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Development and validation of an LC-MS/MS multiplex assay for the quantification of bedaquiline, *n*-desmethyl bedaquiline, linezolid, levofloxacin, and clofazimine in dried blood spots

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ABSTRACT

Dried blood spot (DBS) assays to quantify novel and repurposed drugs for the treatment of rifampicin-resistant tuberculosis (RR-TB) would facilitate pharmacokinetic studies and therapeutic drug monitoring in low-middle income settings, considering their ease of application and simple sample storage requirements. We describe a DBS method for the simultaneous quantification of bedaquiline and metabolite *N*-desmethyl bedaquiline, linezolid, levofloxacin, and clofazimine. The analytes were extracted from the matrix and isolated by solid-phase extraction. Two LC-MS/MS systems were used, optimized for the separate analysis of the more polar compounds (linezolid and levofloxacin), and less polar compounds (bedaquiline, *N*-desmethyl bedaquiline, and clofazimine), employing gradient elution. Electrospray ionization and multiple reaction monitoring were used to quantify the analytes on a Sciex API3200 and an API5500 triple quadrupole mass spectrometer, for the more polar and less polar analytes, respectively. Isotopically labelled internal standards were used to compensate for variability in the quantification of each analyte. The method was validated according to international guidelines and applied to samples from a clinical trial. We performed correlation and agreement analysis of the DBS assay and in-house plasma methods using Deming regressions and Bland–Altman plots. Coefficients of correlation between measured plasma and DBS concentrations ranged from 0.866 (95% CI: 0.817–0.902) to 0.989 (95% CI: 0.985–0.992). More than 67% of the samples showed a difference between the observed and estimated plasma concentrations within 20% of their means, meeting EMA requirements for method reproducibility and demonstrating the interchangeability of our DBS and plasma LC-MS/MS methods.

1. Introduction

Treatment success rates for patients with rifampicin-resistant tuberculosis (RR-TB) have steadily improved from 50 % in 2012 to 63 % in 2022, due in part to the replacement of long injectable regimens with shorter all-oral bedaquiline-based regimens [1]. However, a limited understanding of the dose–response relationships of most RR-TB drugs hinders their optimization in combination therapy [2–4]. Studies investigating the pharmacokinetics (PK) and pharmacodynamics (PD) of anti-TB drugs are important for improved dosing regimens of new and existing anti-TB drugs [5]. Therapeutic drug monitoring (TDM) draws from this knowledge to optimize dosing on an individual level—especially the dosing of drugs that display high interpatient variability and have a narrow therapeutic index (e.g., linezolid)—while also serving as a monitoring tool for drug adherence.

The accurate quantification of anti-TB drugs by validated bioanalytical methods is key to both PK/PD research and TDM. Bioanalytical assays have traditionally been developed in plasma or serum prepared from whole blood [6–10]. Whole blood sampling is invasive, often requires significant blood volumes for sample preparation, and processing and storage of plasma/serum samples can be challenging [11,12], especially in TB endemic areas that often lack the necessary infra-structure [13]. Dried blood spot (DBS) sampling is an attractive alternative because of its simplicity, minimal invasiveness, low blood

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volume requirements, and the possibility of self-sampling [14–16]. Generally, analytes are more stable in DBS than in plasma and serum samples, allowing for simpler storage and transportation measures. This is specifically attractive in remote settings [17].

Using DBS technology, we developed an LC-MS/MS assay for the simultaneous quantification of bedaquiline (BDQ) and its main metabolite *N*-desmethyl bedaquiline (BDQ-M2), levofloxacin (LVF), linezolid (LZD), and clofazimine (CLF). Our multiplex assay simultaneously extracts the analytes from a single DBS disk (10 mm) and isolates them by solid-phase extraction prior to analysis by LC-MS/MS, using stable isotope-labelled internal standards for all analytes. Electrospray ionization and multiple reaction monitoring (MRM) were used to detect and quantify the analytes on a Sciex API3200 and an API5500 triple quadrupole mass spectrometer, for the more polar and the less polar analytes, respectively. We successfully validated our method against international guidelines and conducted correlation studies, demonstrating that our DBS assay can be used interchangeably with existing plasma LC-MS/MS methods.

2. Material and methods

2.1. Chemicals and reagents

High-grade solvents were used. Methanol (LC-MS grade), *N, N*-dimethylformamide (HPLC grade), zinc sulfate heptahydrate (grade not provided), and dimethyl sulfoxide (DMSO) (HPLC grade) were purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile (LC-MS grade) was purchased from Honeywell International Inc. (Charlotte, NC, US), and formic acid (LC-MS grade) and 2-isopropanol (LC-MS grade) were obtained from Merck (Darmstadt, Germany). Deionized water (LC-MS grade) was produced in-house using a Millipore water purification system (Merck-Millipore, Germany). Whatman 903 DBS Protein Saver cards were obtained from Amazon (Seattle, WA, US).

Bedaquiline (BDQ) (100 % purity), clofazimine (CLF) (99.65 % purity), clofazimine-d7 (CLF-d7) (99.2 % purity; 99.7 % isotopic purity), levofloxacin (LVF) (98 % purity), levofloxacin-d8 (LVF-d8) (98.8 % purity; 98.9 % isotopic purity), and linezolid-d3 (LZD-d3) (98.0 % purity; 99.0 % isotopic purity) were purchased from Toronto Research Chemicals (ON, CA). Bedaquiline-d6 (BDQ-d6) (97.6 % purity; 99.6 % isotopic purity) was purchased from Clearsynth (Mumbai, India). *N*-Desmethyl bedaquiline (BDQ-M2) (99.1 % purity) and desmethyl bedaquiline-^{13}C-d3 (BDQ-M2-d3) (93 % purity; isotopic purity confirmed in-house against analyte reference standard) were obtained from Tibotec (Johnson & Johnson Pharmaceutical Research and Development), a division of Janssen Pharmaceutical N.V. (Beerse, Belgium). Linezolid (LZD) (99.6 % purity) was sourced from the United States Pharmacopeia (North Bethesda, MD, US).

2.2. Collection of human whole blood

Ethics approval for the collection of whole blood from human volunteers was granted by the Human Research Ethics Committee (HREC) of the Faculty of Health Sciences, University of Cape Town (295/2021). The blood was collected at the Division of Clinical Pharmacology (University of Cape Town, South Africa) by a phlebotomist on the day before DBS preparation from a consenting donor who had not taken any of the study drugs prior to donation. The blood (used for bioanalytical procedures) was collected into tubes containing tripotassium ethylene diamine tetraacetic acid (K3EDTA) as anticoagulant and stored at 4 °C overnight, prior to sample preparation.

2.3. Analyte stock solutions, calibration standards, and quality controls

Analyte stock solutions were prepared in DMSO at a concentration of 1 mg/mL for both BDQ and BDQ-M2, and at concentrations of 3 mg/mL, 5 mg/mL, and 0.5 mg/mL for LVF, LZD, and CLF, respectively. The stock solutions were used to prepare calibration standards and quality controls (QCs) by two separate preparation procedures to accommodate different stability characteristics of the analytes. One procedure made use of spiking whole blood to the desired concentration levels (LVF and LZD) using working solutions prepared by serial dilution in acetonitrile and methanol (1:1, v/v), whereas the other method entailed spiking of whole blood to attain the highest concentration levels, followed by serial dilution with whole blood to attain the lower concentrations levels (BDQ, BDQ-M2, and CLF). Concentration ranges of the calibration standards were 20.2–0.0741 μg/mL (LVF), 30.9–0.113 μg/mL (LZD), 4.94–0.0181 μg/mL (BDQ), 2.47–0.00905 μg/mL (BDQ-M2), and 2.22–0.00814 μg/mL (CLF). QCs were prepared at high concentration (QCH) (16.5, 25.3, 4.04, 2.02, and 1.82 μg/mL for LVF, LZD, BDQ, BDQ-M2, and CLF, respectively), medium concentration (QCM) (8.27, 12.6, 2.02, 1.01, and 0.908 μg/mL for LVF, LZD, BDQ, BDQ-M2, and CLF, respectively), low concentration (QCL) (0.158, 0.241, 0.0385, 0.0193, and 0.0173 μg/mL for LVF, LZD, BDQ, BDQ-M2, and CLF, respectively), and lower limit of quantification (QC LLOQ) (0.0741, 0.113, 0.0181, 0.00905, and 0.00814 μg/mL for LVF, LZD, BDQ, BDQ-M2, and CLF, respectively).

Multiple aliquots (50 μL) of each calibration standard and QC were spotted onto Whatman 903 Protein Saver cards. These were dried overnight at room temperature and stored at ~ − 80 °C in sealed Ziplock bags containing 3 × 1 g sachets of desiccant.

2.4. Internal standard stock solutions and working solutions

Internal standard stock solutions were prepared in suitable organic solvents. BDQ-d6 and BDQ-M2-d3 were each prepared in DMSO at a concentration of 1 mg/mL; CLF-d7 was prepared in a mixture of methanol and 0.1 % formic acid in water (1:1, v/v) at a concentration of 0.5 mg/mL; LVF-d8 and LZD-d3 were each prepared in methanol at a concentration of 1 mg/mL. Internal standard working solutions for BDQ-d6, BDQ-M2-d3, and CLF-d7 were prepared by the addition of 5.00, 50.0, and 10.0 μL of each internal standard stock solution, respectively, to 995, 950, and 990 μL of a mixture of methanol and acetonitrile (1:1, v/v). The internal standard stock solutions of LVF-d8 and LZD-d3 were used undiluted.

2.5. DBS extraction procedure

The extraction procedure comprised a protein precipitation step followed by solid-phase extraction (SPE). The DBS samples were removed from − 80 °C storage and allowed to reach room temperature. A 10 mm punch was taken from the center of each DBS sample, the latter with a 12 mm diameter, thus retrieving ~ 83 % of each sample. The punches were transferred to suitably labelled 1.5 mL microcentrifuge tubes to which 500 μL extraction solution was added to precipitate the proteins. Various precautions were taken to minimize the effects of carry-over that may result from punching. These included the punching of calibration standards and QCs from low to high concentrations, sequentially. Additionally, when punching unknown samples, the puncher was rinsed with 70 % ethanol in between punching, with a blank paper punch done as a further cleaning step.

The extraction solution, prepared daily, contained the internal standards in a mixture of methanol, water, and ~ 0.3 M $ZnSO_4$ solution (66:30:4, v/v/v). The samples were sonicated for ~ 30 min at a starting temperature of 25 °C. This was followed by SPE on Strata-X extraction cartridges (Phenomenex, 33 μm × 200 mg/3 mL). The extraction cartridges were pre-conditioned with methanol (2 mL), equilibrated with 0.1 % formic acid in water (2 mL), and then loaded with 0.1 % formic acid in water (2.2 mL). An aliquot of 400 μL of the supernatant of each sample was transferred onto the equilibrated SPE columns and passed through by applying positive pressure. The cartridges were washed successively with 0.1 % formic acid in water (2 mL) and 0.1 % formic acid in a mixture of methanol and water (1:4, v/v) (1.5 mL), and

allowed to dry for ~ 10 min. Acetonitrile (250 μL) was added onto each cartridge and after waiting for one minute, the analytes were eluted with methanol (1 mL) into glass tubes. The eluates were dried under a stream of nitrogen gas for ~ 1 h and reconstituted in 200 μL 0.2 % formic acid in a mixture of water, acetonitrile, and DMSO (77.4:17.4:5, v/v/v).

Aliquots of the reconstituted samples (50 μL) were diluted 4-fold with reconstituting solvent (150 μL) and transferred to a 96-well plate for the analysis of the less polar analytes BDQ, BDQ-M2, and CLF on a Sciex API5500 LC-MS/MS (injection volume of 5 μL). The remaining reconstituted samples (150 μL) were transferred *as is* to a second 96-well plate for the analysis of the more polar analytes LVF and LZD on a Sciex API3200 LC-MS/MS system (injection volume of 10 μL).

2.6. Chromatographic condition and LC-MS/MS settings

Two LC-MS/MS systems were used for the analysis of the two groups of analytes. The more polar compounds (LVF and LZD) were analyzed on an Agilent 1290 HPLC interfaced with a Sciex API3200, chromatographically separating the compounds on an Agilent Poroshell 120 SB-C18 column (2.1 mm × 50 mm; 2.7 μm). The less polar compounds (BDQ, BDQ-M2, and CLF) were analyzed on an Agilent 1260 HPLC system coupled to a Sciex API5500 employing an Agilent Poroshell 120 EC-C18 column (2.1 mm × 50 mm; 2.7 μm) for chromatographic separation. Gradient elution as described in Table 1 was used for both systems, with 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile as solvents. Injection needles were rinsed with a mixture of methanol and water (1:1, v/v) and the autosamplers were operated at ~ 8 °C in both cases. Out-flows from both columns were connected to six-port switching valves to enable diversion of the mobile phase to waste between injections, preventing residual polar material from contaminating the ionization sources. Diversion from the injection valve of both systems to the waste valve was activated at 0.1 min, reverting to the injection valve at 2 min and 0.6 min for Sciex API5500 and Sciex API3200, respectively.

Both the Sciex API3200 and API5500 were operated in positive electrospray ionization applying multiple reaction monitoring (MRM) with source temperatures at 500 °C and 550 °C, respectively, and spray voltages at 4000 and 4500 V. Gas settings were optimized to achieve the desired detection sensitivity. The ionization parameters and mass transitions used for MRM are summarized in Table 2. Analyst® versions 1.6.2 (API3200) and 1.7.1 (API5500) software (Sciex™, Germany) were used for data processing.

2.7. Method validation procedures

2.7.1. Classical validation assessments

The method was validated according to the guidelines of the USA Food and Drug Administration (FDA) [18] and the European Medicine Agency (EMA) [19]. The validation batches contained the calibration standards over the selected concentration ranges (in duplicate at each concentration level). QCs were analyzed in six-fold at four concentrations (QCH, QCM, QCL, and QC LLOQ) on three consecutive days to assess the intra-day and inter-day accuracy and precision of the method. Accuracy and precision were calculated by the following formulae:

$$\text{Accuracy } (\%) = \frac{\text{measured concentration}}{\text{nominal concentration}} \times 100$$

$$\text{Precision } (\text{CV}, \%) = \frac{\text{standard deviation}}{\text{mean measured concentration}} \times 100$$

Dilution integrity was assessed for all analytes in one of the validation batches, using six replicates at a concentration approximately 1.6 times higher than the upper limit of quantification (ULOQ). A five-fold dilution was achieved by adding 30 μL of the extracted QC sample, prepared above the ULOQ, to 120 μL of the extracted blank sample. Carry-over was investigated in a double-blank sample that did not contain analytes and internal standards, placed after a standard of highest concentration in the injection sequence. QCs, prepared at LLOQ concentrations in matrix from six different sources, were used to assess the sensitivity of the method as indicated by the signal to noise ratio at these lower concentrations, and the selectivity of the method as indicated by the selective quantification of an analyte in the presence of interfering peaks from endogenous compounds, other analytes, and internal standards.

Recovery and matrix effects were assessed at three QC concentrations (QCH, QCM, and QCL). The matrix effects experiment was conducted according to Matuszewski *et al.* [20] using QC samples prepared in six different lots of dried blood spots. Process efficiency, which represents the effects of matrix components combined with the extractability of the analyte, was investigated by spiking injection solution containing the analytes at the three QC concentrations (QCH, QCM, and QCL). Crosstalk assessments were done to evaluate potential interferences between the MRM transition channels of the analytes and internal standards, by using DBS samples that contained only one of either an analyte or an internal standard. The specificity and robustness of the method were further assessed by testing the potential interference of concomitantly administered medication, specifically anti-retroviral and other TB drugs typically co-administered with the study drugs.

The stability of the analytes—to determine suitable storage conditions and to ensure stability throughout the analytical process—was assessed in stock and working solutions, DBS, and whole blood. These tests included freeze–thaw stability, benchtop stability, and long-term matrix stability.

2.7.2. Additional validation assessments (DBS)

Assessments specific to DBS methods typically include analytical validation assessments such as the investigation of volume effect, hematocrit effect, and carry-over resulting from the DBS preparation specifically [18,19,21]. In addition, a clinical validation assessment (method correlation study) is required to correlate the results obtained for paired samples by application of the DBS assay and a validated reference method.

In this study, we attempted to minimize the volume and hematocrit effects by volumetric sample application and using near-to-whole punches [21]. The blood spots were prepared by accurately depositing 50 μL blood aliquots onto Whatman 903 Protein Saver cards, each aliquot placed into a demarcated circle of 12 mm in diameter. Punches (10 mm in diameter) were then taken to prepare the DBS samples. Furthermore, we included a batch of DBS samples that were produced with a smaller volume of blood (25 μL), to investigate whether the analytical method meets accuracy and precision criteria when using DBS samples produced from less than 50 μL blood.

For the clinical validation assessment (correlation study), we compared our DBS method with validated in-house plasma methods [22–25] by correlating the DBS and plasma results obtained when

Table 1
Gradient elution for optimal chromatographic separation.

LZD and LVF (more polar) Poroshell 120 SB-C18 (2.1 mm × 50 mm; 2.7 μm) at 40 °C			BDQ, BDQ-M2, CLF (less polar) Poroshell 120 EC-C18 (2.1 mm × 50 mm; 2.7 μm) at 40 °C		
Time (minutes)	% Solvent B	Flow-rate (μL/min)	Time (minutes)	% Solvent B	Flow-rate (μL/min)
0.00	20	250	0.00	40	300
0.10	20	250	1.25	45	300
1.00	90	250	4.75	90	300
1.75	90	250	6.00	90	300
2.50	20	250	6.25	40	300
4.00	20	250	9.00	40	300

Conditions displayed on the left are for separation of the more polar analytes, whereas conditions for separation of the less polar analytes are displayed on the right.
Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile.

Table 2
Parameters for transition sources and collision cells.

Analytes	MRM transitions (*m/z*)	Dwell Times (ms)	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)	Collision cell entrance potential (V)
AB Sciex API3200							
Levofloxacin	362.1 → 318.2	40	26	10	27	2	14
Levofloxacin-d8	370.1 → 326.2	40	61	10	29	2	22
Linezolid	338.1 → 296.3	40	81	7	25	2	16
Linezolid-d3	341.1 → 297.3	40	91	8	29	2	14
AB Sciex API5500							
Bedaquiline	555.1 → 58.1	80	116	10	91	8	NA
Bedaquiline-d6	561.1 → 64.1	50	146	10	105	8	NA
N-Desmethyl bedaquiline	541.0 → 480.0	80	161	10	25	30	NA
N-Desmethyl bedaquiline-13C-d3	545.1 → 480.1	50	226	10	27	10	NA
Clofazimine	473.0 → 431.1	80	231	10	51	24	NA
Clofazimine-d7	480.4 → 429.0	50	236	10	61	28	NA

Abbreviations: NA = Not applicable.

applying both the DBS and plasma methods to paired samples. For this purpose, 141 blood samples, for the preparation of both the DBS and the plasma samples, were collected into K3EDTA collection tubes during a phase III clinical trial from consented participants [26]. Blood samples were stored overnight at 4 °C, prior to sample preparation. Approval to conduct the study was given by the University of the Witwatersrand HREC (HREC/REF: 727/2019) and the HREC of the Faculty of Health Sciences, University of Cape Town (UCT HREC ref: 727/2019). The newly developed DBS assay and the in-house plasma methods were used to quantify BDQ, BDQ-M2, CLF, LVF, and LZD in the 141 paired samples. Statistical analysis on the two sets of measurements generated by the DBS and plasma methods was done using the statistical software MedCalc® version 20.111 (Ostend, Belgium). Data distribution analysis of the results was done to determine median ratios and outliers using Box and Whisker Plots. The Deming regression was used to determine the strength of correlation between the DBS and plasma concentrations. Unlike ordinary least squares (OLS) regression, which assumes that only the Y-axis measurements are subject to error, the Deming regression considers measurement errors on both axes, making it a preferred method for evaluating correlations between continuous variables such as drug concentrations [27,28]. The regression equation derived from the correlation was used to calculate estimated plasma concentrations (EPC) from measured DBS concentrations for all analytes [21]:

$$[EPC] = [DBS] \bullet a + b.$$

where [EPC] represents the estimated analyte concentration in plasma, [DBS] represents the measured analyte concentration in DBS, and a and b represent the slope and intercept, respectively.

Bland–Altman plots were used to quantify the magnitude of differences between the measured plasma concentrations and the estimated plasma concentrations (EPC), thus determining the agreement between the DBS and plasma measurement methods. The Bland–Altman analysis requires that the differences between the two measurements are normally distributed. If a normal distribution does not exist, logarithmic transformation may be applied [29,30]. As we used a sufficiently large sample size (141), a normal distribution was assumed [31]. The data were assessed against the method reproducibility criteria of the EMA guidelines, as recommended in the International Association for Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) guidelines [21], i.e., the difference between the two values obtained for a time point had to be within 20 % of their mean difference for at least 67 % of the paired samples. The percentage difference was calculated by the following formula:

$$\text{Difference}\,(\%) = \frac{[PC] - [EPC]}{\text{Mean of } [PC] \text{ and } [EPC]} \times 100$$

where [PC] represents the measured analyte concentration in plasma and [EPC] represents the estimated analyte concentration in plasma.

3. Results and discussions

3.1. Method development

3.1.1. Optimization of the chromatography

Our aim was a method suitable for the measurement of drug levels in DBS samples collected during the BEAT-Tuberculosis trial, a study that treated RR-TB patients with a novel regimen of BDQ, LVF, LZD, and CLF, and compared outcomes with the current standard of care [26].

Owing to the distinct polarity of the analytes, with LVF and LZD being more polar than BDQ, BDQ-M2, and CLF, separate chromatographic conditions were needed to achieve optimal separation and peak shapes. For this purpose, an Agilent Poroshell 120 SB-C18 (2.1 mm × 50 mm, 2.7 μm) column was used to analyze LVF and LZD, whereas BDQ, BDQ-M2, and CLF were better separated and retained on an end-capped Agilent Poroshell 120 EC-C18 (2.1 mm × 50 mm, 2.7 μm) column. Further optimization was achieved with gradient elution specifically designed for each group, preventing carry-over, especially for the less polar analytes (Table 1). Chromatograms for each group are depicted in Fig. 1. A custom injector program was activated on the AB Sciex API5500 instrument to prevent carry-over of highly retained less polar analytes (Table 3).

3.1.2. Optimization of the extraction procedure

We based our extraction procedure on a standard DBS extraction method [32]. The DBS disk is soaked in a solution, accompanied by sonication or shaking for optimal analyte dissolution, to extract the analyte(s). Extraction efficiency is typically enhanced by acidifying the extraction solution or introducing salts to improve analyte solubility or facilitate protein precipitation. An additional clean-up step, such as liquid–liquid or solid-phase extraction, can then be used to optimize the sensitivity.

Our aim was to simultaneously extract all analytes from the DBS. Most analytes were extractable using various solvents such as acetonitrile, methanol, a 1:1 mixture of acetonitrile and methanol, with or without acidification, and a mixture of acetonitrile and methanol in water at different ratios. However, the recovery of CLF was notably low and inconsistent. Subsequent attempts involved liquid–liquid extraction using ethyl acetate, and mixtures of dichloromethane and hexane. The differences in the analytes' pKa values (Table 4) made these attempts exceptionally challenging, specifically for LVF which showed a drastic loss of recovery. The addition of zinc sulfate ($ZnSO_4$), as described by Li *et al.* [33], improved both the recovery and consistency of the CLF extraction alongside those of the other analytes. However, this led to the crucial requirement of eliminating $ZnSO_4$ from the final extract, and hence the use of SPE with Strata-X (Phenomenex, 33 μm × 200 mg/3 mL) extraction cartridges. The procedure outlined in section 2.5

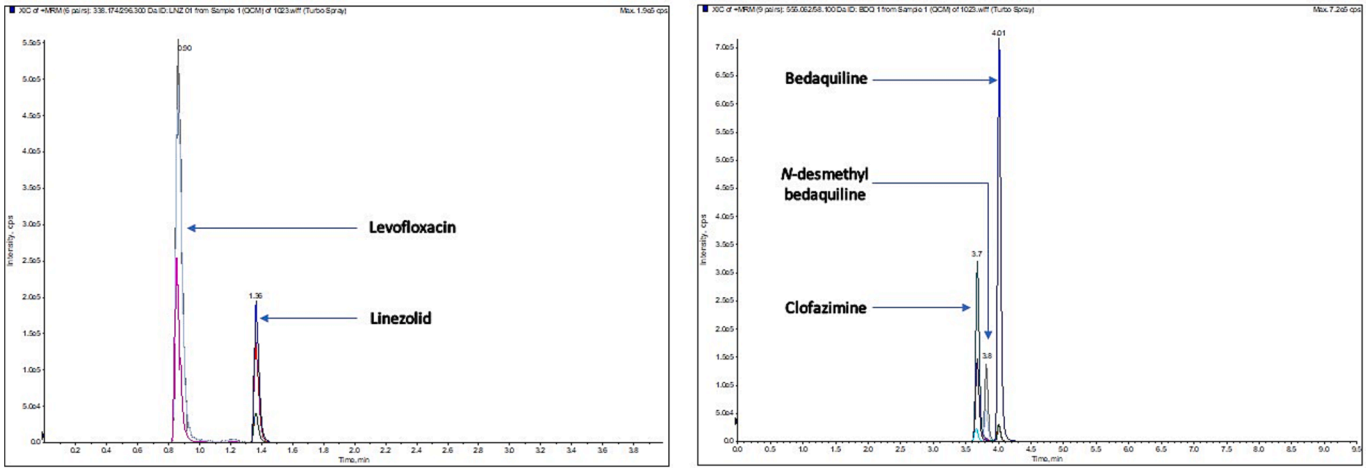


Fig. 1. Representative chromatograms of a quality control sample prepared in dried blood spots at mid-concentration, indicating the separation of more polar linezolid and levofloxacin (left), and less polar bedaquiline, *N*-desmethyl bedaquiline, and clofazimine (right).

Table 3
Custom injector program for API 5500.

Steps	Functions	Parameters
1	Draw	Draw default volume from sample with default speed using offset *Minutes*
2	Wash	Wash needle as per method
3	Inject	Inject
4	Remote	Set remote line to *Start* for 750 ms
5	Wait	4.5 min
6	Valve	Switch to *Bypass*
7	Wait	0.1 min
8	Valve	Switch to *Main Path*
9	Wait	0.1 min
10	Valve	Switch to *Bypass*
11	Wait	0.1 min
12	Valve	Switch to *Main Path*
13	Remote	Set remote line to *Start* for 125 ms

Abbreviations: ms = milliseconds.

yielded a final extract suitable for analyzing both the more polar and less polar analyte groups in the two separate analytical runs.

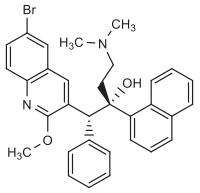
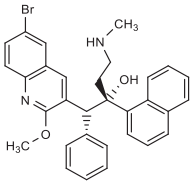
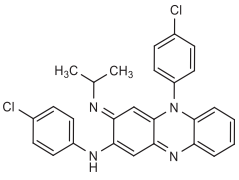
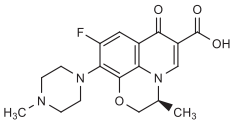
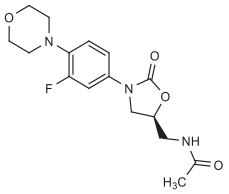
3.2. Method validation

3.2.1. Classical validation assessments

3.2.1.1. Calibration curve, accuracy, and precision. Accuracy and precision criteria were met across the calibration ranges for all analytes. QCs—included at QCH, QCM, QCL, and QC LLOQ—met criteria, measuring within ± 15 % of nominal concentrations (±20 % at LLOQ), with coefficients of variation (CVs) of ≤ 15 % (≤20 % at LLOQ) at all concentration levels. The calibration curves fitted a quadratic regression, weighted by $1/x^2$ for BDQ, BDQ-M2, CLF, and LZD, and by 1/x for LVF, over the concentration ranges of 20.2–0.0741 μg/mL (LVF), 30.9–0.113 μg/mL (LZD), 4.94–0.0181 μg/mL (BDQ), 2.47–0.00905 μg/mL (BDQ-M2), and 2.22–0.00814 μg/mL (CLF).

Dilution integrity—QC DiL samples at pre-dilution concentrations of 33.1, 50.5, 8.09, 4.04, and 3.64 μg/mL for LVF, LZD, BDQ, BDQ-M2, and CLF, respectively—met acceptance criteria for all analytes, except for *N*-desmethyl bedaquiline which showed a precision slightly above the acceptance criterion (Table 5). This slight imprecision might have been

Table 4
Analyte structures and predicted physicochemical properties.

Analytes	Bedaquiline	*N*-Desmethyl bedaquiline	Clofazimine	Levofloxacin	Linezolid
Structures					
Solubility in water (mg/mL)	~0.00	~0.00	~0.00	~2.23	~0.230
pKa (Strongest acid)	13.6	13.6	15.4	5.17	14.6
pKa (Strongest base)	8.91	9.69	5.89	8.39	−1.18
Lop	7.13	6.75	7.3	−1.06	0.637
Molecular formula	$C_{32}H_{31}BrN_2O_2$	$C_{31}H_{29}BrN_2O_2$	$C_{27}H_{22}Cl_2N_4$	$C_{18}H_{20}FN_3O_4$	$C_{16}H_{20}FN_3O_4$
Molecular mass (g/mol)	555.5	541.5	473.4	361.4	337.4

Predicted physicochemical properties calculated from https://chemicalize.com/app/calculation (ChemAxon, accessed 04 April 2022).

Table 5
Summary of within and between-days accuracy and precision for all analytes.

Analytes	Samples	Nominal conc. (μg/mL)	Within-day (n = 6) Accuracy (%)			Precision (CV, %)			Between-days (n = 18) Accuracy (%)	Precision (CV, %)
Bedaquiline	QC LLOQ	0.0181	97.5	81.3	84.4	8.2	7.1	7.3	87.7	11.0
	QCL	0.0385	100.0	105.7	96.3	5.3	4.4	4.1	100.7	5.9
	QCM	2.02	98.6	109.2	95.6	7.7	8.0	8.9	101.2	9.7
	QCH	4.04	111.0	106.1	103.3	7.6	4.0	4.3	106.8	6.1
	QC DiL	8.09	92.2			7.1				
N-Desmethyl bedaquiline	QC LLOQ	0.00905	93.7	85.1	92.6	17.7	10.3	11.6	90.5	13.7
	QCL	0.0193	98.9	102.5	105.2	5.7	6.4	8.8	102.2	7.2
	QCM	1.01	94.4	104.1	100.5	7.1	6.0	9.0	99.7	8.1
	QCH	2.02	113.2	100.0	109.0	8.4	9.9	6.0	107.4	9.3
	QC DiL	4.04	107.1			19.6				
Clofazimine	QC LLOQ	0.00814	101.5	87.4	95.3	12.4	11.7	6.2	94.7	11.7
	QCL	0.0173	104.3	105.6	97.6	7.9	9.7	9.8	102.5	9.3
	QCM	0.908	104.0	109.4	104.9	4.9	5.7	7.7	106.1	6.3
	QCH	1.82	109.2	99.9	104.5	5.4	8.1	5.3	104.6	7.0
	QC DiL	3.64	94.8			12.4				
Levofloxacin	QC LLOQ	0.0741	112.9	104.9	113.1	3.8	8.9	4.0	110.3	6.6
	QCL	0.158	102.9	107.0	105.3	4.0	6.8	7.2	105.0	6.0
	QCM	8.27	95.2	101.9	101.2	4.7	4.7	4.6	99.4	5.4
	QCH	16.5	102.2	96.1	107.0	5.4	3.0	3.2	101.8	5.9
	QC DiL	33.1	105.8			6.5				
Linezolid	QC LLOQ	0.113	99.3	119.8	115.7	5.7	5.9	8.2	111.6	10.4
	QCL	0.241	110.0	106.8	107.4	5.2	6.3	5.2	108.1	5.4
	QCM	12.6	106.3	107.5	107.9	5.3	3.2	3.2	107.3	3.8
	QCH	25.3	104.3	103.9	109.1	1.4	3.1	4.1	105.8	3.8
	QC DiL	50.5	102.6			11.6				

Abbreviations: QC = Quality Control, QC LLOQ = QC Lower Limit of Quantification, QCL = QC Low, QCM = QC Medium, QCH = QC High, QC DiL = QC Dilution, CV = Coefficient of variation.

caused by the complexity of the matrix. The dilution of samples with *N*-desmethyl bedaquiline above the ULOQ must therefore be applied with caution.

3.2.1.2. Sensitivity, selectivity, and carry-over. The sensitivity of the analytical method met the acceptance criteria, with a signal to noise (S/N) ratio of $\geq$ 5.0 obtained for all analytes (Fig. 2). Selectivity was confirmed by the absence of interfering peaks at the retention times of the analytes. Carry-over was within acceptance criteria for all analytes, with no peaks of intensity > 20 % of LLOQ observed in the double-blank samples.

3.2.1.3. Recovery, process efficiency, and matrix effects. The results for recovery, process efficiency, and matrix effects assessments are summarized in Table 6. Owing to the varying polarity of the analytes, the selection of a suitable solvent for optimal extractability presented a significant challenge and we had to forfeit a higher recovery for the sake of the simultaneous extraction of the analytes from the DBS. Additionally, by using larger punches (10 mm) to mitigate the hematocrit effect, the ratio of extraction solution to contact surface was smaller than would have been the case had we used smaller punch sizes, thus compromising the extractability of analytes from the DBS even further. These disadvantages were exacerbated by the fact that we had to, for practical reasons, add the internal standards to the extraction solution, thus limiting their compensatory activity as they were not able to compensate for the recovery of the analytes from the DBS. These challenges resulted in a relatively low recovery for all analytes, with CLF displaying the lowest recovery. However, the low CV values obtained for

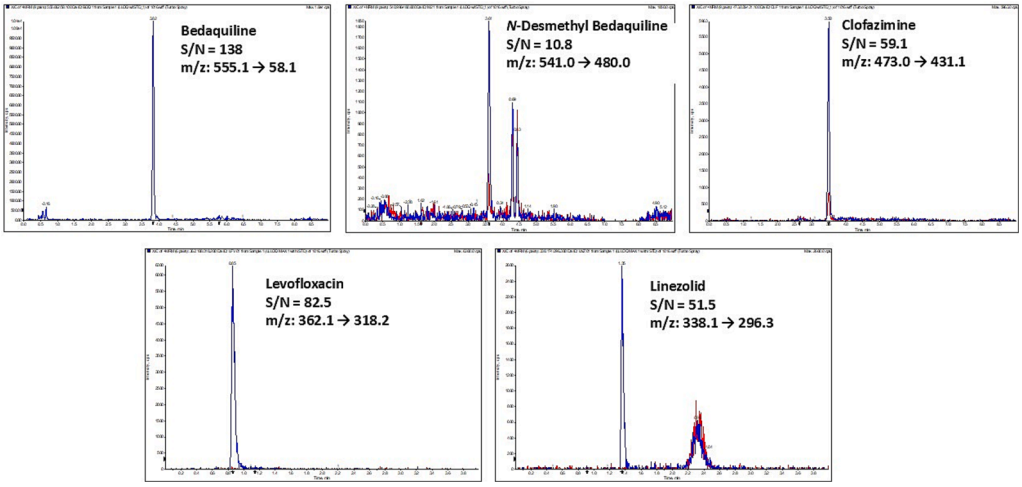


Fig. 2. Representative chromatogram overlays for all analytes of LLOQ and blank samples from the same matrix lot prepared in dried blood spots. Blue represents the LLOQ signal and red represents the blank signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Summary of recovery, process efficiency, and matrix effects results expressed as average (n = 6).

Analyte	Recovery (%) (CV, %)			Process efficiency (%) (CV, %)			Matrix effects (CV, %)			
	QCH	QCM	QCL	QCH	QCM	QCL	QCH	QCM	QCL	Slope
Bedaquiline	35.5 (4.1)	36.5 (6.1)	36.8 (6.3)	34.2 (6.1)	33.5 (8.3)	34.3 (13.1)	4.1	6.1	6.3	4.2
***N*-Desmethyl bedaquiline**	51.0 (4.9)	55.1 (3.0)	59.1 (7.4)	52.9 (3.2)	59.5 (3.4)	61.2 (12.3)	4.9	3.0	7.4	5.0
Clofazimine	16.3 (3.0)	15.2 (3.2)	14.4 (4.3)	16.5 (0.9)	15.2 (3.8)	14.6 (0.6)	3.0	3.2	4.3	3.0
Levofloxacin	81.2 (2.4)	76.0 (2.0)	69.5 (4.8)	80.0 (2.6)	74.1 (0.4)	71.4 (6.2)	2.4	2.0	4.8	2.5
Linezolid	71.4 (2.0)	70.2 (2.9)	71.0 (2.5)	72.1 (1.0)	69.9 (1.4)	67.2 (0.9)	2.0	2.9	2.4	2.1

Abbreviations: QC = Quality Control, QCL = QC Low, QCM = QC Medium, QCH = QC High.
Refer to Table 5 for the concentrations of the analytes in the QCs.

all analytes attest that consistency and robustness of the extraction process were achieved despite these challenges. The recovery (%) of the analytes was similar to their process efficiency (%), indicating a negligible contribution by matrix components. The matrix effects assessment confirmed that matrix components (including hematocrit of which levels can be expected to vary in different lots of blood) did not have a significant effect on the analytical process, with variations (CV, %) of the QCs (peak area ratios) at all levels across the six different matrices ranging from 2.0 % to 7.4 % and the slopes of the six regression lines derived from the QCs in the six different matrices ranging from 2.1 % to 5.0 % (Table 6).

3.2.1.4. Crosstalk and concomitant medication. No interfering peaks that could be ascribed to crosstalk were observed in individual analyte channels. Additionally, assessments showed that none of the drugs likely to be co-administered with the study drugs has any significant effect on the detection and quantification of the analytes.

3.2.1.5. Stability assessments. Stock solutions of LZD, LVF, and CLF prepared in DMSO were shown to be stable for 189, 33, and 86 days, respectively, when stored at ~ − 80 °C, whereas stability at − 20 °C, 4 °C, and room temperature was confirmed for 20 h. Stock solutions of BDQ and BDQ-M2 prepared in DMSO were stable for 34 days when stored at − 80 °C, and for 24 h when stored at − 20 °C, 5 °C, and room temperature. Working solutions that were prepared for LZD and LVF were stable for 84 days at − 80 °C.

The stability of the analytes in the matrix (DBS) under various storage conditions and durations was assessed. BDQ, LVF, and LZD remained stable in the matrix for 313 days when stored at 4 °C, −20 °C, and − 80 °C. CLF was shown to be stable in matrix for 313 days when stored at − 80 °C, and for 53 days when stored at − 20 °C and 4 °C. BDQ-M2 was shown to be stable for 313 days at − 20 °C and − 80 °C, and for 53 days at 4 °C. All analytes were stable in matrix at room temperature for 10 days. Additionally, stability of the analytes was confirmed for a minimum of four hours during the benchtop stability experiment. Sample freezing (fresh vs. frozen) did not influence the accuracy and precision of the assay, and all analytes were stable for at least three freeze–thaw cycles. Auto-sampler stability and reinjection reproducibility were shown for up to 144 h for LZD and LVF, and for 96 h for BDQ, BDQ-M2 and CLF, when samples were kept at 8 °C on the autosampler. All analytes were found to be stable for up to seven days at 4 °C, and 48 h at room temperature when incubated in whole blood prior to spotting. This indicates that, if a venous blood sample is not immediately applied onto the filter paper card, it may still be used to produce a DBS within 48 h if kept at room temperature, or within seven days if stored at 4 °C.

3.2.2. Additional validation assessments (DBS)

3.2.2.1 vol. effect and carry-over. The validation batch that comprised DBS samples produced with a smaller blood volume (25 µL) met the accuracy and precision criteria for all analytes, with accuracy ranging from 85.1 % to 107.9 % and precision (CV, %) ranging from 2.4 % to 12.3 % across all concentration levels for all analytes, indicating the suitability of the method for situations in which only small volumes of blood can be acquired, such as in pediatric studies. Future investigation into free-falling blood collection (finger and heel prick sampling) may require additional assessments of volume effect.

3.2.2.2. Hematocrit effect. The hematocrit effect is a combination of hematocrit-based area bias (caused by viscosity), recovery bias (due to insufficient compensation of the internal standard), and matrix effect bias (generally contributing the least to the hematocrit effect) [34]. The hematocrit-based area bias can be avoided by volumetric application of the blood sample together with whole-punch analysis. This also removes the volume effect which is of specific concern when collecting free-falling blood [21], however, it is a trade-off in simplicity, which ultimately is the aim of DBS sampling [21]. In our study, volumetric sample application and near-to-whole punches were used. Although the punches were only ~ 83 % of the DBS surface, this had no significant effect on the precision and accuracy of the assay.

3.2.2.3. Method correlation: Comparability of DBS and plasma assays. Removal of datapoints.

Paired DBS and plasma concentrations below the limit of quantification (BLQ)—i.e., no quantifiable peaks in either the DBS or plasma sample collected at the same timepoints—were manually removed from the dataset to ensure inclusion of only accurate and reliable data. Twenty-eight values (19.9 %) were discarded for LVF and nine (6.38 %) for LZD. No BLQ concentrations were observed for the other three analytes. Outliers with extreme DBS/plasma ratios—resulting from interpatient and analytical variability, and/or other sources [35]—were identified using the Grubbs test [36] and manually removed to avoid bias in the Deming regression analysis. Six outliers were detected for BDQ, one for both BDQ-M2 and LVF, and none for CLF and LZD (Fig. 3).

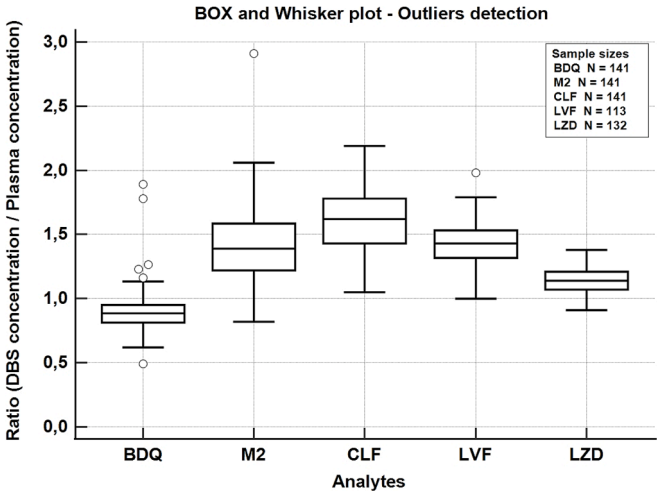


Fig. 3. Box and whisker plots of the ratios of dried blood spots (DBS) to plasma concentrations for all analytes, identifying the outliers as data points outside of the whiskers. Generated automatically using Medcalc® version 20.111.

Data distribution analysis.

Box and Whisker plots showed that the [DBS]/[Plasma] ratio is approximately normally distributed for all analytes. A median ratio of 0.886 (95 % confidence interval [CI]: 0.861–0.905) for DBS to plasma concentrations of BDQ (Fig. 3). A higher concentration of BDQ was thus measured in plasma than in DBS, possibly owing to a superior extractability of BDQ from plasma than from DBS, a possibility that is supported by our in-house recovery data showing a higher recovery for BDQ from plasma than from DBS. Conversely, the ratios of DBS to plasma concentrations of BDQ-M2 (1.39 [95 % CI: 1.33–1.45]), CLF (1.62 [95 % CI: 1.56–1.69]), LVF (1.43 [95 % CI: 1.38–1.48]), and LZD (1.14 [95 % CI: 1.11–1.15]) were all greater than one (Fig. 3), indicating that higher concentrations of these analytes were measured in DBS. Our in-house data confirmed a superior recovery from plasma for all analytes when compared to their recovery from DBS. The higher DBS measurements of these four analytes can therefore not be ascribed to a higher extractability of BDQ-M2, CLF, LVF, and LZD from DBS, but might rather be ascribable to a lower accumulation of these analytes in plasma than in cellular blood components. This possibility is supported for BDQ-M2 by a study that investigated the concentrations of BDQ and BDQ-M2 in plasma and peripheral blood mononuclear cells (PBMCs) [37]. Ngwalero *et al.* found that BDQ concentrations in plasma were approximately 5-fold higher than those of BDQ-M2, whereas BDQ-M2 concentrations in PBMCs were roughly 3-fold higher than those of BDQ. This disparity in intra- and extracellular partitioning may be attributed to the activity of membrane drug transporters involved in uptake and efflux processes [38,39]. The demethylation of BDQ to BDQ-M2 could influence their differential binding affinity to specific transmembrane protein transporters, leading to their distinct distribution patterns.

The lower concentration of LZD in plasma than in DBS corresponds with data published by Ferrone *et al.* [40]. La Marca *et al.* [41] reported an equal concentration of LZD in DBS and plasma in a study with a small sample size. An exploratory multiplex DBS assay that included the analysis of LVF and LZD corroborated the lower accumulation of LVF in plasma than in the cellular blood components of DBS but an increased accumulation of LZD in plasma [42]. The disparity may be ascribed to a small sample size, or as acknowledged by the author, because sample collection was not intended for TDM. We found no published data describing the distribution of CLF, BDQ, and BDQ-M2 in plasma and red blood cell (RBC) components.

DBS versus plasma correlation (Deming).

Deming regression plots (Fig. 4) show a good correlation between the concentrations measured in both matrices with the Pearson correlation coefficients ranging from 0.866 to 0.989 as summarized in Table 7. However, the slopes of the Deming regression lines do not coincide with those of the lines of identity, confirming the difference of analyte concentrations measured in the two matrices, as observed in the data distribution shown in Fig. 3. Furthermore, these findings are reflected in the slopes, indicating that the concentrations of BDQ are higher in plasma than in DBS (slope > 1), whereas the concentrations of BDQ-M2, CLF, LVF, and LZD are lower in plasma than in DBS (slopes < 1). Notably, none of the 95 % CIs for the slopes of the regression lines include the value 1, indicating a proportional difference between the two measurements for all analytes. In contrast, a constant difference was observed for BDQ-M2, CLF, and LZD, as the 95 % CIs for the intercepts of their regression lines do not include the value 0. For BDQ and LVF, the 95 % CIs for the intercepts contain the value 0, indicating no constant difference for these two analytes, suggesting that the two measurements are more aligned, with no systematic offset.

The proportional and, in some cases, constant differences observed between DBS and plasma concentrations in the Deming regression plots may be attributed to interpatient variability in HcT levels and/or differences in the analyte's random distribution between RBC and plasma components. To account for analyte partitioning and HcT variability, an appropriate correction factor is recommended [21]. Our analyses were conducted on near-whole punches to mitigate the need for further HcT

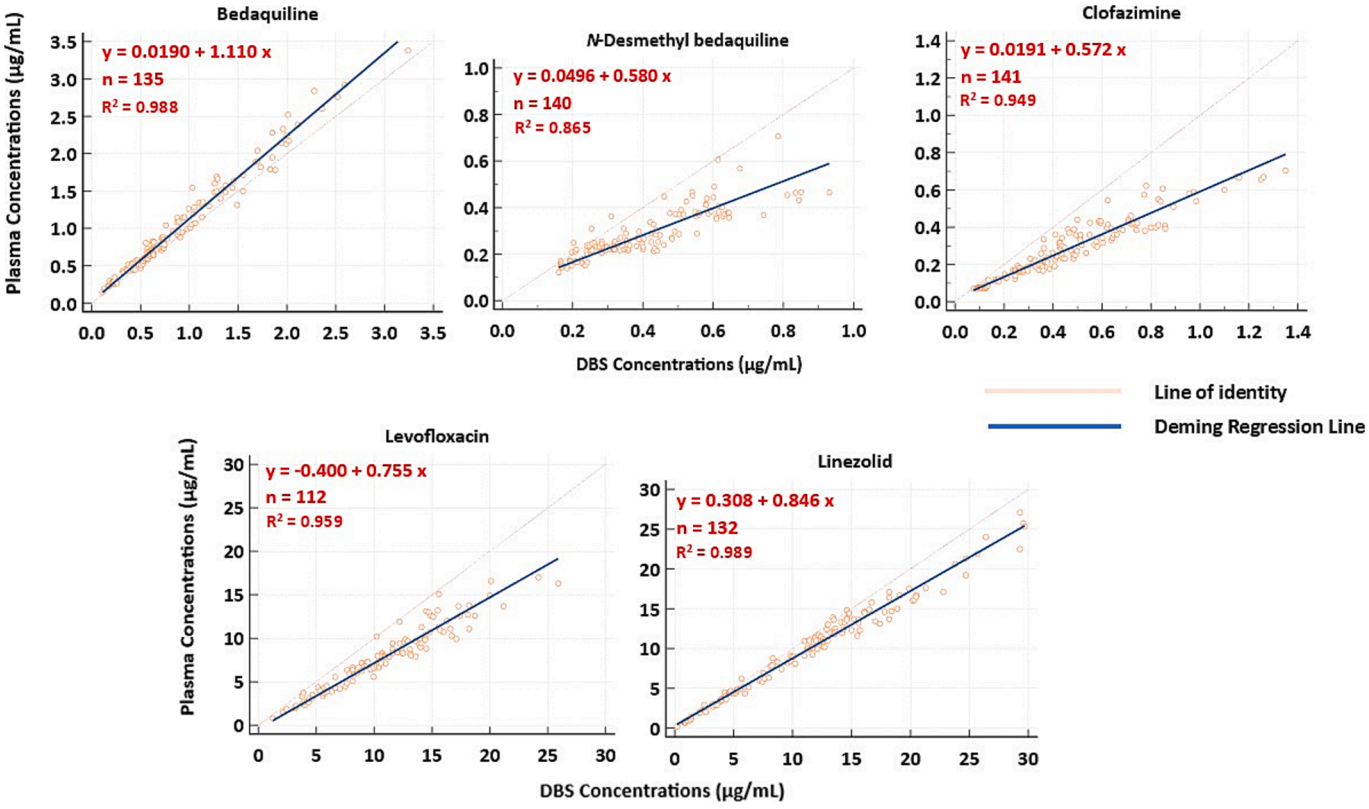


Fig. 4. Deming regressions of plasma concentrations versus dried blood spots (DBS) concentrations for all analytes. The slopes indicate that BDQ concentrations were higher in plasma than in DBS (slope > 1), whereas the concentrations of BDQ-M2, CLF, LVF, and LZD were lower in plasma than in DBS (slopes < 1).

Table 7
Summary statistics of method correlation and agreement.

Analytes (n)	Deming regression					Bland–Altman plot		
	Corr. Coeff (95 % CI)	(DBS vs. PC) Intercept (95 % CI)	Slope (95 % CI)	(EPC vs. PC) Intercept (95 % CI)	Slope (95 % CI)	Mean difference (%) (95 % CI)	EMA LoA (> 67 % ± 20 %)	P-value (H_0: Mean = 0)
Bedaquiline (135)	0.988 (0.983–0.991)	0.0190 (−0.0104–0.0483)	1.11 (1.07–1.15)	−0.000346 (−0.0306–0.0299)	1.00 (0.964–1.04)	−0.4 (−2.04–1.28)	95.5	0.652
***N*-Desmethyl bedaquiline (140)**	0.865 (0.817–0.902)	0.0496 (0.0164–0.0827)	0.580 (0.485–0.675)	−0.00824 (−0.0519–0.0354)	1.03 (0.856–1.20	0.2 (−2.50–2.83)	78.6	0.901
Clofazimine (141)	0.949 (0.929–0.963)	0.0191 (0.00564–0.0326)	0.572 (0.536–0.608)	0.000983 (−0.0157–0.0138)	1.00 (0.939–1.08)	0.7 (−1.71–3.16)	84.4	0.557
Levofloxacin (112)	0.959 (0.940–0.971)	−0.400 (−0.874–0.0743)	0.755 (0.701–0.810)	0.0171 (−0.429–0.463)	0.998 (0.926–1.07)	−1.06 (−3.49–1.38)	89.2	0.393
Linezolid (132)	0.989 (0.985–0.992)	0.308 (0.0712–0.544)	0.846 (0.819–0.873)	0.0313 (−0.212–0.274	0.997 (0.965–1.03)	2.08 (−0.0443–4.20)	93.9	0.0549

Abbreviations: DBS = dried blood spot, EPC = estimated plasma concentration, Corr. Coeff = correlation coefficient, CI = confidence interval, EMA = European Medicines Agency, LoA = limit of agreement, H_0 = null hypothesis.

effect investigation. Process efficiency and recovery outcomes were reproducible across all QC levels, with no matrix effects observed, indicating minimal HcT interference. Therefore, the Deming equation was applied without integration of a HcT factor, to estimate plasma concentrations from DBS measurements. Summary statistics for all analytes are provided in Table 7.

Method agreement based on bias analysis (Bland–Altman).

The Bland–Altman analysis, using a graphical plot, assesses the bias of the mean differences between two measurements and determines an agreement interval within which 95 % of the differences lie [43,44]. If the line of equality (indicating perfect equality between two measurements) is not within the 95 % confidence intervals of the mean difference, a significant bias is indicated [44]. The agreement interval determined by the Bland–Altman analysis does not indicate acceptable limits; these must be defined *a priori* [44], which in this case was based on the EMA requirements for method reproducibility, recommended in the IATDMCT guidelines [21].

Following the calculation of the EPCs by the application of the Deming regression equation, the differences of the plasma concentrations and EPCs were plotted against their means. The differences were expressed as a percentage of the mean of measured and estimated plasma concentrations because a proportional error, fixed to the precision of the assay (CV, %), was observed across the data points for all analytes. No trend or drift were observed across the measurement ranges, either at high or low concentrations, for any of the analytes (Fig. 5). The percentage difference between the plasma concentrations and EPCs (for all paired samples) fell within 20 % of their mean percentage difference for more than 67 % of the paired samples (ranging from 78.6 % to 95.5 % for the five analytes), thus fulfilling the EMA criteria for method reproducibility [21] Notably, the distribution of the percentage differences for LZD exceeded the LoA exclusively at lower concentrations (Fig. 5), indicating potential overprediction of plasma

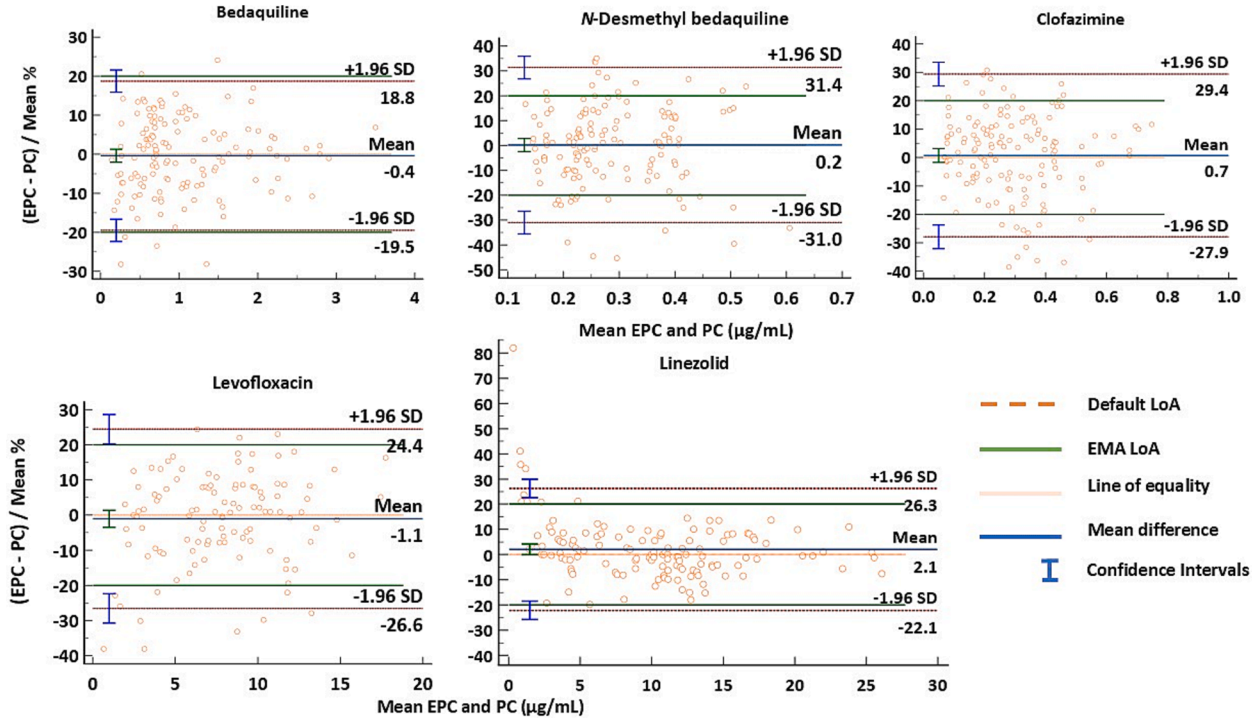


Fig. 5. The Bland–Altman plots indicate the arithmetic mean difference as a percentage; the limits of agreement (LoA): ± 1.96 × SD (standard deviation) away from the mean differences; EMA LoA (± 20 % of the means differences); and the 95 % CI indicating estimated precision. EPC: Estimated Plasma Concentration, PC: Plasma Concentration.

concentrations (EPC) when using the DBS assay, and underprediction at these concentration levels when using the plasma assay. However, the mean percentage difference for all analytes was within the assay criteria for precision, ranging from − 1.1 % to 2.1 %, with the lines of equality (mean = 0) falling within the 95 % CIs of the mean percentage differences, indicating an insignificant bias [44]. P-values calculated by the paired sample *t*-test were > 0.05 for all analytes (0.0549–0.906), indicating no statistically significant differences between the estimated and measured plasma concentrations [29]—see result summary in Table 7.

Our results are not directly comparable with results reported in the literature [41,42,45], possibly due to differences in study design and reporting. There are consistencies, however, between our study and a study conducted by Vu *et al,* investigating the effect of varying hematocrit content (between 20 % and 50 %) on the quantification of LZD in DBS [45]. This study reported no significant effect on assay accuracy and precision. Similarly, no apparent effect on assay accuracy was seen in a study that determined CLF concentrations in DBS samples where the hematocrit content varied from 27 % to 55 % [33].

4. Conclusion

We developed and successfully validated a robust multiplex assay for the quantification of bedaquiline and its main metabolite *N*-desmethyl bedaquiline, linezolid, levofloxacin, and clofazimine in DBS. The clinical applicability of our assay was demonstrated by comparison with in-house plasma methods, supporting its potential use in PK studies, TDM, and as an adherence measure in RR-TB patients. Future research is required to explore the use of this multiplex assay in the routine care of RR-TB patients, specifically in remote areas where the advantages of DBS sampling are desperately needed.

CRediT authorship contribution statement

Gerard Aime Kenfack Teponnou: Writing – original draft, Validation, Methodology, Formal analysis. **Anton Joubert:** Writing – review & editing, Supervision, Methodology. **Saskia Spaltman:** Writing – review & editing, Validation, Methodology. **Marthinus van der Merwe:** Writing – review & editing, Validation, Supervision, Methodology. **Edda Zangenberg:** Writing – review & editing, Visualization, Validation. **Sharon Sawe:** Writing – review & editing, Validation, Methodology, Formal analysis. **Paolo Denti:** Writing – review & editing, Formal analysis. **Sandra Castel:** Writing – review & editing, Validation, Supervision, Methodology. **Francesca Conradie:** Writing – review & editing, Resources, Funding acquisition. **Richard Court:** Writing – review & editing, Supervision, Investigation, Formal analysis. **Gary Maartens:** Writing – review & editing, Visualization, Investigation, Funding acquisition, Conceptualization. **Lubbe Wiesner:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing 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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Data availability

Data will be made available on request.

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