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Research Article

Development and validation of a liquid chromatography tandem mass spectrometry assay for the analysis of bedaquiline and M2 in breast milk

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ABSTRACT

Objective: To develop and validate an assay for the analysis of bedaquiline and its M2 metabolite in human breast milk.

Methods: The analytes were extracted using solid phase extraction following protein precipitation. Quantification was performed with liquid chromatography coupled with tandem mass spectrometry. Chromatographic separation was achieved using gradient chromatography on a Poroshell 120 SB-C18 analytical column at 40 °C, with a flow rate of 350 μL/minute and a total run time of eight minutes. An AB Sciex 3000 mass spectrometer with electrospray ionization in the positive mode was used for detection, employing multiple reaction monitoring scan mode. Bedaquiline-d6 and M2-d3-^{13}C were used as internal standards.

Results: Calibrations curves for bedaquiline and M2 exhibited quadratic (weighted 1/x concentration) regressions over the respective concentration ranges of 0.0780 to 5.00 μg/mL and 0.0312 to 2.00 μg/mL. Inter- and intra-day validation accuracies ranged between 96.7 % and 103.5 % for bedaquiline, and 104.2 % to 106.5 % for M2, with a coefficient of variation below 9.2 % for both compounds.

Conclusion: The developed assay demonstrated selectivity and robustness, enabling differentiation between bedaquiline and M2 within the context of endogenous compounds from six separate lots of breast milk samples. Successful application was observed in the analysis of breast milk samples sourced from patients treated for multidrug-resistant tuberculosis within a clinical study setting.

Introduction

Multidrug-resistant tuberculosis (MDR-TB) is a global health threat, with 161,746 patients treated for the disease in 2021 [1]. Historically, treating MDR-TB has presented significant challenges, such as extended treatment duration and low completion rates, along with a high drug toxicity profile [2]. Recently, the World Health Organization (WHO) has recommended the use of shorter multidrug regimens that include bedaquiline, which has demonstrated improved outcomes when incorporated into MDR-TB treatment regimens [2].

A study conducted by Diacon et al. found that an MDR-TB regimen incorporating bedaquiline resulted in a faster rate of sputum-culture conversion compared to a placebo group (83 days vs. 125 days) [3]. Olayanju et al. reported that including bedaquiline in the treatment regimen for MDR-TB patients in South Africa led to a more than five-fold improvement in outcomes [4]. In a larger study, Borisov et al. also observed a favorable treatment success rate of 71.3 % and a relatively low occurrence of adverse events when using bedaquiline-containing MDR-TB treatment regimens [5].

Bedaquiline is categorized as a diarylquinoline and its mechanism of action involves inhibiting the activity of mycobacterial ATP synthase by binding to the C subunit of the enzyme [6]. It undergoes oxidative metabolism through the cytochrome P450 (CYP) isoenzyme 3A4, leading to the formation of its main metabolite known as the N-monodesmethyl metabolite (M2) [7]. M2 exhibits lower drug exposure in humans compared to the parent molecule, ranging from 23 % to 31 %,

Abbreviations: STD, Calibration standard; CV, Coefficient of variation; CYP, Cytochrome P450; DMSO, Dimethyl sulfoxide; LC-MS/MS, Liquid chromatography coupled with tandem mass spectrometry; LLOQ, Lower limit of quantification; QC, Quality control; SPE, Solid phase extraction; TFA, Trifluoroacetic acid; ULOQ, Upper limit of quantification; MDR-TB, Multidrug-resistant tuberculosis; MRM, Multiple reaction monitoring; M2, N-monodesmethyl metabolite; WHO, World Health Organization.

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and its antimycobacterial activity is four to six times weaker than that of bedaquiline [8]. The concentration of M2 in plasma, rather than bedaquiline, is considered responsible for causing QT prolongation [9].

Co-administration of bedaquiline with potent CYP3A4 inhibitors often results in drug-drug interactions, which can increase the risk of adverse reactions associated with bedaquiline [10]. Compared with some other TB drugs, bedaquiline's unique mechanism of action makes it less susceptible to cross-resistance [6]. Approximately 99.9 % and 99.7 % of bedaquiline and M2, respectively, are reported to bind to plasma proteins [11]. The mean terminal elimination half-life for both bedaquiline and M2 is approximately 5.5 months. This extended elimination half-life is partially due to their slow release from peripheral tissues [11,12]. It should be noted that their long elimination half-life contributes to important adverse effects, such as QT prolongation [12].

There is evidence suggesting that bedaquiline improves outcomes in pregnant women being treated for rifampicin-resistant TB [13]. However, at the start of our study, there was limited data available on the presence of bedaquiline in breast milk, with most information coming from animal studies [14]. There are various factors that can affect the transfer of drugs into breast milk; for example, drugs that are highly lipid-soluble are more likely to be present [15]. Additionally, only unbound drugs can diffuse into breast milk, so drugs with high protein binding may have limited secretion into breast milk. Bedaquiline has a long elimination half-life, which means it can accumulate in breast milk, as observed in both animal [14] and human studies [15]. The level of drug exposure in breastfeeding infants depends on how much breast milk they consume and the concentration of the drug in the breast milk. It is important to quantify TB drug exposure in breastfeeding infants because they could be at risk of drug toxicity or develop drug resistance if plasma drug concentrations are too low.

The World Health Organization (WHO) recommends using bedaquiline to treat children over six years old and pregnant and post-partum women with MDR-TB. However, the safety of using bedaquiline in these populations has not been fully established [2]. Pregnant and post-partum women have historically been excluded from clinical trials involving new drugs, including those for TB. This exclusion has led to a lack of pharmacokinetic and safety data for this vulnerable population [16]. To address this issue, we have developed an assay to determine the concentrations of bedaquiline and its metabolite M2 in human breast milk samples collected during a longitudinal study of women undergoing treatment for MDR-TB. This information will help inform safe breastfeeding practices for women being treated for MDR-TB [15,17].

Experimental

In this study, we have developed and validated a novel analytical method using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to accurately measure the concentrations of bedaquiline and its metabolite M2 in breast milk samples. The assay was then applied to analyze milk samples obtained from breastfeeding women who were undergoing treatment with bedaquiline [15]. Furthermore, we investigated the relationship between the concentrations of bedaquiline and M2 in both breast milk and plasma samples from each participant. The plasma samples were analyzed using a previously validated in-house method for measuring bedaquiline levels [15].

Collection of breast milk samples and its application to clinical studies

The breast milk bank, Milk Matters, in Cape Town, South Africa, kindly donated breast milk that did not contain the analytes of interest. This "blank" breast milk was used to prepare samples for the development and validation of our method. Additionally, clinical breast milk samples were collected from women undergoing bedaquiline treatment for MDR-TB in a longitudinal pharmacokinetic study conducted at King Dinuzulu Hospital in Durban, Kwazulu-Natal [15]. We obtained the necessary ethics approvals from the University of Cape Town Human Research Ethics Committee (HREC: 666/2018, 639/2019, and 120/2021).

Reagents and chemicals

Bedaquiline reference material and the internal standard, bedaquiline-d6, were purchased from Toronto Research Chemicals (Ontario, Canada), while M2 was purchased from Clearsynth (Mumbai, India), and M2-d3-^{13}C was donated by Janssen (Beerse, Belgium). Methanol and trifluoroacetic acid (TFA) were purchased from Anatech (Gauteng, SA) and Sigma-Aldrich (Missouri, USA), respectively. Acetone and acetonitrile were purchased from Honeywell (North Carolina, USA). Formic acid and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). LC-MS/MS grade Millipore water was sourced in-house (Merck-Millipore, Germany).

Extraction procedure

The extraction procedure combined protein precipitation and solid phase extraction (SPE). Breast milk samples, as well as calibration standards and quality control standards, were thawed at room temperature, mixed briefly using a vortex mixer for ~ 10 s, and 100 μL of each sample was transferred into respective 1.5 mL microcentrifuge tubes. Deproteinization was achieved by adding 250 μL of a precipitation reagent (methanol: acetonitrile, 50:50 v/v) containing the internal standards, bedaquiline-d6 and M2-d3-^{13}C, at 125 ng/mL, and vortex-mixed for ~ 30 s at maximum speed. The samples were equilibrated for an hour at room temperature and then centrifuged at 20 238 g for 5 min.

Following deproteinization, solid phase extraction (SPE) was performed using Strata-X (Phenomenex, 33 μm x 200 mg/3 mL) extraction columns which were conditioned with 2 mL methanol and equilibrated with 2 mL water, using a manifold under positive pressure supplied with nitrogen gas. A volume of 350 μL of the supernatant of each sample following protein precipitation was diluted with 1.75 mL LC-MS/MS grade Millipore water and transferred onto the Strata-X columns. The flow-through was discarded and the columns were washed with 2 mL of a LC-MS/MS grade Millipore water, methanol, and acetonitrile mixture (4:3:3, v/v). The columns were then dried by applying positive pressure for ~ 10 min to remove any remnants of water in the columns. The analytes were eluted twice with 500 μL of a methanol and formic acid mixture (99.9:0.1 v/v), collecting the eluent in glass tubes. The samples were dried under nitrogen gas at ~ 40 °C for ~ 30 min. The residues were reconstituted in 250 μL of an acetonitrile, methanol, LC-MS/MS grade Millipore water, and formic acid mixture (50:25:25:0.1, v/v) and vortex-mixed for ~ 30 s. The extracted samples were transferred to a 96-well polypropylene plate for analysis.

Instrumentation

An Agilent 1200 high performance liquid chromatograph was used for analyte and metabolite separation. Baseline separation was essential to reduce crosstalk between bedaquiline and M2. After investigating several columns and mobile phases, the desired chromatographic separation was achieved with an Agilent Poroshell 120 SB-C18 (2.1 mm x 50 mm, 2.7 μm) column at 40 °C using a gradient mobile phase which consisted of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B) at a flow rate of 350 μL/min for a total run time of 8 min. A step gradient was applied which started at 15 % solvent B for 0.5 min, then solvent B was increased to 60 % from 0.6 to 3.00 min, followed by a further increase to 95 % from 3.10 to 4.30 min, and back to 15 % at 4.40 to 8.00 min. The injection volume was 5 μL, and the samples were kept at 8 °C in the autosampler.

A Sciex API 3000 mass spectrometer (AB Sciex™, Germany) with electrospray ionisation in the positive mode, applying multiple reaction monitoring (MRM) scanning at unit resolution was used for detection.

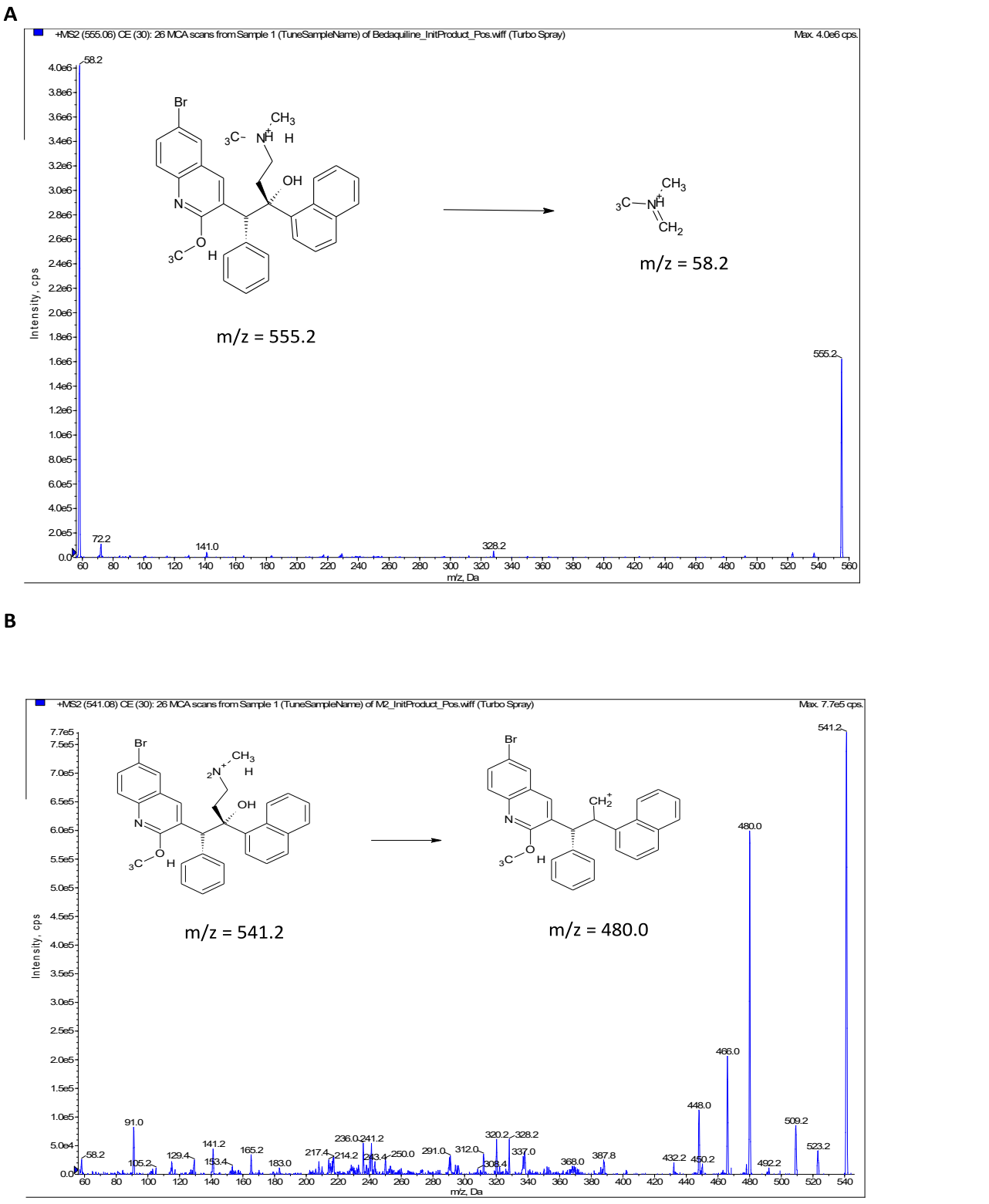

Fig. 1. Fragment mass spectra of bedaquiline (A) indicating the precursor ion at 555.2 and the most prominent product ion at 58.2, and M2 (B) indicating the precursor ion at 541.2 and the most prominent product ion at 480.0.

The transitions of protonated precursor ions to the most prominent product ions for bedaquiline, bedaquiline-d6, M2, and M2-d3-^{13}C were 555.1 to 58.2, 561.1 to 64.1, 541.1 to 480.3, and 545.1 to 480.4, respectively. Fig. 1 shows the fragment mass spectra for bedaquiline and M2. The nebuliser gas, turbo gas, curtain gas, and collision gas for all the analytes were optimised at 8, 8, 10, and 4 arbitrary instrument settings, respectively, while the source temperature was set at 500 °C and the ion spray was 5000 V. The declustering potential for bedaquiline was 50 V, and 61 V for bedaquiline-d6, M2, and M2-d3-^{13}C. The entrance potential to the collision cell was 10 V for all the analyte ions, while the collision energy for bedaquiline was 54 eV, 59 eV for bedaquiline-d6, and 29 eV for M2 and M2-d3-^{13}C. The collision cell exit potential was 2.5 V for

bedaquiline and 4 V for bedaquiline-d6, M2, and M2-d3-^{13}C. The dwell time for all analyte transitions was 70 ms, and the pause time was set at five milliseconds. The instrument was interfaced with a computer running Analyst® version 1.5.2 software (AB Sciex™, Germany).

Method validation

Preparation of calibration standards and quality control samples

Stock solutions for bedaquiline and M2 were prepared in a mixture of water, DMSO, and TFA (10: 90; 0.2 v/v) at 750 μg/mL and 300 μg/mL, respectively. Stock solutions of bedaquiline-d6 and M2-d3-^{13}C were prepared in DMSO at a final concentration of 1 mg/mL for both internal standards. The stock solutions were stored at ~ -80 °C. Working solutions for the preparation of calibration standards (STDs) were prepared in DMSO and serially diluted. One hundred microliters of the stock solutions were individually spiked into 1900 μL blank breast milk to obtain STDs at 0.0780, 0.157, 0.299, 0.584, 1.06, 1.90, 3.52, and 5.00 μg/mL for bedaquiline and 0.0312, 0.0627, 0.120, 0.234, 0.423, 0.762, 1.41, and 2.00 μg/mL for M2. Quality control samples (QC) were prepared by spiking 100 μL of appropriately diluted working solutions to 1900 μL breast milk to produce QC high, medium, low, and LLOQ with concentrations of 4.00, 2.00, 0.195, and 0.0780 μg/mL for bedaquiline and 1.60, 0.800, 0.0780 and 0.0312 μg/mL for M2. Working solutions, STDs, and QCs were stored at ~ -80 °C until used.

For validation, the intra- and inter-day batch accuracy and precision were determined using calibration standards in duplicate and quality controls in six-fold [18].

Stock solution stability

Stock solutions were assessed for the stability of the analytes stored at ~ -80 °C for ~ 160 days for bedaquiline and ~ 71 days for M2. Short-term stability was evaluated at ~ -20 °C and ~ 4 °C for 2 h and at room temperature for ~ 4 h. For all these stability assessments, bedaquiline and M2 stock solutions were diluted to 7.5 μg/mL and 3.0 μg/mL, respectively, and assessed in triplicate on a spectrophotometer at 237 nm for bedaquiline and 236 nm for M2.

Working solution stability

Working solution stability was assessed for ~ 4 days at ~ -80 °C, ~-20 °C, ~4°C, and at room temperature for ~ 4 h at the concentrations of QC high working solution (bedaquiline: 100 μg/mL; M2: 40.0 μg/mL) and QC low working solution (bedaquiline: 1.56 μg/mL; M2: 0.624 μg/mL). Working solutions were diluted in the injection solvent consisting of a mixture of acetonitrile, methanol, water, and formic acid (50: 25: 25: 0.1, v/v) containing 125 ng/mL of the internal standards. Working solutions were tested in six-fold using the LC-MS/MS method by comparing analyte peak area ratios of the test samples and the peak area ratios of the reference samples.

Freeze-thaw stability

Bedaquiline and M2 in breast milk were assessed for three freeze–thaw cycles. Samples stored at ~ -80 °C were thawed for ~ 2 h at room temperature and refrozen for ~ 24 h at ~ -80 °C. QC high and low samples in six-fold were analysed, and accuracy and precision were determined.

Benchtop stability

The stability of bedaquiline and M2 in breast milk left on bench for ~ 4 h at room temperature was assessed using QC high and low samples in six-fold. The samples were analysed with the LC-MS/MS method, and the observed concentrations were compared to the nominal concentrations.

On-instrument stability and reinjection reproducibility

The on-instrument stability was assessed by reinjecting the first validation batch after ~ 35 and ~ 66 h for bedaquiline and M2, respectively. Stability was determined by comparing the peak area ratios of the reinjected QC high and QC low samples to the same QCs obtained during the first injection. This assessment indicates the feasibility of reinjection of an analytical batch due to possible on-instrument interruptions.

Selectivity and Carryover

The method was evaluated for its ability to detect bedaquiline and M2 in the presence of background compounds. Breast milk samples from six different lots were spiked at the LLOQ concentrations and extracted without internal standards. The same matrices were also extracted as blanks (internal standards spiked but no analytes) and the same lots were also analysed as double blank samples (no analytes and internal standards spiked). The samples were analysed with the LC-MS/MS method and the chromatograms were assessed for their signal-to-noise response at the LLOQ, which should be $>$ 5 times the response of the blank samples [18]. The response of the blank samples at the retention time of the analyte should be $<$ 20 % of the LLOQ sample response [18,19].

Carryover was assessed by injecting a double blank sample after the highest calibration standard. Peaks at the retention times of the analytes should be $<$ 20 % of the LLOQ peak [18,19].

Crosstalk

The possible signal contributions between analytes and internal standards were assessed by spiking each analyte separately at the LLOQ and upper limit of quantification (ULOQ), analysed in triplicate without internal standards. Individual blank breast milk for bedaquiline and M2 was spiked at 125 ng/mL and analysed in triplicate. The samples were used to assess the crosstalk between internal standards and analytes. The crosstalk percentage was determined by monitoring bedaquiline ULOQ in the M2 MRM channel and comparing it to the LLOQ peak response of M2. The same was applied for monitoring M2 on the bedaquiline MRM channel. Interfering peaks should be less than 20 % of the LLOQ peak response and less than 5 % of the mean response of the internal standard in the blank sample.

Recovery

The extraction efficiencies of bedaquiline and M2 were assessed in six different lots of breast milk samples at QC high, medium, and low concentrations. Analyte and internal standard peak area ratios of pre-extracted samples were compared to post-extraction spiked samples and the percentage recovery calculated.

Matrix effect assessment

Matrix effect evaluation was done according to methodology described by Matuszewski et al. [20,21]. Six different lots of breast milk were spiked at QC high, medium, and low concentrations and analysed. The peak area ratios at each concentration were used to produce linear regressions for bedaquiline and M2. Slope variability between the matrix sources was evaluated. Variability of $<$ 5 % is considered acceptable [18,19].

Effects of concomitantly administered medication

The following analytes were evaluated for their potential effect on accuracy and precision of the bedaquiline assay: delamanid, DM-6705, levofloxacin, clofazimine, linezolid, pyrazinamide, moxifloxacin, efavirenz, lopinavir, atazanavir, darunavir, dolutegravir, nevirapine, raltegravir, tenofovir, emtricitabine, lamivudine, and abacavir. The potential influence of these compounds on bedaquiline and M2 accuracy and precision was assessed in six-fold by analysing QC high and low samples spiked at appropriate concentrations with a mixture of the analytes as mentioned above and compared to QC high and low samples as a reference.

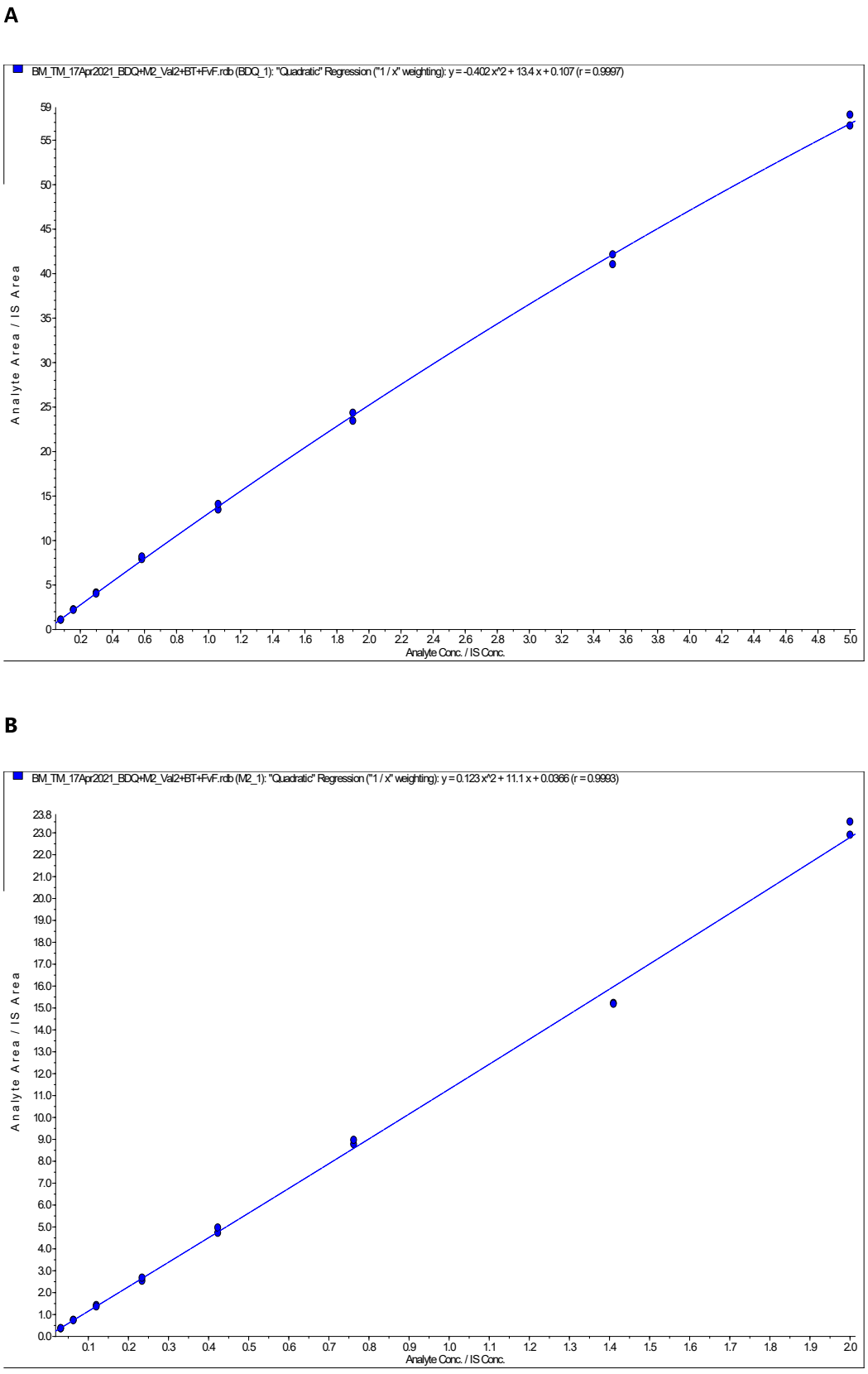

Fig. 2. Representative calibration curves for bedaquiline (A) and M2 (B) in breast milk. The calibration curves were fitted using a quadratic regression model weighted by 1/x with concentration ranges of 0.0780 to 5.00 μg/mL and 0.0312 to 2.00 μg/mL for bedaquiline and M2, respectively. The regression equation is as follows f(x) = ax2 + bx + c; bedaquiline: y = -0.402 x2 + 13.4 x + 0.107 (r = 0.9997) and M2: y = 0.123 x2 + 11.1 x + 0.0366 (r = 0.9993).

Table 1
Bedaquiline and M2 validation summary of QCs.

	Bedaquiline QCs				M2			
	High	Medium	Low	LLOQ	High	Medium	Low	LLOQ
Nominal concentration (μg/mL)	4.00	2.00	0.195	0.0780	1.60	0.800	0.0780	0.0312
Average	4.03	2.04	0.202	0.0754	1.67	0.836	0.0831	0.0328
STDEV	0.139	0.0938	0.00790	0.00477	0.0803	0.0372	0.00351	0.00245
CV(%)	3.4	4.6	3.9	6.3	4.8	4.5	4.2	7.5
%Accuracy	100.6	101.8	103.5	96.7	104.2	104.5	106.5	105.0
N	18	18	18	18	18	18	18	18

Results and discussion

At the time of developing this assay, a reference for the expected ranges of bedaquiline and M2 in breast milk was not available. Therefore, the analytical ranges were based on bedaquiline concentration ranges in animal milk [15]. The calibration ranges for bedaquiline and M2 were chosen to be 0.0780 to 5.00 μg/mL and 0.0312 to 2.00 μg/mL, respectively. We screened a few, randomly selected participant samples prior to the method validation to confirm that the concentrations observed would be within the suggested ranges. The validated calibration curves fitted a quadratic regression (weighted 1/x) over the ranges of 0.0780 to 5.00 μg/mL for bedaquiline and 0.0312 to 2.00 μg/mL for M2. The quadratic equation was chosen since it provided a better fit than the linear equation. Representative calibration curves of bedaquiline and M2 are presented in Fig. 2. The analysis of three validation batches validated the method, and a summary of the accuracy and precision of the QCs are indicated in Table 1. The accuracy (%Nom) and precision (CV%) of bedaquiline QC concentrations at high, medium, low, and LLOQ (N = 18) were between 96.7 % and 103.5 %, and less than 6.3 %, respectively. Furthermore, M2 accuracy and precision were between 104.2 % and 106.5 % and less than 7.5 %, respectively.

Under the conditions mentioned for the stability assessments, all stability results were within the required acceptance criteria [18,19]. Stock solutions of bedaquiline and M2 were shown to be stable in a mixture of water, DMSO and TFA (10:90:0.2, v/v) for 160 and 71 days, respectively, when stored at ~ -80 °C. These periods cover the duration of the method development and validation procedures. Both the analyte and metabolite stock solutions were stable for 24 h at ~ -20 °C and ~ 4 °C, and for ~ 4 h at room temperature. These stability periods enable stock solutions to be used for up to 4 h when spiking working solutions. Working solutions prepared in DMSO at the high and low concentrations were stable for ~ 4 days when stored at ~ -80 °C and up to 4 h at room temperature. Therefore, the preparation time for calibration standards and quality controls did not exceed 4 h on the bench. The analytes were shown to be stable in breast milk for at least three freeze–thaw cycles when thawed at room temperature for ~ 2 h, indicating that sample freezing did not negatively affect the accuracy and precision of the assay.

Bedaquiline and M2 short-term stability in breast milk was determined for ~ 4 h at room temperature, which allows for the extraction of the analytes at room temperature within 4 h. The entire analytical batch of bedaquiline and M2 was reinjected following ~ 35 h for bedaquiline and ~ 66 h for M2 if kept in the autosampler set at ~ 8 °C.

The method was sensitive and able to differentiate between the analyte peaks and the background noise at LLOQ. The mean signal-to-noise ratio of six different lots of breast milk for bedaquiline and M2 was 128 and 83.5, respectively. Representative chromatograms showing the LLOQs overlaid with blank samples are displayed in Fig. 3.

Carryover observed for the analytes was insignificant; for bedaquiline and M2 it was 4.1 % and 7.3 % of the LLOQ, respectively, which is well within the required criteria [18,19].

Special consideration was given to separate bedaquiline and M2 to prevent crosstalk, as depicted in Fig. 4. Crosstalk of less than 2 % was observed.

The average recovery from six different lots of breast milk spiked at QC high, medium, and low concentrations was 57.4 % and 68.3 % for bedaquiline and M2, respectively, with CV(%)s less than 1.1 %.

No significant matrix effects were observed as presented in Tables 2.1 and 2.2. The slope variability between the matrix sources was less than 5 % for both analytes and thus meets the acceptability criteria. This indicates that the method is well-suited to identify the compounds in the presence of interferences in various breast milk lots.

No significant concomitant medication interference was observed. The differences of test samples at QC high and low was less than 12 %, and CVs were less than 5.7 % for both bedaquiline and M2 compared to the reference samples.

The assay was used to analyse breast milk samples from breastfeeding women treated with bedaquiline as reported by Court et al. [15].

Clinical application

The assay was successfully applied to determine the concentrations of bedaquiline and M2 in the breast milk of women treated for MDR-TB [15]. The concentrations ranged from 2.61 to 8.11 μg/mL and 0.273 to 0.814 μg/mL for bedaquiline and M2, respectively. Seven samples from two participants were analysed. We observed that the breast milk concentrations of bedaquiline were significantly higher than the corresponding maternal plasma bedaquiline concentrations taken at the same time points, as described by Court et al. [15]. They also described significantly higher concentrations of bedaquiline in breast milk than in maternal plasma samples taken at the same time points. In the same study, bedaquiline plasma concentrations in the breastfed infant, were also similar to the maternal plasma concentrations, suggesting significant exposure of bedaquiline to breastfeeding infants of mothers treated for MDR-TB, which may have implications for infant safety. Bedaquiline is highly lipid-soluble and protein-bound, which may partly explain the high concentration of bedaquiline in breast milk observed. The high concentrations of bedaquiline previously observed in infant plasma could be explained by the immaturity of the infant CYP3A4 metabolic system to metabolise bedaquiline effectively [15]. The implication of the high breast milk bedaquiline concentrations observed in our study is unclear and requires further exploration.

Conclusion

We have developed and successfully validated an analytical method to quantify bedaquiline and its major metabolite, M2, in human breast milk. Our method is selective and reproducible, and able to differentiate between bedaquiline and M2 in the presence of endogenous components in different lots of breast milk. Stability experiments demonstrated that bedaquiline and M2 were not affected by the conditions associated with the assay.

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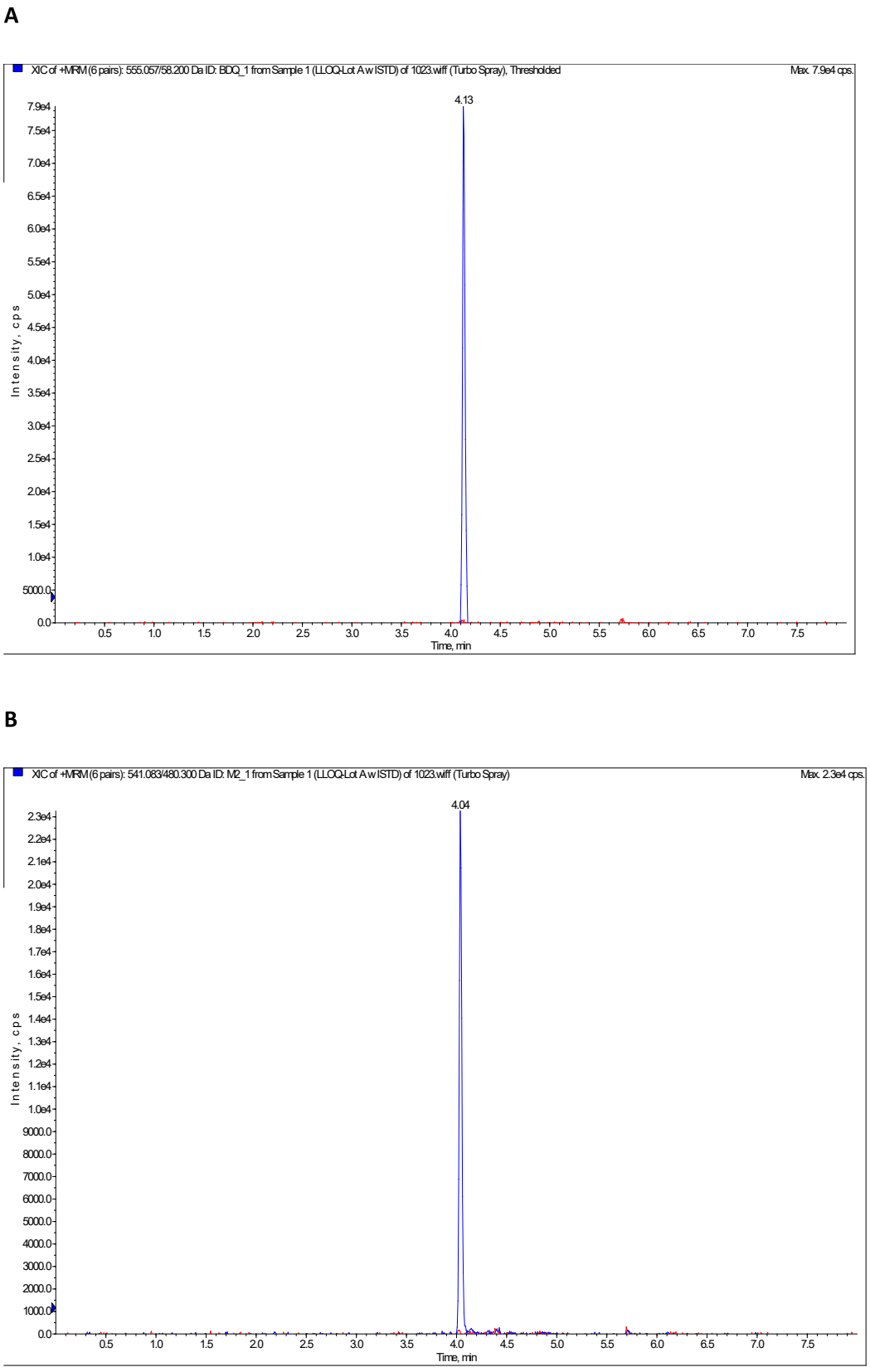

Fig. 3. Overlay example of LLOQ and blank chromatograms (A – bedaquiline, B – M2) of one breast milk lot. The LLOQ is shown in blue, and the blank is in red. Monitored for BDQ and M2 mass transitions 555.1 to 58.2 and 541.1 to 480.3, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

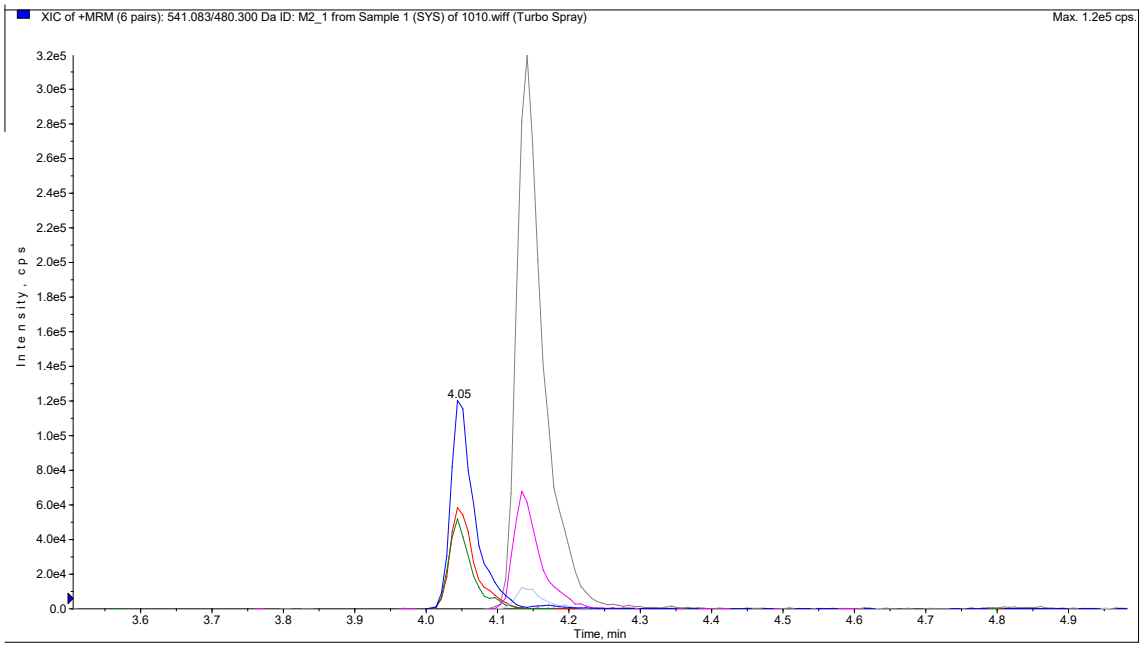

Fig. 4. A zoomed-in view of a chromatogram showing the separation of the analytes: M2 elutes at 4.05 min along with its internal standard, M2-d3-^{13}C; bedaquiline and its internal standard, bedaquiline-d6 elute at 4.15 min. The M2 quantifier and qualifier are represented in blue and red, respectively; green represents M2-d3-^{13}C. Bedaquiline quantifier and qualifier are represented in grey and light blue, respectively; purple represents bedaquiline-d6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2.1
Matrix effects assessment of bedaquiline from six different breast milk sources.

	High Concentration (4.00 µg/mL) Peak Area Ratio	Medium Concentration (2.00 µg/mL) Peak Area Ratio	Low Concentration (0.195 µg/mL) Peak Area Ratio	Area Ratio vs Concentration Regression Slope
Average	66.0	34.9	4.09	16.3
STDEV	1.57	0.714	0.0824	0.414
CV(%)	2.4	2.0	2.0	2.5

Table 2.2
Matrix effects assessment of M2 from six different breast milk sources.

	High Concentration (1.60 µg/mL) Peak Area Ratio	Medium Concentration (0.800 µg/mL) Peak Area Ratio	Low Concentration (0.0780 µg/mL) Peak Area Ratio	Area Ratio vs Concentration Regression Slope
Average	21.2	10.6	1.07	13.2
STDEV	0.761	0.410	0.0202	0.497
CV(%)	3.6	3.9	1.9	3.8

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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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