatus and conditions employed are displayed below (Table 1).

Validation parameters

Linearity. Standard solutions were prepared from the stock solution utilizing mobile phase as a diluent to prepare working standards of 1 μg ml^{-1}, 2 μg ml^{-1}, 5 μg ml^{-1}, 10 μg ml^{-1}, 25 μg ml^{-1} and 50 μg ml^{-1} concentrations. These samples were separately analysed in triplicate. A standard curve of the peak area *versus* concentration (μg ml^{-1}) was constructed and the linearity of bedaquiline tested by linear regression analyses of the graph to obtain the following linear regression equation:

$$y = mx + c$$

where y = the peak area of the analyte; m = slope; x = the drug concentration (μg ml^{-1}); $c = y$ − intercept.

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Table 1 Instrumentation and HPLC chromatography conditions for the detection of bedaquiline

Parameter	Conditions
Instrument	A PerkinElmer Flexar HPLC system, consisting of a binary pump, autosampler, column oven and a UV/Vis detector. The system was equipped with Chromera® software for data acquisition and analysis (PerkinElmer Inc., Waltham, Massachusetts, USA)
Column	Phenomenex Kinetex C18 RP LC column with dimensions of 150 × 4.6 mm and particle size of 5 μm
Mobile phase	An organic phase of (A): (methanol : acetonitrile (85 : 15)) and an aqueous phase of (B): (triethylamine (10 ml) and potassium phosphate buffer (0.15 mM) adjusted with orthophosphoric acid to a pH of 7.4). Ratio of 95 : 5 (A : B). The phosphate buffer was filtered using a vacuum pump through a 0.45 μm membrane
Flow rate	1 ml min^{-1}
Detection wavelength	275 nm
Injection volume	20 μl
Run time	5 min
Column temperature	25 °C
Elution technique	Isocratic

Precision

Intraday precision. Three samples (1 μg ml^{-1}, 10 μg ml^{-1}, and 50 μg ml^{-1}) were used to determine intraday precision. Samples were injected in triplicate in the morning and afternoon (8 hours apart) on the same day. The acceptance criteria for intra-day precision are set to be a % relative standard deviation (RSD) ≤ 2.0.

Interday precision. Three, low, medium and high-quality control (QC) samples from points on the line of regression (1 μg ml^{-1}, 10 μg ml^{-1}, and 50 μg ml^{-1}) were used to determine inter-day precision. Samples were injected in triplicate for three consecutive days. The acceptance criteria for intra-day precision are set to be a % relative standard deviation (RSD) ≤ 2.0.

Accuracy. The method was used to detect bedaquiline in physiological fluid *e.g.* simulated pericardial fluid (phosphate-buffered saline (PBS)) during *ex vivo* simulation studies. PBS with sodium lauryl sulphate (SLS) was used to simulate the fluid during the *ex vivo* simulation studies. For the quality of analytical results, three different standard concentrations were prepared by spiking PBS (pH 7.4) containing 0.2% sodium lauryl sulphate (SLS) using a 25 μg ml^{-1} standard solution to obtain the following final concentrations: 8 μg ml^{-1}, 10 μg ml^{-1}, and 12 μg ml^{-1}. These concentrations are three different levels of target concentration (80%, 100% and 120%) as per the ICH guideline. The samples were analysed in replicates of 6. The acceptance criteria for accuracy are between 98 and 102%.[16]

Robustness. The robustness of the method was performed on 1 μg ml^{-1}, 10 μg ml^{-1}, and 50 μg ml^{-1} standard samples by adjusting mobile phase, temperature, and flow rate. The mobile phase was adjusted by ±1% of the organic component, temperature was adjusted by ±1 °C and flow rate was adjusted by ±0.1 ml min^{-1} and tested to note its variation in % recovery. Samples were injected 6 times. The change in R_t and the area change were evaluated (RSD ≤ 2%).

Stability. The stability of the analyte in the mobile phase was tested by placing 1 μg ml^{-1}, 10 μg ml^{-1}, and 50 μg ml^{-1} samples at 25 °C, 4 °C and −20 °C, tested weekly until significant change occurred. The acceptance criteria for recovery are between 98 and 102%. This allows the analyst to know the time frame a sample can be kept from the day of preparation until the time it degrades by 2%.

Results

RP-HPLC method development and validation for bedaquiline

Method development. For this method, various mobile phase ratios of 90 : 10, 85 : 15 and 95 : 5 (A : B) containing (A) methanol/acetonitrile (85 : 15) : (B) phosphate buffer pH 7.4 were all tested. A mobile phase ratio of 95 : 5 (A : B) was the only ratio to show a peak within a run time of 5 min, and was therefore selected for method validation. The lack of a chromatogram within the 5 min run time was found to be due to a retention time shift of up to 0.55 min per 1% change of mobile phase from organic to aqueous phase, as shown in the robustness section. The mobile phase ratio of 95 : 5 (A : B) was chosen since it produced a narrow and symmetrical peak with a retention time of 4.17 minutes that allowed a run time of less than 5

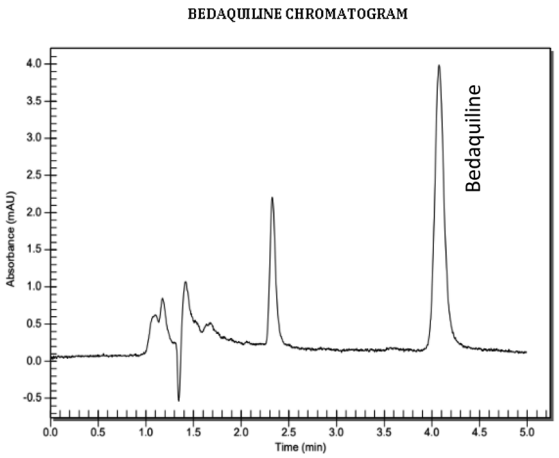
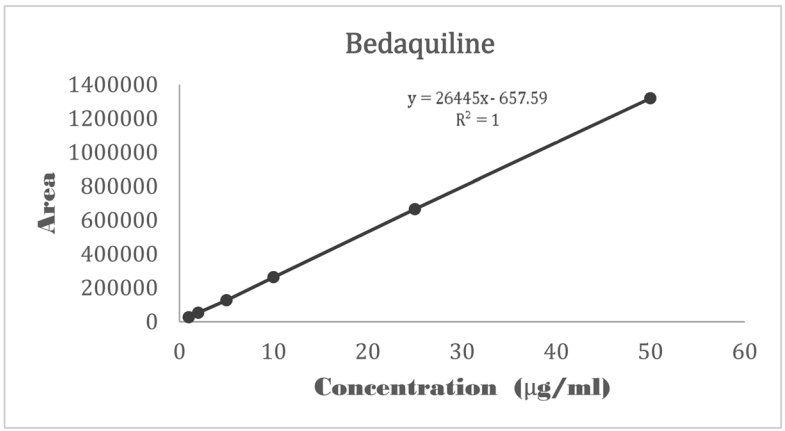


Fig. 2 RP-C18 HPLC chromatogram with a run time of 5 minutes and a peak with a retention time of 4.17 min for bedaquiline (1 μg ml^{-1}) using a mobile phase of 95 : 5 (A : B) (A) organic phase (containing methanol 85 : 15 acetonitrile) : (B) phosphate buffer at a flow rate of 1 ml min^{-1} and a detection wavelength of 275 nm (20 μl, 25 °C).

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(a)



(b)

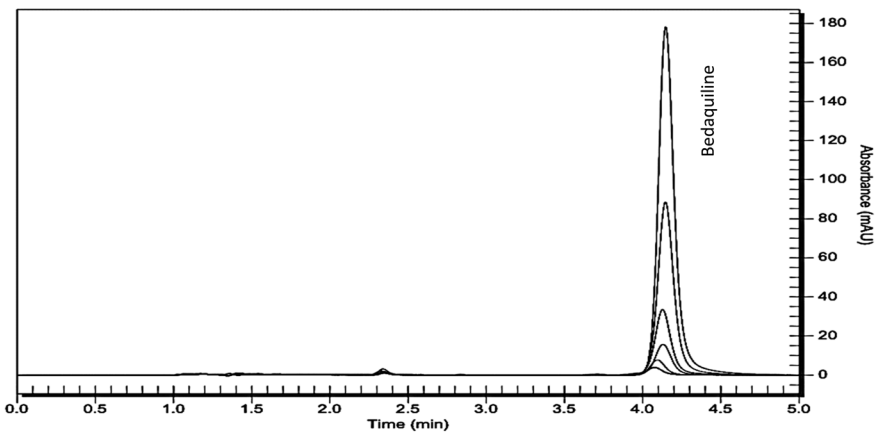


Fig. 3 Linearity of bedaquiline (a) a standard curve of bedaquiline ($n = 3$) (each standard was injected 3 times), (b) overlay of bedaquiline standard curve chromatograms ($R_t = 4.17$ minutes).

minutes (Fig. 2), low pressure, and a lesser chance of crystallization. As the buffer ratio increased in the system, crystallization was observed. Organic solvents were predominantly utilised due to the hydrophobic nature of bedaquiline. The lipophilicity of the molecule caused it to precipitate in mobile phases with a high aqueous content. To prevent crystallization in the column, the column was first equilibrated with 95 : 5 (A : B) (A) methanol/acetonitrile : (B) water for 10 minutes. Thereafter, the water was replaced with the prepared phosphate buffer and equilibrated for 30 minutes at 95 : 5 (A : B).

Table 2 Linearity of bedaquiline established from three independent standard curves using the established RP-C18 HPLC method. Each analyte concentration was performed in triplicate

Concentration ($\mu g\ ml^{-1}$)	Std curve 1	Std curve 2	Std curve 3	Overall mean	
	Mean peak area ± SD	Mean peak area ± SD	Mean peak area ± SD	Peak area ± SD	% RSD
1	28 972.74 ± 722.63	25 370.32 ± 205.15	26 550.40 ± 147.99	26 964.49 ± 1499.55	5.56
2	57 405.93 ± 709.35	50 550.11 ± 233.54	51 765.03 ± 157.07	53 240.35 ± 2986.97	5.61
5	126 958.76 ± 999.31	124 289.06 ± 1010.07	129 325.74 ± 279.57	126 857.85 ± 2057.45	1.62
10	253 987.42 ± 405.98	267 336.73 ± 1456.12	268 280.89 ± 432.23	263 201.68 ± 6526.85	2.48
25	669 440.35 ± 5174.52	676 459.28 ± 290.18	651 068.14 ± 384.35	665 655.92 ± 10 705.73	1.61
50	1 300 634.92 ± 10 707.34	1 344 631.17 ± 5026.61	1 313 333.77 ± 1835.96	1 319 533.29 ± 18 488.61	1.40

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Method validation

Linearity. Linear regression of the standard curves displayed the suitability of the method within the prescribed concentration range (1–50 μg ml^{-1}) for bedaquiline ($R^2 = 1$) (Fig. 3a and b, Table 2). The LOD and LOQ were determined to be 0.05 μg ml^{-1} and 0.15 μg ml^{-1} (Fig. 4a and b), respectively using signal-to-noise ratio (S/N)[17] and 0.62 μg ml^{-1} and 1.90 μg ml^{-1} using the standard deviation of response and slope calculation method.[18]

Precision. The inter-day and intra-day precision showed reproducibility over 3 days in the detection of bedaquiline, with a % RSD of <2% for all selected standard concentrations, which is within the acceptance criteria (Table 3).

Accuracy. The method was efficient, with an overall recovery of 99.69% with an RSD of 0.47% ($n = 6$) after a spike of the PBS, containing 0.2% SLS, with 25 μg ml^{-1} bedaquiline, as presented in Table 4.

Robustness. The developed method is consistent, showing repeatability and specificity in the established running conditions. The robustness of the HPLC method was investigated by small variations in the following conditions: flow rate, temperature, and the organic mobile phase component (Table 5). A slight increase in temperature did not alter the retention time (% RSD < 2%) and did not alter the peak area detected for bedaquiline significantly, with a recovery range of 96.0–97.8%. However, the retention time decreased by 3.2% and the area detected was slightly less stable with a slight temperature decrease, with a recovery range of 92.0–96.8%. As the flow rate decreased (by 0.1 ml min^{-1}), elution occurred later by a difference of 8.51%. The % recovery increased to a range of 104.3–

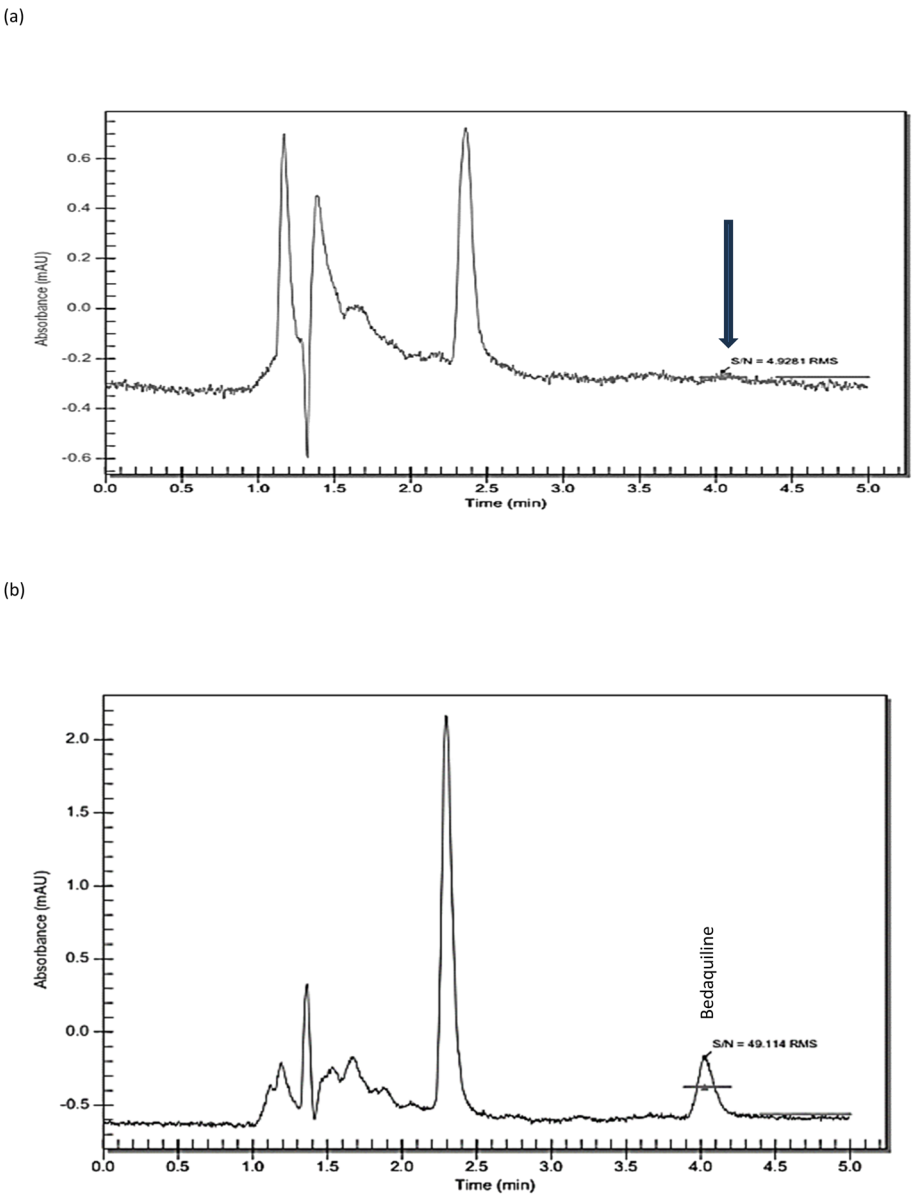


Fig. 4 RP-C18 HPLC chromatogram for the specific and selective detection of bedaquiline to determine the LOQ (10× S/N ratio). (a) Blank peak signal-to-noise ratio for bedaquiline ($R_t = 4.17$ minutes) (S/N ratio of 4.9) (b) a diluted concentrate to obtain peak 10× signal-to-noise ratio ($R_t =$ 4.17 minutes, S/N ratio of 49, 10 times the S/N ratio of blank).

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Table 3 Precision determined for the established HPLC method from three independent standard concentrations. Each analyte concentration was performed in triplicate[a]

		Intraday precision		Interday precision			
		Day 1		Day 2		Day 3	
		Mean peak		Mean peak		Mean peak	
Drug	Concentration (μg ml^{-1})	Area ± SD	% RSD	Area ± SD	% RSD	Area ± SD	% RSD
BDQ	1	362 608.88 ± 250.47	0.095	362 272.36 ± 235.70	0.142	362 473.30 ± 183.72	0.114
	10	1 732 359.35 ± 686.47	0.197	1 732 700.69 ± 1352.44	0.206	1 730 004.29 ± 3884.79	0.128
	50	3 416 987.88 ± 2095.96	0.043	3 411 933.39 ± 2309.15	0.031	3 411 276.29 ± 1137.87	0.041

[a] BDQ: bedaquiline; % RSD: % relative standard deviation.

Table 4 Accuracy of the established HPLC method from three independent concentrations. Each analyte concentration was performed in sextuplicate[a]

Drug	Level, %	Concentration (μg ml^{-1})	Amount recovered ± SD (μg ml^{-1})	Recovery ± SD%	RSD (%)
BDQ	80	8.00	7.96 ± 0.015	99.45 ± 0.186	0.55
	100	10.00	10.02 ± 0.027	100.24 ± 0.267	0.24
	120	12.00	11.92 ± 0.005	99.37 ± 0.039	0.63

[a] BDQ: bedaquiline; SD: standard deviation; RSD: relative standard deviation.

114.1%. As the flow rate increased (by 0.1 ml min^{-1}), elution occurred earlier by a difference of 10.86%. The % recovery decreased to a range of 89.7–96.2%. As the organic component in the mobile phase decreased, the peak eluted later by a difference of 13.55%. The % recovery decreased slightly to a range of 97.2–98.4%. As the organic component in the mobile

Table 5 Robustness determined for the established HPLC method from three independent standard concentrations by varying flow rate, temperature, and organic mobile phase component. Each analyte concentration was performed in sextuplicate[a]

	Bedaquiline				
Condition	Mean peak area ± SD	Amount tested/ recovered (μg ml^{-1})	% recovery	Mean R_t ± SD	Mean R_t % RSD
FR: 1 ml min^{-1}	22 149.03 ± 357.53	0.86 ± 0.04	—	4.16 ± 0.014	—
MP: 95 : 5	224 328.14 ± 3339.72	8.51 ± 0.15	—		
T: 25 °C	1 126 410.03 ± 3203.44	42.62 ± 0.15	—		
FR change: 0.9 ml min^{-1}	23 132.39 ± 282.05	0.90 ± 0.04	104.3 ± 4.44	4.52 ± 0.039	8.51
	246 750.78 ± 278.72	9.36 ± 0.04	110.0 ± 0.43		
	1 285 761.89 ± 4065.01	48.65 ± 0.18	114.1 ± 0.37		
FR change: 1.1 ml min^{-1}	19 794.46 ± 394.04	0.77 ± 0.04	89.7 ± 5.19	3.71 ± 0.024	10.86
	210 510.93 ± 2006.28	7.99 ± 0.10	93.9 ± 1.25		
	1 084 096.45 ± 2869.08	41.02 ± 0.13	96.2 ± 0.32		
T change: 24 °C	20 318.04 ± 131.28	0.79 ± 0.03	92.0 ± 3.80	4.13 ± 0.014	1.07
	213 138.20 ± 1753.37	8.08 ± 0.09	95.0 ± 1.11		
	1 090 081.38 ± 1627.26	41.25 ± 0.09	96.8 ± 0.22		
T change 1: 26 °C	21 477.79 ± 116.04	0.84 ± 0.03	97.1 ± 3.57	4.03 ± 0.011	3.20
	215 379.26 ± 248.68	8.17 ± 0.03	96.0 ± 0.37		
	1 101 493.03 ± 963.25	41.68 ± 0.06	97.8 ± 0.14		
MP change: 94 : 6 (A : B)	21 520.981 ± 227.78	0.84 ± 0.03	97.2 ± 3.57	4.72 ± 0.034	13.55
	219 065.3141 ± 352.46	8.31 ± 0.04	97.7 ± 0.48		
	1 108 243.451 ± 1460.23	41.93 ± 0.08	98.4 ± 0.19		
MP change: 96 : 4 (A : B)	21 022.98616 ± 68.23	0.82 ± 0.03	95.1 ± 3.66	3.76 ± 0.011	9.66
	224 131.9622 ± 197.77	8.50 ± 0.03	99.9 ± 0.35		
	1 138 549.174 ± 2307.75	43.08 ± 0.11	101.1 ± 0.26		

[a] FR: flow rate; *T*: temperature; MP: mobile phase; R_t: retention time; % RSD: % relative standard deviation from normal conditions.

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Table 6 Stability of the established HPLC method from three independent standard concentrations. Each analyte concentration was performed in sextuplicate[a]

		Bedaquiline					
	Concentration (μg ml^{-1})	1		10		50	
	Temperature °C	25	4	25	4	25	4
W0	Rec (μg ml^{-1})	0.98	0.78	10.13	8.76	50.87	42.74
	% RV	—		—		—	
W1	Rec (μg ml^{-1}) ± SD	1.06 ± 0.08	0.79 ± 0.01	10.55 ± 0.42	8.78 ± 0.02	52.85 ± 1.98	42.23 ± 0.51
	% RV	107.80	100.97	104.12	100.28	103.89	98.81
W2	Rec (μg ml^{-1}) ± SD	1.07 ± 0.09	0.86 ± 0.08	11.60 ± 1.05	9.47 ± 0.71	55.84 ± 4.97	44.77 ± 2.03
	% RV	108.82	110.68	114.44	108.20	109.77	104.75
W3	Rec (μg ml^{-1}) ± SD	1.27 ± 0.29	0.88 ± 0.10	14.48 ± 4.35	10.24 ± 1.48	66.14 ± 15.27	49.04 ± 6.30
	% RV	128.54	113.09	142.92	116.98	130.01	114.74

[a] W0: initial concentration; W1: week 1; W2: week 2; W3: week 3; Rec: recovery; % RV: % recovery. Error bars = SD.

phase increased, the peak eluted earlier by a difference of 9.66%. The % recovery was found to be 95.1–101.1%, where the change favoured higher concentrations of 10 μg ml^{-1} and 50 μg ml^{-1} (% RSD < 2%).

Stability. Bedaquiline showed good stability when stored at 4 °C and room temperature (25 °C) in the first week of storage. From week 2 onwards, an increase in concentration was observed due to the high percentage of methanol's low vapour pressure causing it to volatilize into air (Table 6). Bedaquiline demonstrated better stability in the fridge (4 °C) as compared to at room temperature. Both compounds may be stored for at least one week before analyses at either room temperature (25 °C) or fridge (4 °C).

Application of the validated method. After method validation, the method was tested for suitability of quantification of bedaquiline, which was loaded into a nanosystem during *ex vivo* animal studies. These studies involved the investigation of the diffusion kinetics of a bedaquiline-loaded nanosystem across porcine pericardium. Porcine pericardial tissue was collected from the University of the Witwatersrand Central Animal Unit (ethics waiver numbers 04-06-2020-O and 02-04-2024-O), snap frozen in liquid nitrogen and stored at −70 °C. The nanosystem was developed through a one-step ionic gelation probe sonication method.[19] After the pericardial tissue was thawed in PBS (pH 7.4), the tissue was sectioned into 7 pieces (1 cm^2 each) and mounted in the flow-through cells of a PermeGear 7-In-Line Flow-through Diffusion system (PermeGear Inc, Hellertown, USA). The bedaquiline-loaded nanosystem (1 ml : 1 mg ml^{-1}) was added to the donor compartments of each flow-through diffusion cell. PBS containing 0.2% (w/v) sodium lauryl sulphate (SLS) was pumped through the receptor compartments at a rate of 1.5 ml h^{-1} at 37 °C and samples collected in a fraction collector 2-hourly for 24 hours. The collected samples were filtered and subsequently diluted 50 : 50 with mobile phase and analysed using the

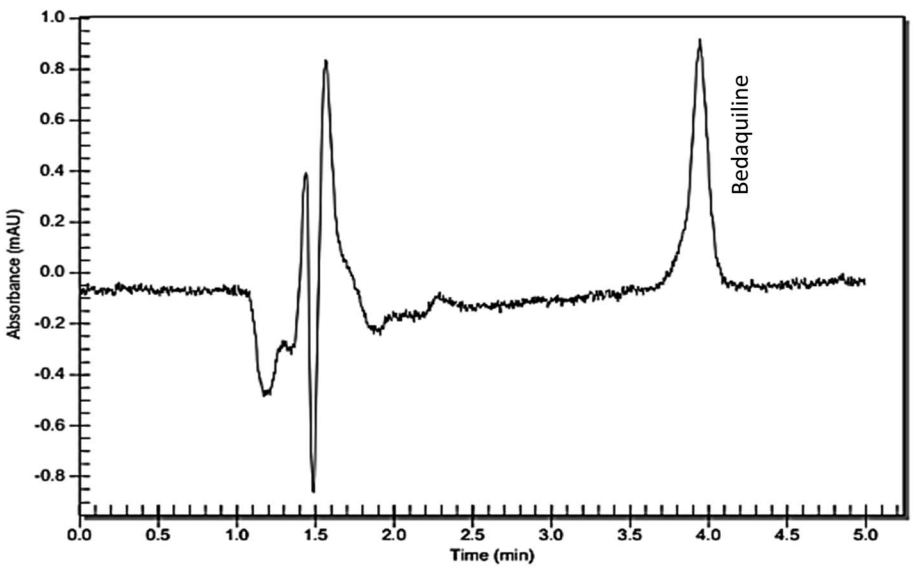


Fig. 5 RP-C18 HPLC chromatogram showing bedaquiline detection (R_t = 4.01 min) from bedaquiline-loaded nanosystem samples that diffused across excised porcine pericardium.

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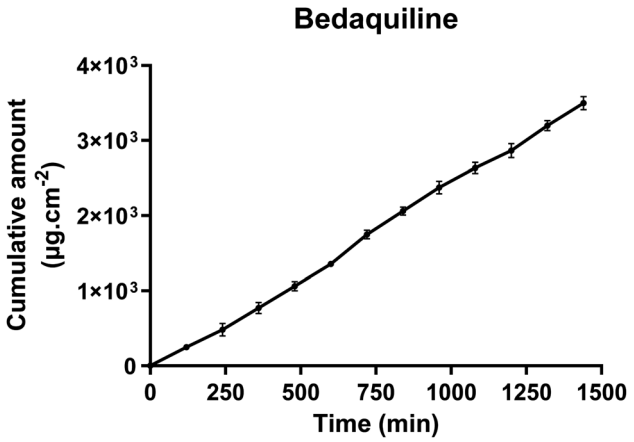


Fig. 6 The diffusion kinetics of bedaquiline from bedaquiline-loaded nanoparticles across excised porcine pericardium ($n = 3$).

Table 7 Quantification of bedaquiline in a bedaquiline-loaded nanosystem over a period of 24 hours during a preliminary *ex vivo* diffusion study across porcine pericardium ($n = 3$)

Time (min)	Cumulative amount µg cm^{-2}
0	0
120	249.10 ± 19.23
240	481.21 ± 83.39
360	770.01 ± 75.06
480	1062.59 ± 61.05
600	1353.63 ± 18.56
720	1746.78 ± 59.46
840	2061.49 ± 55.14
960	2376.51 ± 85.39
1080	2635.83 ± 73.74
1200	2864.26 ± 95.30
1320	3199.81 ± 68.99
1440	3499.49 ± 87.15

developed HPLC method, without the need for pre-treatment. Fig. 5, 6 and Table 7 show the chromatogram and cumulative amounts (µgcm^{-2}) *versus* time (min) graph and values determined from a preliminary diffusion study across excised porcine pericardium over a period of 24 hours. The method proved that it could successfully detect and quantify the amount of bedaquiline that diffused across the pericardium without any precipitation of the drug occurring in the receptor compartments after diffusion. Subsequent to this preliminary study showing the suitability of the method, a full study was performed on bedaquiline-loaded nanosystems and the diffusion characteristics across both excised porcine and human pericardium, which was submitted for publication.[19] This study indicated that injected bedaquiline-loaded nanosystems may thus be considered for further studies in the treatment of tuberculous pericarditis.[19]

Discussion

The results from this study indicated that a simple and cost-effective HPLC method with UV detection was developed and validated for the detection and quantification of bedaquiline present in simulated pericardial fluid (pH 7.4). The method was precise, accurate, rapid and sensitive, with bedaquiline eluting at a retention time of 4.17 min. All validation parameters were found to be within the acceptable limits as specified by the regulatory guidelines.[16] A search of the literature indicated very few HPLC methods were available for the detection of bedaquiline. Most methods employ the use of a high organic solvent in the presence of an acidic buffer.[20,21] A pH of 7.4 was selected to suit the pH of the simulated pericardial fluid samples to be collected. The buffer was initially made with triethylamine, which decreased retention time and strengthened peaks by reducing tailing.[22] The buffer was then adjusted to pH 7.4 using orthophosphoric acid.

A pH of 7.4 was suitable due to its proximity with the pK_a value of 8.91 of bedaquiline.[23] Although bedaquiline is slightly more soluble in an acidic pH,[24] the use of a high ratio of organic phase in the method neutralises this solubility limitation. In *ex vivo* and *in vitro* studies, the use of SLS prevents precipitation of bedaquiline from samples. Body fluids are known to contain surfactants therefore, mimicking the presence of surfactants in *ex vivo* and *in vitro* studies further demonstrates the practicality of the method.[25]

Bedaquiline is a water-insoluble drug that can only be eluted in a highly organic mobile phase; therefore a 95 : 5 organic solvent : buffer ratio was furthermore required to elute the drug. Methanol was initially used as the primary solvent at a ratio of 95 : 5 methanol : buffer. The run time was set at 5 min and no elution of bedaquiline was observed in that time frame. Acetonitrile was gradually added to the methanol to make a 95 : 5 methanol : acetonitrile organic phase and slowly, the ratio of acetonitrile was increased by 5 (to make 90 : 10 and 85 : 15 methanol : acetonitrile organic phase). A bedaquiline peak was observed at a ratio of 85 : 15 methanol : acetonitrile organic phase, with the mobile phase 95 : 5 (organic phase : buffer) and the retention time of bedaquiline was observed within 5 min.

The peak was found to be symmetrical and consistent, thereafter the method validation was conducted according to the ICH guidelines. The developed method is comparable to established methods with a rapid elution time, very low LOD (0.05 µg ml^{-1}) and LOQ (0.15 µg ml^{-1}) as compared to the already established methods, with high sensitivity. It is unique in its ability to quantify bedaquiline is media with pH 7.4, allowing the easy quantification of bedaquiline in body fluids.

Flow rate variation was found to significantly affect the recovery of bedaquiline as compared to the effects of temperature and mobile phase variation for the validated method. The developed method in this study is acceptable and sensitive considering no other method could be found in the literature to detect bedaquiline at a physiological pH of 7.4.

Conclusion

A method was successfully developed and validated to detect bedaquiline in physiological fluid (pH 7.4) *e.g.* pericardial fluid using an isocratic HPLC-UV method. It can be used to detect the drug when studies are performed utilizing bedaquiline-loaded nanosystems as compared to the current methods available, which are more suitable for the detection of bedaquiline in oral

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formulations and acidic (gastric pH) conditions. The method was found to be sensitive, accurate, precise, and reliable. It was successfully employed in the detection of bedaquiline using simulated pericardial fluid in an *ex vivo* pericardial diffusion study of bedaquiline-loaded nanoparticles across the pericardium. This study further provides the prospective applicability of the method for the qualitative and quantitative analysis of bedaquiline in physiological fluids with a pH of 7.4 *i.e.*, blood samples.

Abbreviations

M2	2 major metabolites *N*-monodesmethyl
M3	*N*-Didesmethyl-bedaquiline
pK_a	Acid dissociation constant
TB	Tuberculosis
MDR	Multi-drug resistant
XRD	Extensively drug resistant
ICH	International Conference on Harmonisation
HPLC	High performance liquid chromatography
BDQ	Bedaquiline
RSD	Relative standard deviation
LOD	Limit of detection
LOQ	Limit of quantification
SD	Standard deviation
MIC	Minimum inhibitory concentration

Data availability

Ayodele *et al.*, RP-C18-HPLC-UV method development and validation for the detection and quantification of bedaquiline at physiological pH 7.4. The authors declare that the data supporting the findings of this study are available within the paper. Should any data files be needed in any other format, they are available from the corresponding author upon reasonable request.

Author contributions

Simisola Ayodele: writing – original draft; writing – review & editing, methodology; investigation, Armorel D. van Eyk: investigation; methodology; supervision; writing – original draft, Pradeep Kumar: conceptualization; investigation; supervision; writing – review & editing, Yahya E. Choonara: conceptualization; funding acquisition; project administration; supervision; writing – review & editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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