

https://dspace.org/

Validation and application of an online extraction and liquid chromatography tandem mass spectrometry assay for the analysis of delamanid and its DM6705 metabolite in human breast milk

Buyisile Mkhize; Richard Court; S Castel; Anton Joubert; Francesca CONRADIE ...

ELSEVIER SCIENCE BV
https://hdl.handle.net/10539/44042
WOS

Downloaded from a DSpace repository. https://dspace.org/

Journal of Pharmaceutical and Biomedical Analysis 246 (2024) 116225



Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis





Validation and application of an online extraction and liquid chromatography tandem mass spectrometry assay for the analysis of delamanid and its DM-6705 metabolite in human breast milk

Buyisile Mkhize [a], Richard Court [a], Sandra Castel [a], Anton Joubert [a], Marthinus van der Merwe [a], Gary Maartens [a], Francesca Conradie [b], Lubbe Wiesner [a,*]

[a] Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Cape Town, South Africa
[b] Department of Clinical Medicine, University of the Witwatersrand, South Africa

ARTICLE INFO

Keywords:
Delamanid
DM-6705
Drug-resistant tuberculosis
Breast milk
Liquid chromatography tandem mass spectrometry
Online solid phase extraction

ABSTRACT

We developed and validated a bioanalytical assay to quantify delamanid and its key metabolite (DM-6705) in breast milk and aimed to quantify the secretion of these compounds in breast milk. Due to the hydrophobic nature of the analytes, special care was taken during sample preparation to prevent the formation of fatty deposits during protein precipitation. This was followed by online solid phase extraction and liquid chromatography with tandem mass spectrometry for detection. A Restek Viva BiPh C18 column (1.0 mm×50 mm, 5 μm) was used for extraction while chromatographic separation was performed using a Waters Xterra MS C18 (2.1 mm×100 mm, 5 μm) analytical column with an isocratic mobile phase consisting of acetonitrile, methanol, and 5 mM ammonium carbonate. The mass spectrometric detection of the analytes was performed using an AB Sciex 3200 mass spectrometer employing electrospray ionisation in the positive mode with multiple reaction motoring of the relevant precursor and product ions. Delamanid-d4 and OPC-14714 were used as internal standards. A quadratic (weighted 1/x concentration) regression was used to fit calibration curves for delamanid and DM-6705 over the concentration range of 10.0 – 1000 ng/mL. The intra- and inter-day validation accuracies of the quality control samples were between 92.1% and 98.3% for delamanid, and 97.0% and 102.8% for DM-6705. The percentage coefficient of variation (precision) was less than 7.8%. To our knowledge, this is the first report describing the concentrations of delamanid and DM-6705 in the breast milk of patients treated for rifampicin-resistant tuberculosis.

1. Introduction

Delamanid, a prodrug belonging to the nitro-dihydro-imidazooxazole class, has shown promise in improving efficacy in treatment regimens for rifampicin-resistant tuberculosis (RR-TB) [1]. Delamanid has been quantified in several different matrices including plasma, lung tissue, cerebrospinal fluid (CSF), and hair [2–4].

Delamanid is usually dosed orally at 100 mg twice daily, with oral bioavailability improved 2.7-fold if administered with a standard meal compared to the fasting state [5]. Regardless of food intake, peak plasma delamanid concentrations are reached at approximately 4 hours post-dose, with a large volume of distribution (2100 L) [5]. Delamanid is mainly metabolized by albumin to its primary metabolite, DM-6705; the cytochrome P450 metabolic pathway has a lesser role [6,7]. Both delamanid and its metabolites are highly protein bound (>98.7%) [7]. The half-life of delamanid and DM-6705 ranges between 30 and 38 hours [8], and 217–350.5 hours, respectively [9]. Delamanid requires activation by deazaflavin-dependent nitroreductase in the *Mycobacterium tuberculosis* (*M. tb*) complex [10] to initiate its antimicrobial action. Once activated, delamanid inhibits the synthesis of mycolic acid, a vital process for *M. tb* growth, thereby impeding survival of the bacterium [11].

In male mice, a single 3 mg/kg dose of delamanid resulted in a plasma Cmax of 431 ng/mL at 4 hours, with a 5.9-hour elimination half-life [2]. The area under the concentration time curve $(AUC)_{0-48}$ and AUC_{∞} were 5673 and 5697 ng·h/mL, respectively. Lung tissue concentrations were three times higher than in plasma, indicating significant penetration of delamanid [2].

* Corresponding author.
E-mail address: lubbe.wiesner@uct.ac.za (L. Wiesner).

https://doi.org/10.1016/j.jpba.2024.116225
Received 12 March 2024; Received in revised form 24 April 2024; Accepted 13 May 2024
Available online 16 May 2024
0731-7085/© 2024 Published by Elsevier B.V.

In a study by Tucker *et al.*, delamanid pharmacokinetics in the central nervous system were compared between humans with TB meningitis and rabbits with induced TB meningitis. Human participants received 100 mg delamanid twice daily, while rabbits were given a single 5 mg/kg dose [3]. Despite low CSF/plasma ratios (less than 0.18) in both, human participants with extensively drug-resistant TB meningitis showed clinical improvement and survival. This suggests that, despite relatively low CSF concentrations, delamanid may play a crucial role in managing RR-TB meningitis [3].

Reckers *et al.* measured delamanid in hair samples from 12 RR-TB patients, observing concentrations ranging from 0.004 ng/mg to 0.264 ng/mg (median: 0.012 ng/mg) [4]. The metabolite DM-6705 showed concentrations from 0.412 ng/mg to 12.041 ng/mg (median: 2.682 ng/mg) [4]. Higher DM-6705 concentrations were attributed to its higher pKa, allowing better penetration into hair. Notably, there was no correlation (r=0.05) between delamanid and DM-6705 concentrations in the participants' hair samples [4].

To explore the safety of breastfeeding infants of mothers treated for drug-resistant TB, it is important to quantify the concentrations of key TB drugs in breast milk. The extent of drug exposure in breast milk is affected by the actual drug concentration in the breast milk, and by the quantity of breast milk consumed by the infant. Delamanid exposure in the breast milk of mothers treated for drug-resistant TB has not previously been described. The transfer of delamanid into breast milk has been demonstrated in lactating rats, where the concentration of delamanid in rat milk was four times higher than in blood [5]. A study involving pregnant women with RR-TB demonstrated positive birth outcomes and good tolerance to a treatment regimen including bedaquiline, delamanid, and linezolid [1], but breast milk drug concentrations were unfortunately not measured. It is possible that delamanid exposure to infants via breast milk could be harmful or potentially prophylactic against the development of drug-resistant TB in breastfeeding infants.

We describe the development and validation of a novel analytical method using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to measure the concentrations of delamanid and its primary metabolite, DM-6705, in human breast milk. The method was employed to analyse breast milk samples from breastfeeding women treated with delamanid in the Building Evidence for Advancing New Treatment for Tuberculosis (BEAT-Tuberculosis) study, an open-label, multi-centre, randomised controlled trial comparing a novel MDR-TB treatment regimen, including delamanid, with standard of care. We also compared maternal plasma and breast milk delamanid concentrations. Plasma concentrations were determined using a previously validated in-house method.

2. Experimental

2.1. Collection of breast milk samples

Breast milk samples that did not contain delamanid and DM-6705 were donated by the Milk Matters breast milk bank in Cape Town, South Africa - these samples were used for sample preparation during method development and validation.

We collected breast milk samples from post-partum women treated for drug-resistant TB who were participants in the BEAT-Tuberculosis trial at Jose Pearson hospital in the Eastern Cape region of South Africa. The samples were stored at ~-80°C. We performed pharmacokinetic sampling in two participants after approximately four weeks of treatment at the following time points: pre-dose, 2, 4, 6, 8, and 24 hours post-dose. The study was approved by the University of Cape Town Human Research Ethics Committee (HREC: 727/2019, 639/2019, and 120/2021).

2.2. Reagents and chemicals

Reference materials of delamanid, DM-6705, and the internal standard for the metabolite (OPC-14714) were donated by Otsuka (Tokyo, Osaka, and Naruto, Japan), while deuterated delamanid, used as internal standard for delamanid, was purchased from ClearSynth (Mumbai, India). Ammonium hydroxide, ammonium bicarbonate, dimethyl sulfoxide, and N,N - dimethylformamide were purchased from Sigma-Aldrich (MO, USA). Formic acid was purchased from Labchem (PA, USA). Methanol, acetonitrile, and acetone were purchased from Anatech (Gauteng, South Africa). LC-MS/MS grade water was prepared in-house using a Millipore Synergy (Merck-Millipore, Germany) water purification system.

2.3. Preparation of calibration standards, quality controls and internal standards

Stock solutions of delamanid and DM-6705 were prepared in N,N - dimethylformamide (DMF) at a concentration of 500 μg/mL. Working solutions for calibration standards (STDs) were prepared by adding 20 μL of each of the delamanid and DM-6705 stock solutions to 3960 μL of 0.1% formic acid in DMF, resulting in the first working solution with a concentration of 2.50 μg/mL for delamanid and DM-6705. Serial dilutions were then performed to obtain a range of working solutions, with the lowest concentration being 0.0250 μg/mL for delamanid and DM-6705. All working solutions were prepared fresh on the day they were used for the validation experiments. The calibration STDs in breast milk were prepared at concentrations of 10.0, 20.0, 40.0, 100, 240, 500, 750, and 1000 ng/mL.

The same volume of stock solutions was spiked into 4960 μL of 0.1% formic acid in DMF to prepare the working solution for quality controls (QCs) at the high concentration level. Serial dilutions were then carried out to obtain QC working solutions at various concentration levels. Breast milk concentrations at QC high, medium, low, and LLOQ for delamanid and DM-6705 were 800, 400, 25.0, and 10.0 ng/mL, respectively. The calibration STDs and QCs were prepared fresh on the day they were used for each validation experiment. For sample analysis, stock solutions, working solutions, STDs, and QCs were stored at ~-80°C until required for use.

Delamanid-d4 and OPC-14714 internal standard solutions were prepared in dimethyl sulfoxide (DMSO) and methanol, respectively, at 1000 μg/mL. The respective internal standards were then diluted in matrix to form internal standard working solutions.

2.4. Extraction procedure

2.4.1. Sample pre-treatment and precipitation

Breast milk samples were thawed at ~30°C for 15 minutes. After thawing, the samples were mixed by inversion using a revolving rotator at room temperature for 5 minutes and then returning the tubes to ~30°C. For the preparation of STDs and QC samples, 20 μL of working solutions in 0.1% formic acid in DMF was transferred into 1.5 mL microcentrifuge tubes. Subsequently, 50 μL breast milk was added to the tubes and mixed by hand with five inversions of each tube. The samples were vortex-mixed for 10 seconds at a medium speed setting.

The double blank, blank, and study samples were prepared the same way as the STDs and QCs by the addition of 20 μL of 0.1% formic acid in DMF. Fifty microliters of the internal standard working solution was added to all the samples and mixed using a vortex mixer at setting 6 (medium speed) for 10 seconds.

The samples were allowed to equilibrate for 5 minutes. A volume of 400 μL of cooled (~4°C) precipitation reagent (acetonitrile and methanol, 50:50, v/v) was added, followed by vortex-mixing at the maximum speed for 20 seconds. The samples were equilibrated on ice for 30 minutes to allow sufficient time for protein precipitation and for the analytes to partition into the extraction solvent. Subsequently, the samples

Table 1
Flow parameters applied to Pump 2, to create the gradient to wash and condition the online extraction column.

Time (min)	Flow rate (µL/min)	A (%)	B (%)
0.00	250	80.0	20.0
0.50	250	10.0	90.0
2.00	250	10.0	90.0
2.50	250	0.00	100
3.50	250	0.00	100
4.00	250	80.0	20.0
7.00	250	80.0	20.0

Table 2
The six port switching valve program (see also the diagrams in Fig. 1).

Step	Total Time (min)	Valve Position
0	0	Position A
1	2.80	Position B
2	3.40	Position A

were centrifuged at approximately 20000 x g for 5 minutes. Two hundred microliters of the supernatants were transferred to the wells of a 96-well polypropylene plate and 50 µL of a 0.1% formic acid in water solution was added to each well. The samples were then ready for solid phase extraction and LC-MS/MS analysis.

2.4.2. Online solid phase extraction and chromatography

A Restek Viva BiPh C18 column (1.0 mm×50 mm, 5 µm) was used for online solid phase extraction (online SPE) of the analytes while a Waters Xterra MS C18 column (2.1 mm×100 mm, 5 µm) was used for chromatography of the extracted material. Both columns were maintained at a temperature of ~40°C. Two Agilent HPLC pumps, an Agilent 1100 Binary Pump (pump one) and an Agilent 1100 LC Binary Pump (pump two) were used to supply two different mobile phases to the two columns during a total run time of 7 minutes.

Pump one delivered an isocratic mobile phase consisting of 5 mM ammonium bicarbonate: methanol: acetonitrile: ammonium hydroxide (20:40:40:0.2, v/v) at a constant flow rate of 300 µL/minute.

Pump two delivered the gradient mobile phase contained acetonitrile and water with 0.1% formic acid described in Table 1, at a constant flow rate of 250 µL/minute.

A six-port switching valve was employed to direct the flow of the mobile phases from the pumps at different time intervals to the columns. The program for the activation of the valve is detailed in Table 2 and a schematic representation of the online extraction procedure is depicted in Fig. 1.

With the switching valve in position A, a run was started by the injection of 5 µL sample into the flow from pump 2, directed to the extraction column at a flow rate of 250 µL/minute. The analytes were retained while polar matrix components were eluted to waste. At 2.80 minutes the valve switched to position B, directing the mobile phase delivered by pump 1 at 300 µL/minute through the extraction column, transferring the analytes to the analytical column. At 3.40 minutes, when both the analytes have eluted from the extraction column, the valve switched back to position A, allowing the isocratic chromatography of the analytes to proceed on the analytical column, while the gradient flowing from pump 2 (see Table 1) was directed through the extraction column and from there to waste, to clean the

Table 3
Compound related settings for the MRM detection of the analytes and internal standards.

	Delamanid	Delamanid-d4	DM-6705	OPC-14714
Precursor ion mass (*m/z*)	535.2	539.1	466.2	458.0
Product ion mass (*m/z*)	352.0	356.0	352.0	295.0
Dwell time (ms)	50.0	50.0	50.0	50.0
Declustering potential (V)	66.0	61.0	61.0	71.0
Entrance potential (V)	8.00	7.00	8.00	12.0
Collision energy (eV)	29.0	31.0	27.0	31.0
Collision cell exit potential (V)	24.0	22.0	22.0	18.0
Pause time (msec)	5.007			

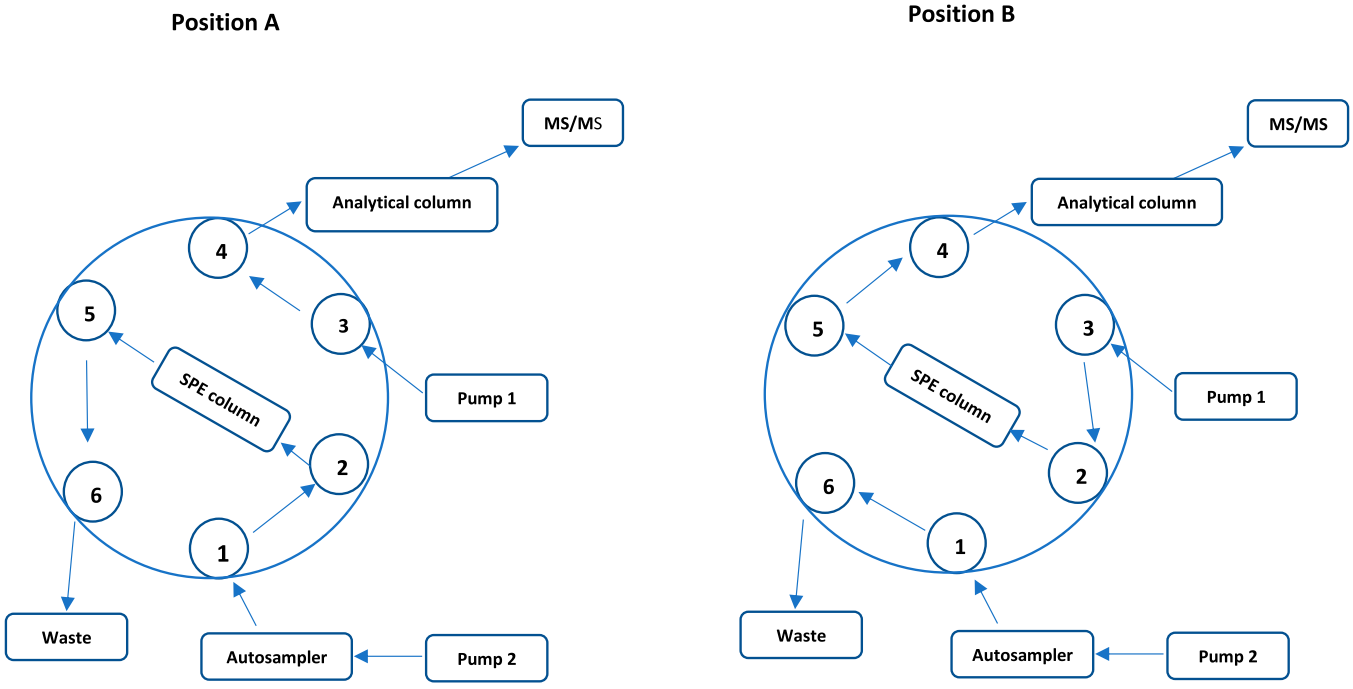


Fig. 1. : A diagram showing the solvent flow from pumps 1 and 2 regulated by the two switching positions of the six port switching valve used to direct the flow to the SPE and analytical columns.

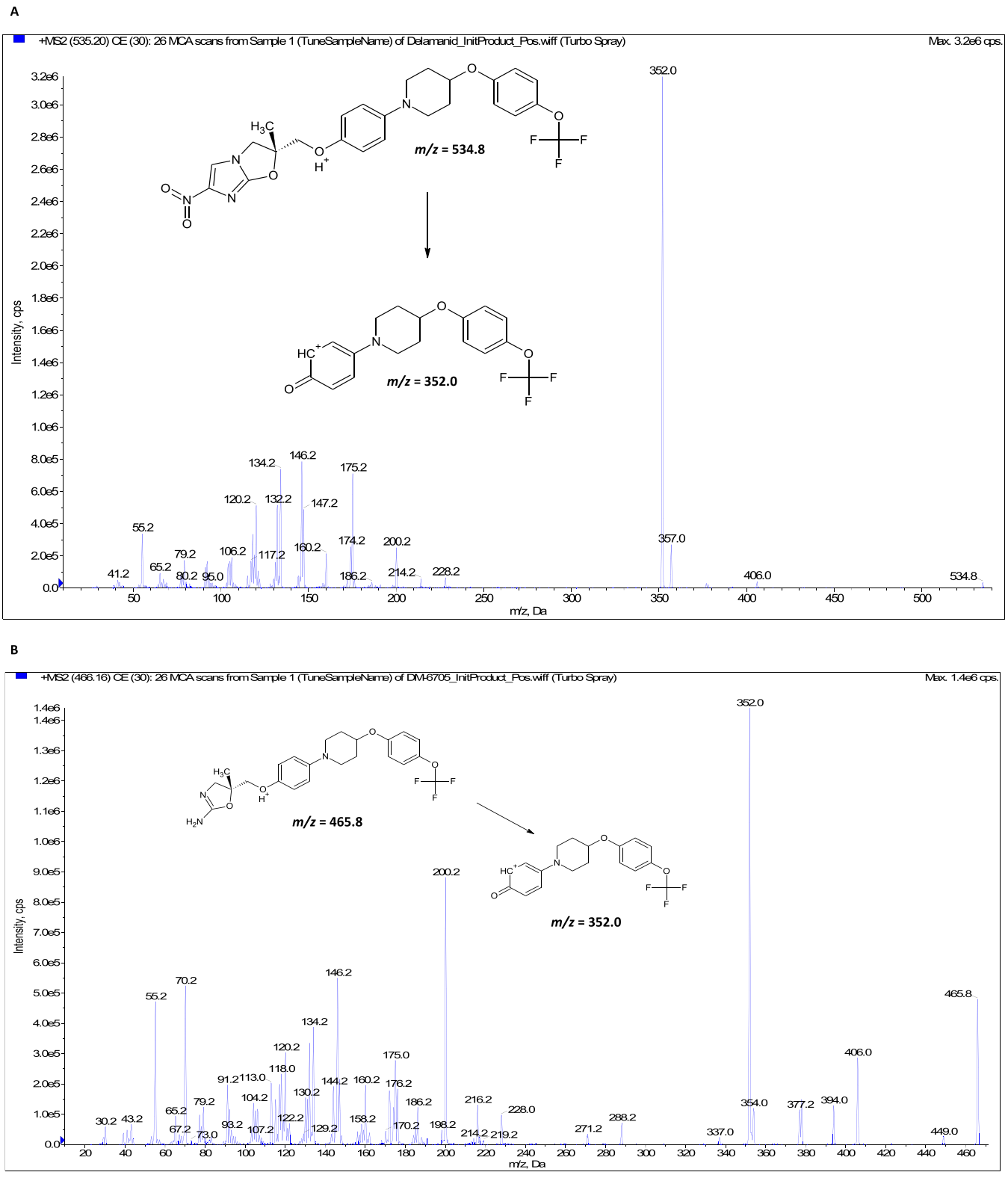


Fig. 2. : Mass spectra fragmentation of delamanid (**A**) showing precursor and product ions at *m/z* 535.2 and 352.0, respectively. DM-6705 (**B**) precursor and product ions are at *m/z* 466.2 and 352.0, respectively.

extraction column of more hydrophobic material following the elution of the analytes. After the total run time of 7 minutes, both columns were conditioned for the next injection.

2.5. Mass spectrometry detection

Mass spectrometric detection of the analytes was performed on a Sciex API 3200 triple quadrupole mass spectrometer, interfaced to the online SPE and chromatographic system by electrospray ionization in the positive mode. The spray voltage was set at 5000 V for both analytes

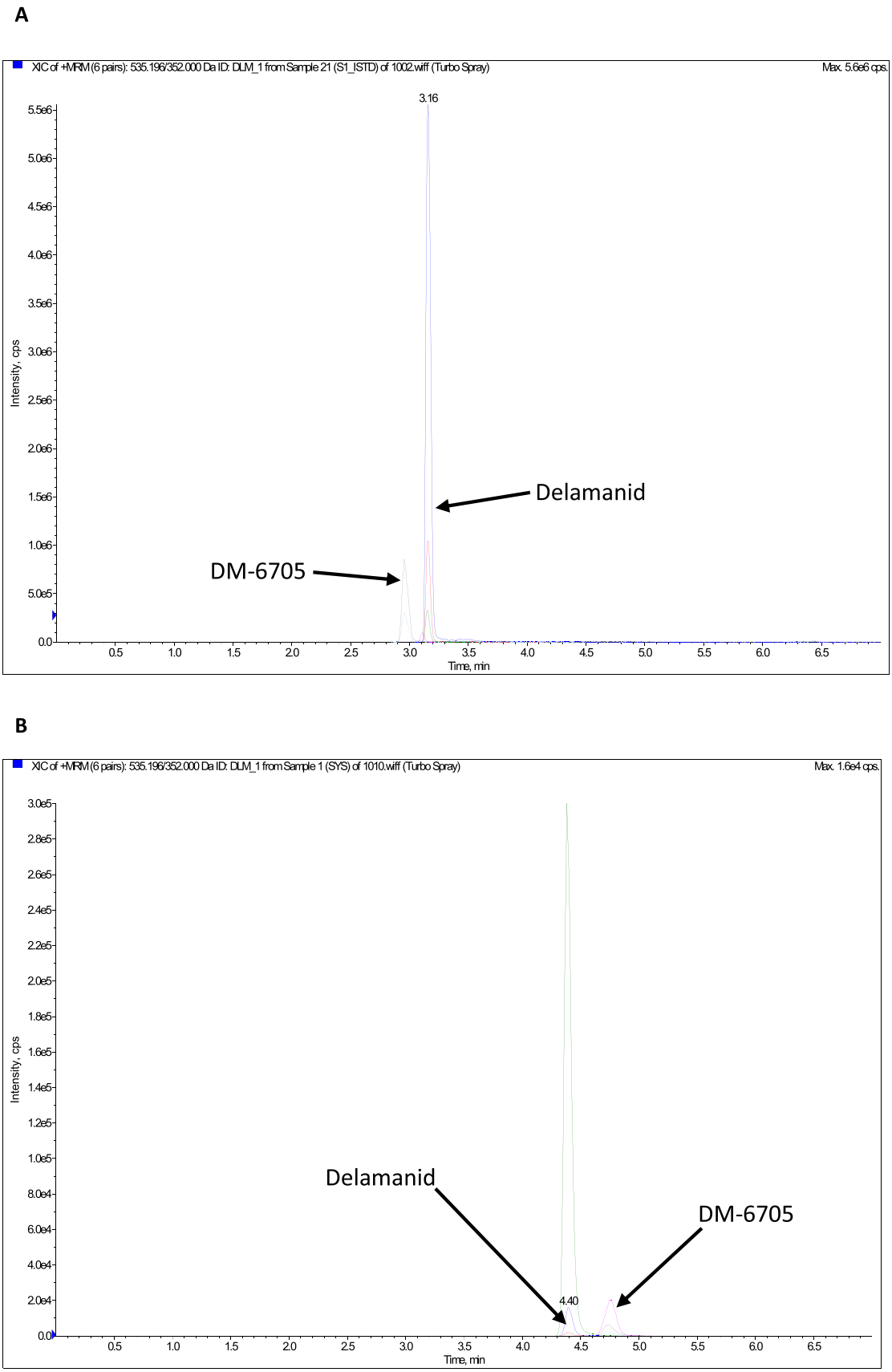


Fig. 3. : A: An example of an online SPE chromatogram showing the analytes eluting on the SPE column. **B:** An example of the complete online extraction and chromatography of delamanid and DM-6705.

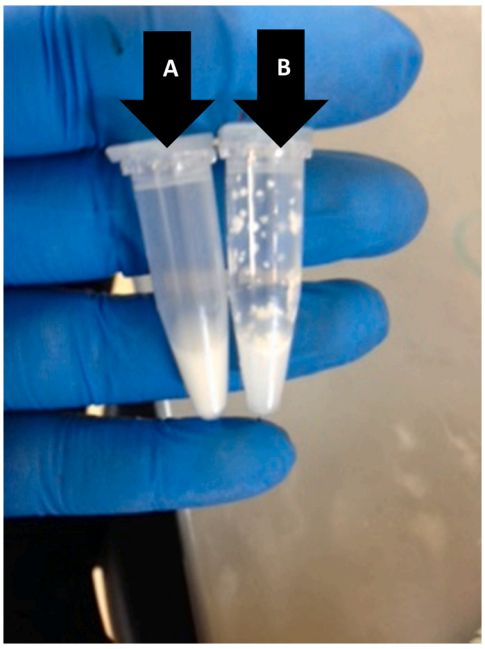


Fig. 4. : Examples of breast milk samples vortex-mixed at medium (A) and high (B) settings. The high-setting example resulted in fat globules separating and adhering to the sides and lid of the tube.

with the source temperature at 500°C and the turbo gas, nebulizer gas and curtain gas at 50, 60 and 40 arbitrary units, respectively. All compound related settings, enabling the optimal detection of the analytes and internal standards by multiple reaction monitoring (MRM) at unit resolution, are indicated in Table 3. The fragmentation patterns of the analytes are presented in Fig. 2**.** The quantitation of analyte concentrations was performed using Analyst® software from Sciex™.

2.6. Method validation

2.6.1. Assessment of precision and accuracy

Intra- and inter-day precision and accuracy were assessed by analysing STDs in duplicate at each calibration point and QCs in six-fold over three days in three separate batches. Each of the STDs was used to define the calibration equation. The regression equation was calculated and the calibration curve constructed based on the peak area ratios of the analyte to internal standards [12,13].

2.6.2. Assessment of analyte stabilities

Stock solutions and working solutions were assessed for at least 4 hours at room temperature and 24 hours at ~4°C and ~-20°C. Stock solutions for delamanid and DM-6705 were also assessed at approximately −80°C for 376 and 370 days, respectively. The stability of the analytes in matrix during three freeze and thaw cycles were evaluated by thawing QC samples at room temperature for ~4 hours and refreezing them for ~24 hours at ~-80°C. The stability of the analytes during sample preparation was assessed by placing QC samples on bench at room temperature for approximately 4 hours, before sample preparation and analysis. For both these stability assessments, QC high and QC low samples were used in six-fold, and were then included in a validated batch, and the observed concentrations compared to the nominal concentrations. During validation, working solutions, STDs and QCs were prepared fresh for each validation analysis. However, for sample analysis, the working solutions, STDs, and QCs used were stored at ~-80°C

Table 4.1
Delamanid recovery varying between breast milk samples with different fat content.

	High (8000 ng/mL)			Medium (4000 ng/mL)			Low (500 ng/mL)		
	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)	Area ratio	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)	Area ratio	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)	Area ratio
Matrix 1	1510000	1790000	0.844	679000	2000000	0.340	76600	2030000	0.0377
Matrix 2	1860000	2010000	0.925	931000	2150000	0.433	115000	2270000	0.0507
Matrix 3	1880000	1890000	0.995	890000	1990000	0.447	119000	2260000	0.0527
Matrix 4	1920000	1920000	1.00	939000	2010000	0.467	106000	2130000	0.0498
Matrix 5	1810000	1990000	0.910	916000	2030000	0.451	108000	2230000	0.0484
***Matrix 6**	848000	1810000	0.469	434000	1950000	0.223	54400	1980000	0.0275
Average	1638000	1901667	0.861	798167	2021667	0.395	96500	2150000	0.0449
STDEV	414111	90425	-	203281	68240	-	25454	123774	-
CV(%)	*25.3	4.8	-	*25.5	3.4	-	*26.4	5.8	-
N	6	6	6	6	6	6	6	6	6

* Matrix 6 had a high fat content and readily formed fat globules upon excessive vortex mixing; CV(%) of the analyte peak areas are above the acceptable criteria

Table 4.2
DM-6705 recovery varying between breast milk samples with different fat content.

	High (8000 ng/mL)			Medium (4000 ng/mL)			Low (500 ng/mL)		
	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)	Area ratio	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)	Area ratio	Analyte Peak Area (counts)	Internal Standard Peak Area (counts)	Area ratio
Matrix 1	1010000	619000	1.63	470000	667000	0.705	40300	685000	0.0588
Matrix 2	1150000	631000	1.82	569000	660000	0.862	52700	690000	0.0764
Matrix 3	1160000	639000	1.82	532000	676000	0.787	51900	708000	0.0733
Matrix 4	1220000	630000	1.94	541000	670000	0.807	48200	704000	0.0685
Matrix 5	1110000	647000	1.72	528000	669000	0.789	47600	682000	0.0698
***Matrix 6**	637000	612000	1.04	322000	646000	0.498	31100	650000	0.0478
Average	1047833	629667	1.66	493667	664667	0.743	45300	686500	0.0660
STDEV	212951	12770	-	90104	10501	-	8230	20666	-
CV(%)	*20.3	2.0	-	*18.3	1.6	-	*18.2	3.0	-
N	6	6	6	6	6	6	6	6	6

* Matrix 6 is very fatty – less analyte was recovered; CV(%) of the analyte peak areas are above the acceptable criteria

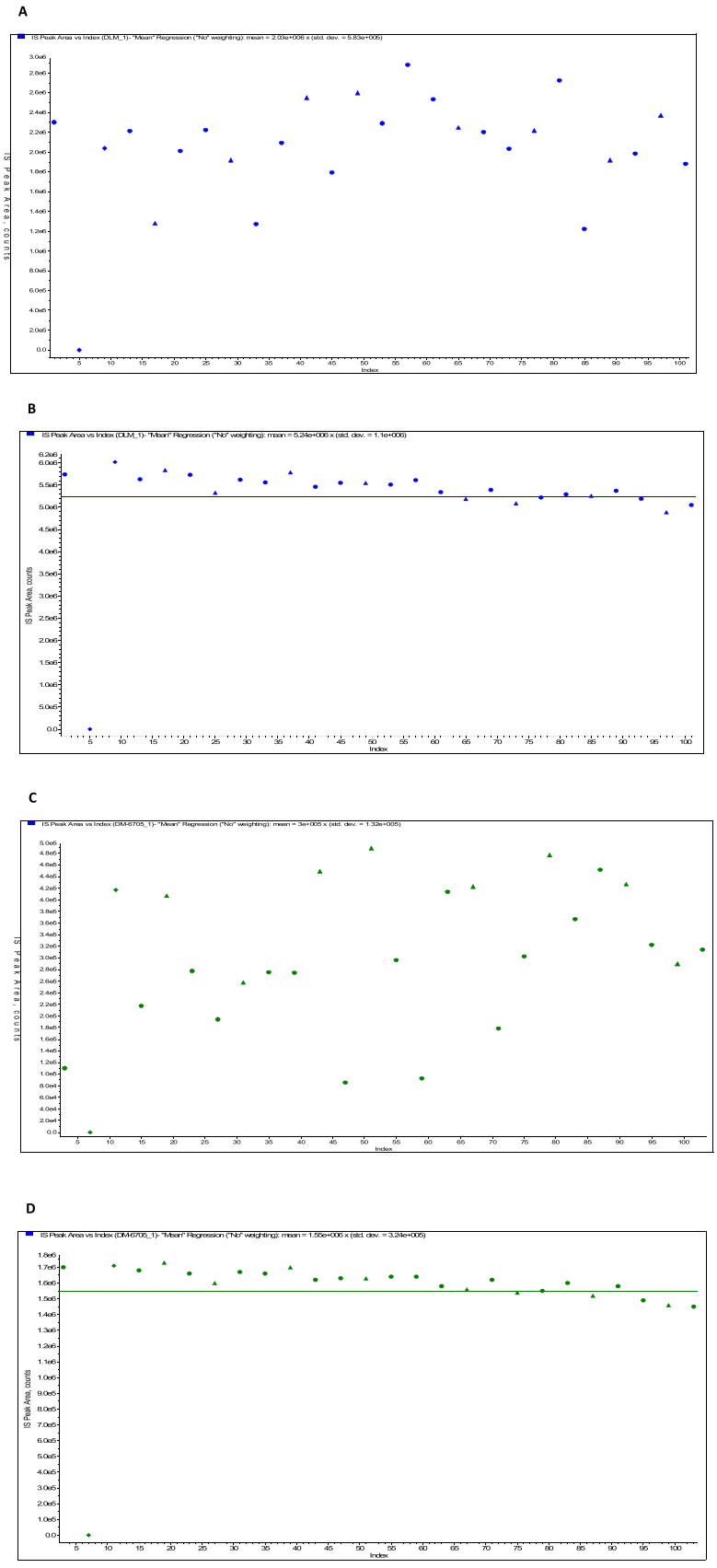


Fig. 5. : An example of the internal standard peak area variability in a batch: Panels **A** and **C** show the intensity fluctuating of delamanid-d4 and OPC-14714, respectively, prepared in precipitation solution. Panels **B** and **D** show a stable signal of delamanid-d4 and OPC-14714, respectively, following their dilution in matrix before application to the precipitation step.

Table 5
Summary of the precision and accuracy results of the QCs for the three validation batches.

	Delamanid				DM-6705			
	QCs							
	High	Medium	Low	LLOQ	High	Medium	Low	LLOQ
Nominal concentration (ng/mL)	800	400	25.0	10.0	800	400	25.0	10.0
Average	787	391	23.7	9.21	789	391	24.2	10.3
STDEV	20.1	17.1	0.756	0.427	18.5	15.7	1.88	0.587
CV(%)	2.6	4.4	3.2	4.6	2.3	4.0	7.8	5.7
%Accuracy	98.3	97.8	95.0	92.1	98.6	97.8	97.0	102.8
N	18	18	18	18	18	18	18	18

prior to analysis.

On-instrument stability was evaluated by reinjecting the first validation batch for delamanid and DM-6705 after ~48 and ~24 hours, respectively. Stability was determined by comparing peak area ratios of high- and low-reinjected QC samples to the peak area ratios of the same QCs obtained during the first injection. Reinjection reproducibility was evaluated to determine if reanalysis is possible during instrument interruptions.

2.6.3. Selectivity and carryover

Selectivity was evaluated to determine if the method can differentiate and quantify delamanid and DM-6705 in the presence of interfering components. Six different lots of breast milk samples were prepared at the LLOQ concentration extracted both with and without the addition of internal standards. In addition, double-blank samples (containing no analytes or internal standards) and blank samples were included from the six different lots of breast milk.

Double-blank samples were injected after the highest calibration standard allowing the detection and evaluation of any potential signal carryover from previous injections.

2.6.4. Crosstalk

Each analyte (delamanid and DM-6705) was separately spiked at the LLOQ and upper limit of quantification (ULOQ) concentrations to examine the signal contribution between the analytes and internal standards. These were then analysed in triplicate without adding internal standards. Crosstalk between delamanid and DM-6705 was investigated by monitoring the percentage of delamanid at the ULOQ concentration in the DM-6705 detection channel and comparing it to the LLOQ peak response of DM-6705. Similarly, DM-6705 was monitored in the delamanid detection channel.

Blank matrix was extracted in triplicate and individually spiked with each of the internal standards at the working concentration of the assay (1.25 μg/mL for delamanid-d4 and 0.625 μg/mL for OPC-14714). These samples were used to assess crosstalk between the internal standards and the analytes. The aim was to ensure that the response of interfering peaks was below 20% of the LLOQ peak response and below 5% of the mean response of the internal standard in the blank sample [12,13].

2.6.5. Recovery and process efficiency

Online SPE coupled to chromatography can be regarded as two-dimensional chromatography, therefore, assessments of recovery and process efficiency must be done by regarding the deproteination step as sample preparation procedure.

Reference samples for recovery assessment were prepared by spiking analytes at QC high, medium, and low concentrations into six different lots of breast milk supernatants after deproteination. These were analysed by online SPE and chromatography as described, and compared to the same breast milk lots in which the same concentrations were spiked before deproteination. For process efficiency assessment, reference samples were prepared by spiking the same concentrations in the precipitation solution containing the internal standards and comparing it analytically to the six breast milk lots used for the recovery assessment.

2.6.6. Assessment of matrix effects and the effect of concomitant medication

The methodology described by Matuszewski *et al.* was used to evaluate matrix effects [14,15]. Six different lots of breast milk spiked at QC high, medium, and low concentrations were analysed. The peak area ratios at each concentration of the analytes were used to generate a linear regression for each matrix lot. Slope variability (CV(%)) between the matrix sources was assessed and should not exceed 5% [12,13].

The ability of the analytical method to reliably quantitate the analytes in the presence of co-administered drugs was assessed. We tested drugs, which were co-administered during the BEAT-Tuberculosis trial, including: bedaquiline, levofloxacin, clofazimine, linezolid, efavirenz, lopinavir, atazanavir, dolutegravir, nevirapine, tenofovir, emtricitabine, and abacavir. High and low QC samples in six-fold were tested, and the mean peak area ratios observed in test samples were compared to the reference samples.

3. Results and discussion

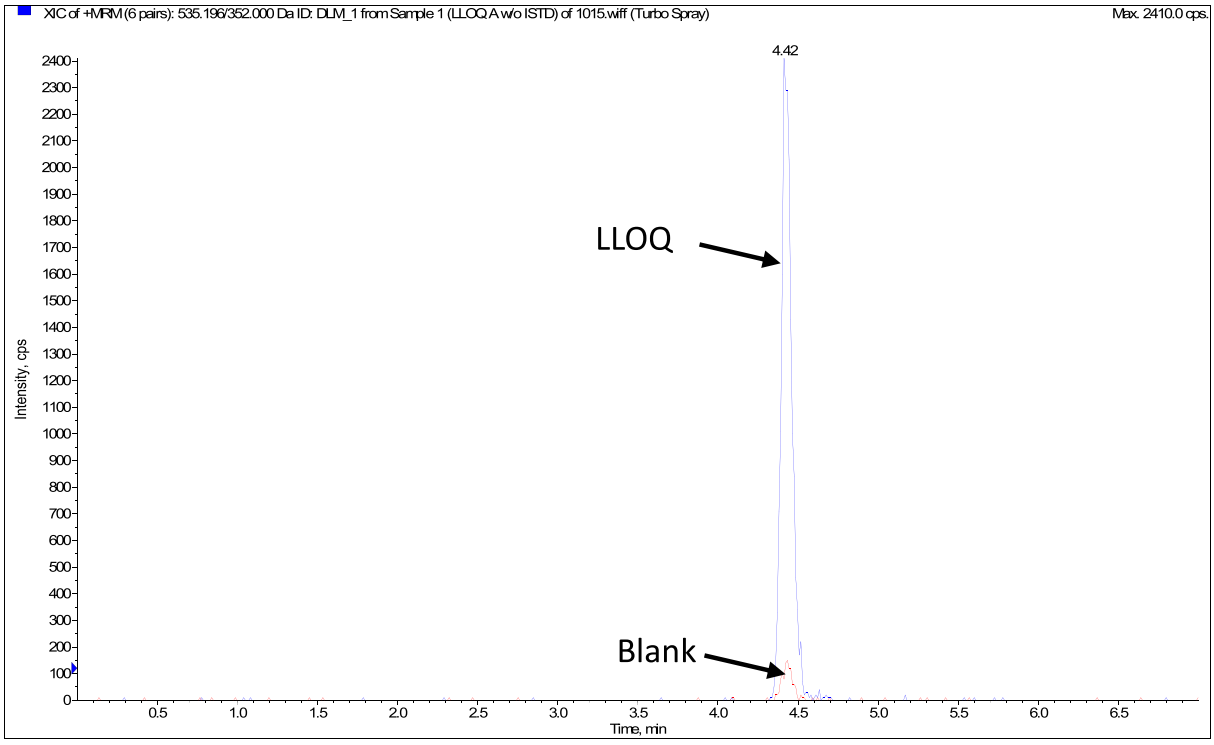
3.1. Method development

The mass spectrometry parameters for delamanid and DM-6705 were optimized as described in Table 3 and **Section 2.5** , and applied in a chromatography procedure, using a Waters Xterra MS C18 column (2.1 mm×100 mm, 5 μm). A mobile phase gradient of acetonitrile, methanol, water, and 0.1% formic acid was used over 6 minutes. The LC-MS/MS method was successfully used to analyse calibration standards, showing linearity across a range of 10.0 – 1000 ng/mL for both compounds, when injected in pure form dissolved in injection solvent. The method was then employed to optimize analyte extraction from human breast milk, addressing challenges specific to the matrix.

Before extracting analytes, breast milk samples underwent deproteinization with a 50:50 methanol and acetonitrile precipitation. Solid phase extraction using Phenomenex STRATA-X cartridges followed, activated with 2 mL methanol, and equilibrated with 2 mL water. Deproteinized samples (~350 μL) were applied and no significant breakthrough occurred. Washing used a 10:90 methanol and water mixture (2 mL). Elution employed 1.75 mL methanol; after evaporation, the residue was reconstituted in a 0.25 mL solution of 5 mM ammonium bicarbonate: methanol: acetonitrile: ammonium hydroxide (20:40:40:0.2, v/v) followed by LC-MS/MS analysis. This process yielded unacceptable results: Delamanid and DM-6705 chromatography after SPE on a Phenomenex STRATA-X cartridge showed acceptable peak shape for delamanid but inconsistent peak areas. DM-6705 exhibited unacceptable peak shape and variable areas. Matrix components co-eluting with analytes likely caused these issues, prompting changes in SPE conditions, including the use of C18 cartridges and volume reductions, but problems persisted. A more comprehensive extraction method was then considered.

Online SPE is commonly used to optimize hydrophobic interaction extraction methods, offering high sensitivity and more flexibility in the adjustment of analyte interaction with the solid phase so that closely similar matrix components can be separated from the analytes [16].

A



B

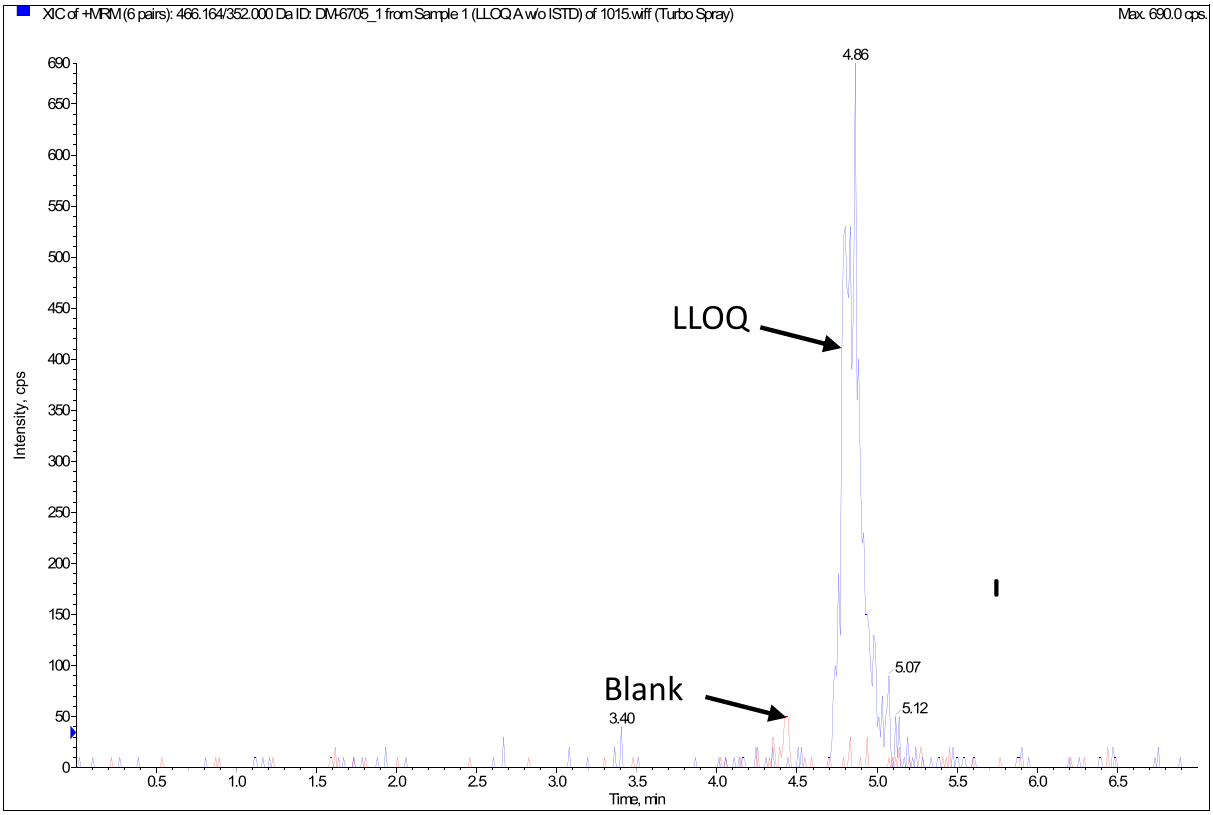


Fig. 6. : An example of the chromatography of a breast milk sample, in which the chromatograph of the LLOQ (10.0 ng/mL) is overlayed by that of a matrix blank (6 **A** – delamanid, 6**B** – DM-6705). Delamanid and DM-6705 were monitored at the mass transitions of *m/z* 535.2–352.0 and 466.2–352.0, respectively.

Table 6
Matrix effects assessment of delamanid and DM-6705 from six different breast milk sources.

	Delamanid	DM-6705
Area Ratio vs Concentration Regression Slope		
Matrix 1	826	5680
Matrix 2	809	5540
Matrix 3	745	5570
Matrix 4	785	5540
Matrix 5	763	5760
Matrix 6	810	5770
Average	790	5640
STDEV	31.2	107
CV(%)	**3.9**	**1.9**

Deproteinized human breast milk samples containing delamanid and DM-6705 underwent online SPE using a Restek Viva BiPh C18 column (5 μm, 1.0 mm×50 mm) for extraction and a Waters Xterra MS C18 column (2.1 mm×100 mm, 5 μm) for chromatography in a total run time of 7 minutes. Standard HPLC equipment was employed with an additional binary pump and a six-port valve. In brief, analytes were retained on the extraction column, enabling more polar compounds to elute to waste. During the analytes' elution time-window, the valve directed flow to the analytical column. Fig. 3**A** shows the elution of the analytes from the extraction column, guiding the precise timing of flow for a short elution time-window to direct the analytes onto the analytical column. The resulting chromatogram in Fig. 3**B** exhibited excellent peak shapes and high repeatability of peak areas. More hydrophobic interfering compounds retained on the extraction column were washed off after the analytes' elution window by applying a cleaning gradient, preparing the column for the next sample. This development resulted in the online SPE and chromatography method described in Section **2.4.2.**, which was employed for the validation and analysis of subject samples.

To ensure consistency aliquoting of representative samples from different matrix lots, it was crucial to thaw stored breast milk samples for extended times at ~30°C, simulating physiological conditions and promoting sample homogeneity. Visual inspection revealed that, irrespective of fat content, breast milk samples thawed at ~30°C exhibited greater consistency than those thawed at room temperature. After thawing, samples were homogenised by gentle inversion on a revolving rotator for 5 minutes and the analytes proved to be stable under these conditions.

The fat content of a sample significantly influences analytical results, particularly depending on the sample preparation technique employed. Vortex-mixing, used post-thawing and during STDs and QC sample preparation, yielded varied results based on breast milk fat content. Extensive vortex-mixing of fatty samples caused fat globules to adhere to tube surfaces and lids (see Fig. 4), whereas the same procedure with less fatty samples resulted in minimal adherence. Due to the lipophilic nature of delamanid and DM-6705, we hypothesized that analytes may associate with fat globules, reducing recovery. This is corroborated by the data presented in Tables 4.1 and 4.2, demonstrating lower analyte and internal standard recovery in highly fatty matrices. The CV(%) of the analyte peak areas were above 15% at the QC high, medium, and low concentrations for both delamanid and DM-6705. To address this, we investigated optimal vortex-mixing durations and intensities for consistent sample integrity, regardless of fat content, testing durations of 10, 20, and 30 seconds and low, medium, and high intensity levels. We found that medium intensity vortex-mixing for 10 seconds produced the most favourable outcome.

Despite these preparative improvements and even when using the same lot of matrix, fluctuations persisted in the internal standard intensity of analysed samples. Quantification of QC samples with calibration STDs prepared in the same matrix lot, revealed accuracy and precision issues with multiple calibration STDs and QCs falling outside acceptance criteria. This clearly indicated that the internal standards were unable to compensate for matrix effects. Introducing internal standards as solutions in acetonitrile and methanol (50:50, v/v) during the deproteination step was the approach initially taken. However, preparing internal standards by dilution into the matrix and adding them to the sample before deproteination significantly mitigated this issue, aligning the behaviour of analytes and internal standards. Fig. 5**A** and **C** illustrate internal standard intensity fluctuations, while Fig. 5**B** and **D** demonstrate consistent internal standard intensity when prepared in the matrix.

In summary, achieving sample integrity, consistency, and repeatability in the extraction of delamanid and its metabolite from breast milk, required the introduction of online SPE, thawing the samples at ~30°C and applying mild mixing techniques. Furthermore, using this matrix, it is important to add the internal standards as solutions in breast milk rather than introducing them with the organic solvent used for protein precipitation.

3.2. *Method validation*

As summarised in Table 5, the overall accuracy and precision of the inter- and intra-day validation were proven by the analysis of three validation batches and evaluating the accuracy and precision of duplicate calibration STDs and six-fold QCs according to the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [12,13]. The calibration curves fitted quadratic (weighted 1/concentration) regressions over a 10.0 – 1000 ng/mL range for delamanid and DM-6705. The accuracies of QC (n=18) high, medium, low, and LLOQ concentrations were between 92.1% and 98.3% for delamanid and between 97.0% and 102.8% for DM-6705. The precision (CV(%)) of QC high, medium, low, and LLOQ concentrations ranged from 2.6% to 4.6% for delamanid and 2.3% and 7.8% for DM-6705.

Delamanid and DM-6705 stock solutions remained stable for 376 and 370 days at ~-80°C, respectively. Delamanid and DM-6705 stock solutions were stable for at least 4 hours at room temperature and for 24 hours at ~4°C and ~-20°C. Working solutions of delamanid and DM-6705 were stable at ~-80°C for 13 and 14 days, respectively, and for 24 hours at ~4°C and ~-20°C. The analytes in working solutions were stable for at least 4 hours at room temperature, which covered the preparation of STDs and QCs.

The analytes were stable in breast milk during three freeze-thaw cycles; the percentage difference for delamanid was −3.1% at QC high and −11.4% at QC low. DM-6705 percentage difference was −1.7% and −7.1% at QC high and low, respectively. The precision for both analytes was below 3.6% at the high and low concentration levels. The measured QC concentrations were within 15% of the nominal concentrations, indicating acceptable accuracy.

Delamanid and DM-6705 were stable in breast milk at room temperature for ~4 hours, indicating sample preparation should not exceed 4 hours (duration of test) with the samples left on the bench. The percentage difference of delamanid was between −0.6% and −11.0% at QC high and low, respectively, and the DM-6705 percentage difference was between 0.9% and −9.4% at QC high and low, respectively. The CV(%) of delamanid at QC high was 2.7% and 4.7% at QC low, and DM-6705 CV(%) was 2.1% at QC high and 5.5% at QC low.

Assessing absolute autosampler stability, it was shown that, if the samples are kept in the autosampler at ~8°C, both delamanid and DM-6705 remain stable if the entire batch is reinjected within 48 hours.

The method exhibited good selectivity, as it effectively differentiated delamanid and DM-6705 in breast milk in the presence of other matrix components and background noise at the LLOQ concentration level. The average signal-to-noise ratio was 216.3 and 35.2 for delamanid and DM-6705, respectively, when spiked in six lots of breast milk. Representative chromatograms of LLOQs overlaid with chromatograms of blank samples are illustrated in Fig. 6. No carryover was observed for both analytes.

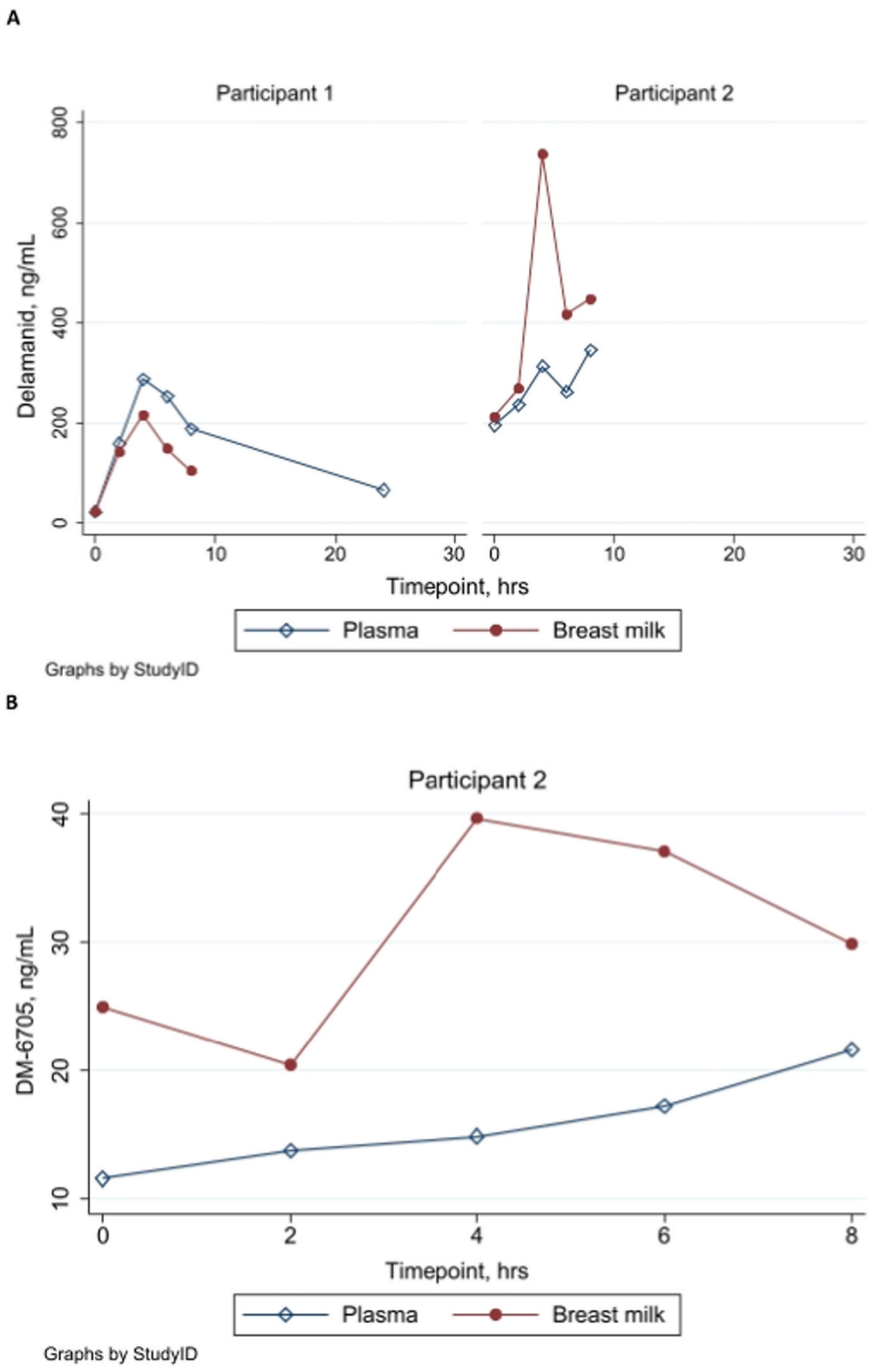


Fig. 7. Pharmacokinetic profiles of the analytes, delamanid (7 **A**) and DM-6705 (7**B**), in breast milk and plasma of two BEAT-Tuberculosis trial participants. We quantified the concentrations of delamanid in the plasma of participants 1 and 2 (7 **A**), while the concentration of DM-6705 in the breast milk of participant 1 was below LLOQ for all samples (7**B**).

Insignificant crosstalk or contribution of less than 5.8% was observed for delamanid-d4 in the delamanid channel. No significant interfering peaks of OPC-14714 were observed in the DM-6705 channel for the blank sample or in the internal standards channel for each analyte sample without internal standards, at ULOQ, indicating that there is no crosstalk or contribution of the analytes to the internal standards.

The mean recovery of delamanid and DM-6705 was 107.8% and 125.3%, and the CV(%) was 0.9 and 3.0, respectively. The mean process efficiency from six different lots of breast milk was 112.5% and 121.9% with a CV(%) of 4.8% and 4.1% for delamanid and DM-6705, respectively. This indicates that the presence of matrix and extraction efficiency had no negative effect on the analytes.

The analytes were differentiated from endogenous components present in six different lots of breast milk, which we evaluated. Using the Matuszewski method for matrix effects assessment, the slope variability CV(%) was 3.9% and 1.9% for delamanid and DM-6705, respectively; the matrix, therefore, does not significantly affect the precision of the assay. The matrix effects results of delamanid and DM-6705 are displayed in Table 6.

The presence of concomitant medications did not significantly affect the quantification of delamanid and DM-6705. For both delamanid and DM-6705 the measured percentage difference in the presence and absence of concomitant medications at QC high and low was below 3.7%, and the CV(%) was less than 4.7%.

3.3. Application to a clinical pharmacokinetic study

The analytical method was applied to measure delamanid and DM-6705 concentrations in breast milk samples from two post-partum participants treated with delamanid for drug-resistant tuberculosis in the BEAT-Tuberculosis trial. Breast milk concentrations of delamanid ranged from 22.8 to 737 ng/mL (n=2), and breast milk DM-6705 concentrations were between 20.5 and 39.7 ng/mL (n=1). In context, the delamanid plasma concentrations at the same time points ranged from 2.60 to 345 ng/mL (n=2), and the DM-6705 plasma concentrations were between 11.6 and 21.6 ng/mL (n=1). The observed concentrations were above the LLOQ (10.0 ng/mL) for delamanid and DM-6705. The results are depicted as pharmacokinetic profiles in Fig. 7. The breast milk concentrations of delamanid in participant 2 were higher than in plasma, which is similar to previous descriptions of animal studies where delamanid concentrations in breast milk were reported to be significantly higher than corresponding plasma concentrations [5]. Participant 1 had low breast milk and plasma delamanid concentrations and concentrations were lower in breast milk than in plasma - DM6705 was not detected in plasma or breast milk, suggesting poor adherence.

Our aim was to develop a robust and accurate method for the determination of delamanid and DM-6705 in breast milk and apply our method to clinical trial samples. Observed concentrations of the analytes in breast milk samples were within the validated calibration range, except for participant 1 where all the samples were below LLOQ for the metabolite, and correlation between DM-6705 concentrations in breast milk and plasma could therefore not be determined. Participant 2 had higher concentrations of delamanid and DM-6705 in breast milk than in plasma. Our study was limited by a small sample size.

4. Conclusion

We developed and validated a novel LC-MS/MS assay using online SPE and analysed delamanid and DM-6705 concentrations in breast milk. The method demonstrated good reproducibility and selectivity, enabling the accurate measurement of the analytes in the presence of a complex matrix. The method was validated according to FDA and EMA guidelines [12,13], over the concentration range of 10.0 – 1000 ng/mL. The combination of online SPE and LC-MS/MS analysis offers a reliable and efficient approach to quantify delamanid and DM-6705 in breast milk, providing invaluable pharmacokinetic insights into potential effects on breastfeeding infants.

Corresponding author statement

I declare this work is original and has not been submitted to any other journal for publication.

CRediT authorship contribution statement

Richard Court: Writing – review & editing, Supervision, Methodology, Investigation. **Buyisile Mkhize:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Anton Joubert:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Sandra Castel:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Gary Maartens:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Marthinus van der Merwe:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **LUBBE WIESNER:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Francesca Conradie:** Writing – review & editing, Resources, Investigation, 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.

Acknowledgements

Overall support for the International Maternal Pediatric Adolescent AIDS Clinical Trials Network (IMPAACT) was provided by the National Institute of Allergy and Infectious Diseases (NIAID) with co-funding from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) and the National Institute of Mental Health (NIMH), all components of the National Institutes of Health (NIH), under Award Numbers UM1AI068632 (IMPAACT LOC), UM1AI068616 (IMPAACT SDMC) and UM1AI106716 (IMPAACT LC), and by NICHD contract number HHSN275201800001I. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

[1] R. Acquah, E. Mohr-Holland, J. Daniels, J. Furin, M. Loveday, V. Mudaly, A. Reuter, Outcomes of children born to pregnant women with drug-resistant tuberculosis treated with novel drugs in Khayelitsha, South Africa: a report of five patients, Pediatr. Infect. Dis. J. 40 (5) (2021) e191–e192, https://doi.org/10.1097/INF.0000000000003069.

[2] Y. Hirao, T. Koga, N. Koyama, Y. Shimokawa, K. Umehara, Liquid chromatography-tandem mass spectrometry methods for determination of delamanid in mouse plasma and lung, Am. J. Anal. Chem. 6 (2015) 98–105, https://doi.org/10.4236/ajac.2015.62009.

[3] E.W. Tucker, L. Pieterse, M.D. Zimmerman, Z.F. Udwadia, C.A. Peloquin, M.T. Gler, S. Ganatra, J.A. Tornheim, P. Chawla, J.C. Caoili, B. Ritchie, S.K. Jain, V. Dartois, K.E. Dooley, Delamanid central nervous system pharmacokinetics in tuberculous meningitis in rabbits and humans, -19, Antimicrob. Agents Chemother. 63 (10) (2019) e00913, https://doi.org/10.1128/AAC.00913-19.

[4] A. Reckers, S. Huo, A. Esmail, K. Dheda, P. Bacchetti, M. Gandhi, J. Metcalfe, R. Gerona, Development and validation of a liquid chromatography-tandem mass spectrometry method for quantifying delamanid and its metabolite in small hair samples, J. Chromatogr. B 1169 (2021) 122467, https://doi.org/10.1016/j.jchromb.2020.122467.

[5] EMA. *Delamanid (Deltyba): Summary of Product Characteristics*. 2014. Available from: ⟨https://www.ema.europa.eu/en/documents/product-information/deltyba-epar-product-information_en.pdf⟩. [accessed 2023 20 March].

[6] Y. Shimokawa, K. Sasahara, N. Koyama, K. Kitano, M. Shibata, N. Yoda, K. Umehara, Metabolic mechanism of delamanid, a new anti-tuberculosis drug, in human plasma, Drug Metab. Dispos. 43 (8) (2015) 1277–1283, https://doi.org/10.1124/dmd.115.064550.

[7] K. Sasahara, Y. Shimokawa, Y. Hirao, N. Koyama, K. Kitano, M. Shibata, K. Umehara, Pharmacokinetics and metabolism of delamanid, a novel anti-tuberculosis drug, in animals and humans: importance of albumin metabolism in vivo, Drug Metab. Dispos. 43 (8) (2015) 1267–1276, https://doi.org/10.1124/dmd.115.064527.

[8] M.T. Gler, V. Skripconoka, E. Sanchez-Garavito, H. Xiao, J.L. Cabrera-Rivero, D. E. Vargas-Vasquez, M. Gao, M. Awad, S.K. Park, T.S. Shim, G.Y. Suh, M. Danilovits, H. Ogata, A. Kurve, J. Chang, K. Suzuki, T. Tupasi, W.J. Koh, B. Seaworth, L. J. Geiter, C.D. Wells, Delamanid for multidrug-resistant pulmonary tuberculosis, N. Engl. J. Med. 366 (23) (2012) 2151–2160, https://doi.org/10.1056/NEJMoa1112433.

[9] A.J. Garcia-Prats, M. Frias, L. van der Laan, A. De Leon, M.T. Gler, H.S. Schaaf, A. C. Hesseling, S. Malikaarjun, J. Hafkin, Delamanid added to an optimized background regimen in children with multidrug-resistant tuberculosis: results of a phase I/II clinical trial, -21, Antimicrob. Agents Chemother. 66 (5) (2022) e02144, https://doi.org/10.1128/aac.02144-21.

[10] M. Fujiwara, M. Kawasaki, N. Hariguchi, Y. Liu, M. Matsumoto, Mechanisms of resistance to delamanid, a drug for Mycobacterium tuberculosis, Tuberculosis 108 (2018) 186–194, https://doi.org/10.1016/j.tube.2017.12.006.

[11] E. Purwantini, B. Mukhopadhyay, *Conversion of NO_2 to NO by reduced coenzyme F_{420} protects mycobacteria from nitrosative damage.* Proc. Natl. Acad. Sci. 106 (15) (2009) 6333–6338, https://doi.org/10.1073/pnas.0812883106.

[12] US-FDA, Drug administration, FDA guidance for industry: Bioanalytical method validation, draft guidance, US Department of Health and Human Services, FDA, 2018. ⟨https://www.fda.gov/media/70858/download⟩ (Available from).

[13] EMA. *European Medicines Agency: Guideline on bioanalytical method validation*. 2011. Available from: ⟨https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf⟩. [accessed 2023 05 April].

[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride

in human plasma at picogram per milliliter concentrations, Anal. Chem. 70 (5) (1998) 882–889, https://doi.org/10.1021/ac971078+.
[15] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (13) (2003) 3019–3030, https://doi.org/10.1021/ac020361s.
[16] S. Rodriguez-Mozaz, M.J.L. de Alda, D. Barceló, Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography–mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water, J. Chromatogr. A 1152 (1-2) (2007) 97–115, https://doi.org/10.1016/j.chroma.2007.01.046.